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Learning and memory in *Octopus vulgaris*: search of the underlying biological machinery

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Abstract

The cephalopod mollusc *Octopus vulgaris* is known for the richness of behavioral repertoire, neural and behavioral plasticity, and complex cognition rivaling higher Vertebrates.

Animals are known to learn over a variety of tasks, equipped with different sensory-motor systems, i.e. visual and tactile, and able to recall the outcomes of their experience for long term (e.g., 'one month' Sutherland, 1957; 'some months' Sanders, 1970).

In the present study, a fear conditioning training protocol was adopted to evaluate *O. vulgaris* behavioral responses to an artificial stimulus to be avoided. Behavioral outcomes have been tested for the effect of a protein synthesis inhibitor on memory acquisition and retention, and for changes in the pattern of expression of genes potentially involved in memory formation.

Applying the 3Rs principle, I used samples from a previous study, thus limiting the number or live animals humanely killed for the aims of this PhD project.

My experiments and analysis allowed to:

i. Identify that cycloheximide-induced protein synthesis inhibition did not alter the octopus ability to acquire an avoidance learning task. However, octopuses ability to retain and recall the memory was impaired;

ii. Data available to me did not allow to rule out a state-dependent effect of cycloheximide injection that somehow affects memory recall and octopuses ability to learn;

iii. Identify 24 target genes, nine memory-related genes and 15 epigenetic modifiers, from *O. vulgaris* transcriptome, and studied their gene expression profile in relation with learning and memory consolidation.

1. Introduction

Learning, memory and gene expression

In every-day life animals behave on the basis of a continuous interaction with the internal and external environment. The continuously acquired individual experience brings to a further refinement of behavior. Any decision and action/reaction in response to a stimulus (either internal or external) relies on previous experiences and to the capacity that the individual has to adapt and benefit from those. Plasticity of biological systems is the overarching phenomenon allowing animals to adapt to environment and experience-based situations.

Among the various forms of plasticity, learning is known as the ability to acquire new information from experience and to change individual behavior in response to the acquired knowledge. The capacity to retain this information over time is memory.

Learning and memory are closely related concepts. As mentioned above, learning is the acquisition of skill or knowledge, while memory is the expression of what acquired. Based on the extent of memory recall, the distinction between a transient and labile, short-term component and a long persistent one has to be attributed to William James; depending on the strength of the information storage he named the ability we have to recall events and/or experiences, respectively, primary and secondary memories (James, 1890). Based on several thousand of studies, we now know that the strength of the information acquired through experience can result in a transient and labile memory (short-term memory, STM), a still weak but more endurable memory, lasting minutes (intermediate-term memory, ITM) or long-lasting memory (LTM; e.g., De Zazzo & Tully, 1995; Kandel, 2001b; Steidl et al., 2003; Tomsic et al., 2009).

Declarative memory and nondeclarative memory are two major classifications of long-term memory systems. Declarative memory allows us to consciously recollect events and facts. It is generally indexed by our ability to explicitly recall or recognize those events or facts. Nondeclarative memory, in contrast, is accessed without consciousness or implicitly through performance rather than recollection. Even though implicit memory is more robust and, once established, it is less sensitive to “emotional” modulation (Izquierdo, 2002; Quillfeldt, 2015), for both implicit and explicit memory, the extent of memory recall is also based on its duration. Declarative memory (semantic and episodic memory) corresponds to explicit memories that are conscious and – in the case of humans - verbally transmittable. Nondeclarative memory represents an implicit and nonverbal type of

memory that is acquired subconsciously. Although most declarative memory "contents" are acquired explicitly, and most nondeclarative memories are acquired implicitly, this dichotomy is an oversimplification and ultimately not accurate. For example, declarative memories can be acquired subconsciously (e.g., memories of an emotionally intense event or subliminal priming effects), and nondeclarative memories can be acquired with conscious engagement (e.g., learning of motor movements playing sports or a musical instrument, e.g. Brem et al., 2013).

Another fundamental "characteristic" of the learning and memory process is that the information acquired through experience (learning) has to pass a "consolidation" period, during which the trace is labile and could be lost. After iterated repetition of training, memory undergoes to the consolidation process following which it can even last a lifetime (e.g., Müller & Pilzecker, 1900).
Thus, learning and memory is accomplished through three subsequent phases: acquisition, consolidation and retention. Acquisition is the initial process, where new experience is acquired because of perception and "understanding" of a situation/task/stimulus and its behavioral outcome. The consolidation phase allows memory trace to stabilize. It is widely recognized that consolidation requires *de novo* protein synthesis, considered a distinctive hallmark carrying from short-term to long-term memory (Davis & Squire, 1984). Retention refers to the recall of the memory stored. During the retention phase, re-exposure to the training context can induce two opposite processes: memory reconsolidation or memory extinction. The two processes can be mutually exclusive, depending on the features of the learning context (e.g., Pedreira & Maldonado, 2003; Pérez-Cuesta & Maldonado, 2009).
Memory is strictly linked to time, attention, and emotional valence of stimuli/context. Evidence exists that neural circuits implicated with these functions are overlapping with areas involved in processing of memory functions.

STM is an essential characteristic of "learning" organisms, and allows the maintenance of information over a short period of time. According to William James (1890), STM involves a conscious maintenance of sensory stimuli over a short period of time after which they are not present anymore. On the other hand, LTM (secondary memory, *sensu* James) involves the reactivation of past experiences that were not consciously available between the time of encoding and retrieval. This led to the assumption from Hebb in the 1940s that STM and LTM are based on separate neural systems.

STM engages repeated excitation of a cellular compound, LTM leads to structural changes on the synaptic level, which are preceded by consolidation processes that are thought to be highly dependent on specific brain functions.

As reviewed by Brem et al. (2013), LTM refers to the mechanism by which acquired memories gain stability or are strengthened over time; these are also resistant to interference. As mentioned above, two components of LTM are described in the literature and frequently included under the term “declarative memory” – episodic and semantic memory. They rely mostly on given brain areas. Episodic memory refers to contents that can be located within a spatiotemporal context, such as holiday memories or autobiographical events. On the other hand, semantic memories are independent of context and are not personally relevant. They consist of general and factual world knowledge. However, “nondeclarative” memory functions, such as procedural memory also involves LTM consolidation processes.

Successful long-term storage includes several steps starting with the encoding of information, followed by short-term storage and consolidation from STM to LTM, as well as repeated reconsolidation. During consolidation, memories can undergo changes that can be quantitative (enhancement, strengthening) and qualitative (review in Brem et al., 2013). During acquisition, the impact, the duration and/or the extent of the experience the individual is facing may induce different levels of memory recall, also based on the temporal effectiveness and stability of memory recall.

For example, by comparing massed (stimuli are presented with very short inter-trial intervals to the animal up to – for example – criterion for learning is achieved) and spaced (stimuli are presented with time-distance or during blocks of trials ‘spaced’ in time) training in honeybees Menzel and colleagues (2001) tested memory retention using inter-training intervals (ITI) between stimulus presentation of 30 sec, 3 min, and 10 min during training; Authors tested memory-recall after 30 min, one day and three days afterwards. Honeybees trained using 30 sec ITI showed the best recall capability after 30 min, but this rapidly decreased on the third day; animals trained with the longestt inter trial interval performed a better memory recall after 30 min and consolidated these memories reaching almost 100% retention on the third day after training.

This brings to the view that short inter-trial intervals induces only STM formation, while longer ITI allow long-term memories stabilization (Menzel, 2001). Similar results were obtained in other species (e.g., *C. elegans:* Beck & Rankin, 1997; *Aplysia*: Botzer et al., 1998).

Although the physical nature of memory trace is still unclear, our understanding of the mechanisms that underlie memory and cognition has extensively improved in the last four decades, especially thanks to the contribution of Professor Eric Kandel (Kandel, 2001; Levenson & Sweatt, 2006). The studies of Kandel and colleagues allow to state that memories in different organisms require the same molecular machinery and biological processes suggesting that memory is an ancient and evolutionarily conserved phenomenon from invertebrates to vertebrates (Levenson & Sweatt, 2006).

It is out of the aims of this PhD thesis to provide a throughout overview of the cellular and molecular mechanisms known to be involved in STM and LTM and those occurring during consolidation and reconsolidation (for review Asok et al., 2019; Kandel et al., 2014; Sakakibara, 2008) .

In brief and from a molecular perspective, short-term memory involves the covalent modifications of pre-existing proteins, mainly activated through c-GMP pathways, while long-term memory requires activation of c-AMP pathways that activate translocation of kinases in the nucleus that in turns stimulate *de novo* protein synthesis (e.g., Davis & Squire, 1984; Y. Lee et al., 2008; Lefer et al., 2013). Studies in *Aplysia* revealed that the short-term facilitation is not blocked by inhibitors of transcription or translation (Schwartz et al., 1971), while these selectively blocked the induction of the long-term changes (Castellucci et al., 1989). Furthermore, protein and RNA synthesis are critically required in a well-defined time window to induce the long-term memory formation (e.g., Davis & Squire, 1984).

In most recent years, studies on gene expression during consolidation have been carried out to increase our knowledge of the overall process. In several species data available confirm that the long-term behavioral and cellular changes require the expression of genes and proteins not required for the short-term. Long-term-sensitization, for example, is associated with the growth of new synaptic connections that facilitate the response, while the ones linked with inhibitory responses are actively reduced (e.g., Bailey et al., 1988a, 1988b; Bailey et al., 1996; Bailey & Chen, 1983). This confirms the original hypothesis by Ramon y Cajal (Santiago y Cajal, 1894) that memories established through experience-dependent modulation of synaptic strength and structure.

The conversion between STM and LTM at neuronal level coincides with the conversion of the short- to the long-term forms of synaptic plasticity and the signaling pathway associated is conserved from

mollusks to vertebrates (review in Kandel, 2001). This pathway depends on the PKA and MAP kinases activation and translocation into the nucleus, and on the phosphorylation and activation of CREB-1 (for example). CREB-2, a memory suppressor and a CREB-1 inhibitor, is thus inactivated. The transcription factor CREB-1 induces the transcription of immediate response genes. A small subset of genes (immediate-early genes) encoding for transcription factors, are rapidly activated and transcribed because of the learning experience, their induction does not need *de novo* protein synthesis, but is mediated by pre-existing transcription factors (Abraham et al., 1992; Hawk & Abel, 2011; Tischmeyer & Grimm, 1999). The protein products obtained after this first "wave" of transcription, guide the expression of a larger set of target genes, finally driving to endurable changes in synaptic transmission through the synthesis of the proteins needed for the growth of new synapses, and the increase of synaptic strength (Bailey et al., 1996). LTM is therefore formed through multiple waves of transcription, in vertebrates and invertebrates (e.g., Freudenthal & Romano, 2000; Lee et al., 2008; Lefer et al., 2013). Interfering with the molecular processes that allow memory formation through the use of transcription inhibitors before and after conditioning, Lefer and colleagues demonstrated that storage of information into long-term memory requires two waves of transcription in honeybees, an early transcription wave (triggered during conditioning) and another wave starting several hours after learning (Lefer et al., 2013). This study identified two time windows during which transcription appeared to be a fundamental process for LTM formation, since transcription suppression impaired memory in a quantitatively and qualitatively way (Lefer et al., 2013).

Figure 1 schematizes the three phases providing few examples taken from studies available from the target organism of this PhD, namely the cephalopod mollusc *Octopus vulgaris*.

In the long history of the search of neural correlates of learning and memory, the common octopus appears as one of the main "characters". This is possibly linked to the huge work promoted by J.Z. Young in the search of a model of the brain (Marini et al., 2017; Young, 1964).

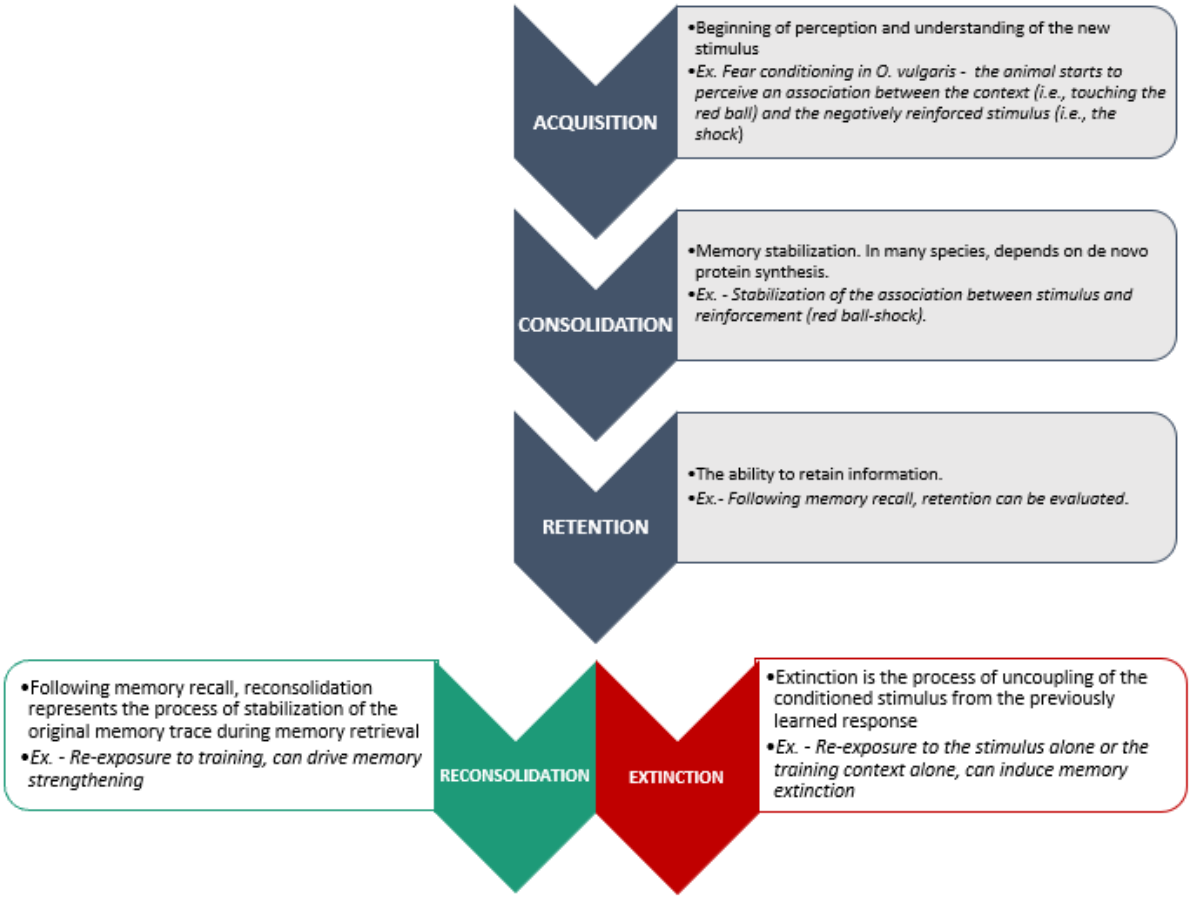


Figure 1. A schematic diagram of learning and memory process. See text for details

Knowledge on the complex set and pathways of changes in gene expression occurring during learning and memory processes is rapidly emerging.

Figure 2 is only one example of this complexity (see also Table 1). It is out of the aims of this PhD thesis summarize the available literature related to the molecular machinery implicated in learning and memory processes (for review see for example: Asok et al., 2019; Cavallaro et al., 2002; Martinez et al., 2007).

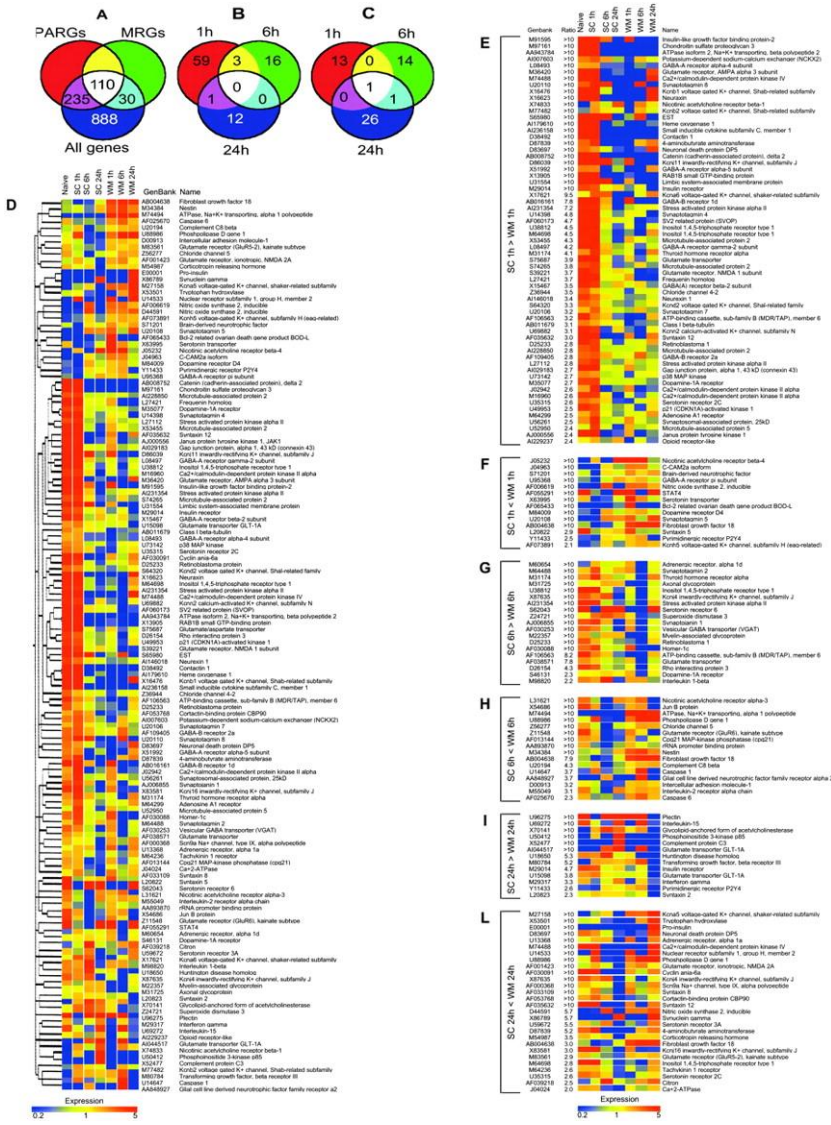


Figure 2 Differential gene expression during water maze learning in mice, with Venn diagrams of differentially expressed hippocampal genes. Genes differentially expressed in naive and swimming control animals 1, 6, and 24 h after training. Reproduced after Cavallaro et al. (2002) with permission.

Epigenetic modifications during learning and memory process

The epigenetic machinery in metazoans is associated with developmental processes of determination and differentiation. During these processes, epigenetic marking of the genome restricts the expression of genes involved in maintaining pluripotency and promotes the expression of genes necessary for the determination and maintenance of the differentiated state. In this way the epigenetic code helps differentiated cells to “remember their phenotype” (Levenson & Sweatt, 2006). In 1956, the developmental biologist Conrad Waddington demonstrated that an environmental stimulus could lead to the inheritance of an acquired characteristic (Waddington,

1956). Waddington understood that to study the inheritance of acquired characteristics, one had to focus on the forms of developmental plasticity that were already present in the population of interest, thus to induce a path already possible in evolution (Noble, 2015). Historically, epigenetic mechanisms have been mainly studied in the context of cellular and organismal development.

Active histone tagging and DNA methylation are known to be critical for the correct functioning of the 'mature' central nervous system (Rudenko & Tsai, 2014).

In neurons, the epigenetic machinery is not restricted to the developmental function of neuronal differentiation and cell-fate determination, but it is also involved in the induction of synaptic plasticity and memory formation and consolidation (e.g., Day & Sweatt, 2011; Miller et al., 2008). Several evidences proved that epigenetic mechanisms have been co-opted in the nervous system to guide cellular responses to environmental stimuli, spanning from maternal behavior, psychological or physical stress, drug exposure, to learning (see for example: Borrelli et al., 2008; Day & Sweatt, 2010; Hackman et al., 2010; Kaminsky et al., 2008; Sweatt, 2013; Weaver et al., 2004). Epigenetic modifications must be stable to preserve the information, but also dynamic to react to environmental stimuli via specific remodeling (Gräff & Mansuy, 2008). These modifications comprise DNA methylation and post-translational modifications of histone tails, including acetylation, phosphorylation, methylation and ubiquitynation.

The first study on the role of epigenetic modifications during memory formation has been carried out in *Aplysia* studying the sensorimotor synapse (Guan et al., 2002). Two forms of synaptic plasticity can be induced in *Aplysia*: 5-HT stimulation brings to long-term facilitation, while FMRF-amide induces long-term depression. At the time of the work by Guan and colleagues it was known that CREB1 was indirectly capable of altering chromatin structure through the recruitment of the CREB binding protein (CBP), which acetylates lysine residues on histone tails (Chrivia et al., 1993). Using chromatin immunoprecipitation assays, Guan and colleagues demonstrated that when a sensory neuron receives stimuli alterations in chromatin structure are induced at the promoter of the genes needed for long-term synaptic plasticity. Other experiments using serotonin stimulation *in vivo* revealed that the chromatin remodeling enzymes (CBP) and TATA-binding protein (TBP) laid at the C/EBP promoter and acetylation of histone H3 and histone H4 occurred while the gene was transcribed. Changes in the epigenome shepherded synaptic plasticity (Guan et al., 2002).

In *Aplysia*, huntingtin has been revealed to be involved in transcriptional regulation of bdnf and other genes important for long term synaptic plasticity through the binding of CBP (Choi et al., 2014).

Several studies in rodents revealed the importance of epigenetic modifications in memory formation and consolidation. The pharmacological approach appeared extremely effective at contributing to this knowledge. Table 1 includes some examples from the literature based on studies in vertebrates and invertebrates.

Table 1. Examples of learning and memory studies in vertebrates and invertebrates with focus on the underlying cellular and molecular mechanisms, including epigenetic modifications.

	Learning paradigm/ Neurophysiological experiment	Species	Cellular and/or molecular 'mechanisms'	Reference
Invertebrates	Post tetanic potentiation and neuronal correlates of habituation and dishabituation	*Aplysia californica*	Short-term plastic changes were not blocked by inhibitors of translation	(Schwartz et al., 1971)
	Sensitization	*Aplysia californica*	Long-term sensitization in Aplysia increases the number of presynaptic contacts to the motor neuron.	(Bailey et al. 1988b)
	Sensitization in isolated preparation	*Aplysia californica*	Long-term sensitization, but not short-term sensitization, was blocked by protein synthesis inhibitor	(Castellucci et al. 1989)
	Sensitization in animal and isolated preparation	*Aplysia californica*	Intermediate-term memory requires PKA activation and protein synthesis, but not gene transcription	(Sutton et al., 2001)
	Cell cultures and electrophysiological experiments (LTF/LTD)	*Aplysia californica*	Histones acetylation is involved in memory formation	(Guan et al., 2002)
	Habituation and associative learning	*Chasmagnathus granulathus*	Massed training brings to ITM which does not require transcription activation; spaced training brings to LTM depending on transcription	(Freudenthal & Romano, 2000)
	Odor avoidance conditioning	*Drosophila melanogaster*	LTM consolidation depends on protein synthesis	(Tully et al., 1994)
	PER conditioning	*Apis mellifera*	Longer is ITI, better are acquisition and retention. Consolidation is dependent on protein synthesis	(Menzel, 2001)
	Olfactory conditioning	*Apis mellifera*	LTM formation requires two waves of transcription	(Lefer et al., 2013)
Vertebrates	Electrophysiological experiments (LTP)	*Rattus norvegicus*	Immediate early genes are transcribed following LTP induction	(Abraham et al., 1992)
	Fear conditioning	*Rattus norvegicus*	One hour after fear conditioning, a significant increase in acetylation of histone H3 is observed	(Levenson et al., 2004)
	Fear conditioning	*Rattus norvegicus*	DNMT gene expression is upregulated in the hippocampus following contextual fear conditioning	(Miller & Sweatt, 2007)
	Fear conditioning	*Rattus norvegicus*	Genes participating to memory consolidation are actively transcribed following H3K4 trimethylation their promoter	(Gupta et al., 2010)

One hour after fear conditioning in rats, significant increases in acetylation of histone H3 was observed after training (but not H4) and estimated through western blot analysis of area CA1 (Levenson et al., 2004). Injection of sodium butyrate, an HDAC inhibitor, prior to contextual fear conditioning enhanced long-term memory formation in the animals. The effects of sodium butyrate and TSA, another HDAC inhibitor, have been evaluated also on LTP (Long Term Potentiation, a cellular analogue of LTM) and on basal synaptic transmission of Schaffer-collateral synapses. The induction of LTP significantly increased in slices treated with TSA or sodium butyrate (Levenson et al., 2004).

DNA methylation in mammals is laid down by DNMT1 (a maintenance methyltransferase) and by DNMT3a and DNMT3b, *de novo* methyltransferases responsible for establishing new methylation patterns. Intra-CA1 infusion of DNMT inhibitor (5-AZA or zeb) immediately after contextual fear conditioning training blocked consolidation.

Thus, DNA methylation appears necessary for memory consolidation (Miller & Sweatt, 2007). However, the effects of DNMT inhibition on memory is not permanent. Animals previously treated with a DNMT inhibitor are capable, after retraining, of forming a memory equal in strength to control animals (Miller & Sweatt, 2007). Mice exposed to fear training showed an increase in DNMT3A and DNMT3B mRNA in area CA1. Furthermore, RT-qPCR and methylation-specific quantitative real time PCR experiments demonstrated that fear conditioning induced increased PP1 methylation, lowering its mRNA levels in CA1 area. PP1 is involved in chromatin remodeling in mammals (Miller & Sweatt, 2007).

Histone methylation is also involved in memory stabilization. Fear learning triggers the increase in H3K4 trimethylation at gene promoter regions of zif268 and bdnf, two genes participating in the consolidation (Gupta et al., 2010). Histone methylation is also involved in regulating behavior in honeybees, such as foraging behavior (Anreiter et al., 2017).

In sum, consolidated findings proved that neural activation through learning and memory or by electrophysiological stimulation leads to rearrangements of neural architecture bringing to changes

in the strength of synapses, increase the release of neurotransmitters and enhance the expression of post-synaptic receptors acting on gene expression. The epigenetic machinery helps the establishment of the genetic pattern of expression needed for learning and memory.

2. Learning and memory in cephalopods: a short overview on studies on the underlying molecular machinery

The molluscan class Cephalopoda comprises the subclass of Nautiloidea (e.g., *Nautilus*) the sole living cephalopod with external shell, and the subclass of Coleoidea, i.e., cuttlefish, squid and octopus.

The passage from the shell-protected ancient cephalopods, to the vulnerable-soft-bodied, smart and agile coleoids is linked to a significant body, habits and nervous system rearrangement that occurred during the evolution of the taxon (Amodio et al., 2019; Packard, 1972).

Cephalopods central nervous system is distinguished from that of other invertebrates for the relative size (comparable to that of vertebrates), the number of neurons and a highly centralized neuroanatomical organization (Williamson & Chrachri, 2004; Young, 1971)

The coleoids nervous system is probably the most sophisticated among invertebrates (Chrachri & Williamson, 2004) and their behavioral responses have been observed and studied since antiquity. Aristotle first reported about their ability to camouflage through the change of the skin patterns and inking in response to fear. He also noted octopus' explorative behavior that pushed it outside its den, making of it an easy prey (Aristotle, 1910).

Scientific interest for these animals can be dated back early in history of science. However, it is largely recognized that the most significant boost of research around these organisms originated by initiative of scientists working at the Stazione Zoologica and by the work promoted by Professor J.Z. Young (review in: De Sio, 2011; De Sio et al., 2020; Marini et al., 2017; Ponte et al., 2013). Amongst cephalopods, octopus was chosen as an ideal experimental organism by J.Z. Young and collaborators aimed at investigating neural correlates of learning: octopus showed its "value for the study of behavior" (Young, 1971). The animal resulted to easy acclimatized to captive conditions and recovered well from brain surgery (review in Hochner et al., 2006); the same did not appear in the case of *Sepia officinalis* (Sanders & Young, 1940).

It is without doubt that these animals provide exceptional substrate for the understanding of the emergence of genomic and neural novelties (for review see: Albertin & Simakov, 2020; Shigeno et

al., 2018) in evolution and sophisticated cognitive abilities (Amodio et al., 2019; Edelman & Seth, 2009). Further interest for this species was gained thanks to the adoption of controlled handling, maintenance, and training procedures (review in De Sio et al., 2020; Marini et al., 2017). In recent years, and thanks also to the sequencing of cephalopod genomes (e.g. Albertin et al., 2015; Da Fonseca et al., 2020; Zarrella et al., 2019) studies on these animals found a renewed attention (Figure 3).

Figure 3. Treemap of Clarivate Zoological Record's for a query on the scientific papers published over the last five years on *Octopus vulgaris* ('Organizations-Enhanced').

Learning and memory has been explored in these animals utilizing several training paradigms (for review see Borrelli & Fiorito, 2008; Marini et al., 2017; see also Table 2 for a short summary).

The relatively simple "brain" of *O. vulgaris* and the corresponding richness of the behavioral repertoire and sophisticated learning capabilities, render this cephalopod an ideal organism to investigate the underlying molecular machinery involved in behavioral and neuronal plasticity (Zarrella et al., 2015). A comparative overview of the nervous system of these animals is available in Nixon & Young (2003), and suggested functional analogies of the nervous system with vertebrates is presented by Shigeno et al. (2018). In brief, the nervous system of *O. vulgaris* is estimated to count over 500 million neural cells, 40% of those (200 million) compose the central brain (i.e. optic lobes, OL; supra-esophageal mass, SEM; sub-esophageal mass, SUB. SEM and SUB are organized in lobes and regions (see: Young, 1963, 1971, Ponte & Fiorito, 2015). A schematic overview of *Octopus vulgaris* brain and its organization in lobes is provided in Appendix 1.

Unlike other mollusks as the gastropod *Aplysia californica*, which contains relatively few large and identifiable neurons, cephalopods' brain contains half a billion nerve cells, the majority being very small (about 5 microns or less). Furthermore, while the well-studied defensive *Aplysia* gill-withdrawal reflex is an example of learning and memory at circuit level (Kandel, 2001), cephalopods possess a very large dedicated neuronal network operating in association to the circuitry controlling the learned behavior (Chrachri & Williamson, 2004; Shigeno et al., 2018).

Table 2. A tabularized summary of forms of learning recognized in cephalopods. References are to reviews including studies on these animals.

TYPE OF LEARNING	DESCRIPTION	References
NON ASSOCIATIVE LEARNING	Animal's response to a stimulus changes in the absence of any associated reinforcement (reward or punishment)	
Habituation	Progressive decline in responding to a repeatedly presented neutral stimulus.	(reviewed in Borrelli & Fiorito, 2008)
Sensitization	Progressive reduction of threshold for induction of appropriate response to a repeated presentation of a non-neutral stimulus	(reviewed in Borrelli & Fiorito, 2008)
ASSOCIATIVE LEARNING	It is the animal's ability to understand, through experience, that relations occur between events in the environment	
Classical conditioning	The use of a neutral stimulus (conditioned stimulus), originally paired with one that drives a response (unconditioned stimulus), brought to the association of the conditioned stimulus to the conditioned response	(Borrelli, 2007; Sanders, 1975b; Zarrella, 2011)
Operant or instrumental conditioning	The strength of a behavior is modified by reinforcement or punishment. An association is formed between a behavior and a consequence for that behavior.	(Sanders, 1975a; Fiorito & Scotto, 1992)
OTHER FORMS OF LEARNING		
Problem solving	The process of figuring out how to achieve a goal	(review in: Sanders, 1975a; Borrelli & Fiorito, 2008)
Spatial learning	An animal acquires "consciousness" of its environment and it can use this knowledge to get a reward or to escape a punishment/danger.	(review in: Sanders, 1975a; Borrelli & Fiorito, 2008)
Social or observational learning	Observing and modeling another individual's behavior, usually a conspecific.	(Fiorito & Scotto, 1992)
Perceptual learning	It is the process by which experience drives the sensory system improvement to respond to environmental stimuli	(Sanders, 1975a)

Historically, squid giant axon has been one of the best studied nerve cells in neuroscience (Williamson & Chrachri, 2004) and around 1960s a great number of publications referred about the effect of a plethora of agents on electrical properties of its membrane (e.g., Armstrong & Binstock, 1964, 1965; Hoskin & Rosenberg, 1964; Kishimoto & Adelman, 1964; Moore et al., 1964; Rojas & Luxoro, 1963; Tasaki & Luxoro, 1964). On the other hand, complex properties of the nervous system have been investigated in cephalopods (for review see Brown & Piscopo, 2013). In addition, a long series of experiments on the neural correlates of learning and memory in cephalopods has been carried out for decades (review in Sanders, 1975; Marini et al., 2017). Most of these studies have been based on selective ablation of parts of the "brain" (Boycott & Young, 1955). This allowed J.Z. Young to identify the circuit underlying neural and behavioral plasticity of *O. vulgaris* when visual and tactile sensory motor systems are considered (Young, 1991, 1995). The neural matrices are considered analogous to the limbic lobe of mammalian brains (for review see also Shigeno et al., 2018), with a pivotal role being assumed by the vertical lobe (VL). Removal of the vertical lobe impairs memory, in some cases also LTM (review in Sanders, 1975) including observational learning (Fiorito & Chichery, 1995). Along with VL removal, also medial superior frontal transection impairs but does not block the octopuses to avoid a crab during an associative learning task (Boycott & Young, 1955).

In the cuttlefish the VL appears with a slightly different neural organization, when compared with the octopus. Lesions in the ventral part of the VL of *S. officinalis* led to marked impairment in the acquisition of spatial learning, whereas lesions in the dorsal part of the VL impaired its long-term retention (Graindorge et al., 2006).

In recent years, the cellular analogue of long term memory has been studied in *O. vulgaris*: slice preparation in analogy to what available for the mammalian hippocampus has been developed by Fiorito, Hochner and coworkers (Hochner et al., 2003). Electrophysiological recordings from *O. vulgaris "*brain"-slice preparations demonstrated the existence of a long term potentiation (LTP) similar to that of vertebrates, confirming not only a structural but also a functional similarity (Hochner et al., 2003). When compared with *S. officinalis s*ynaptic plasticity in VL involves different neural cells (glutamatergic cells for octopus; cholinergic cells for cuttlefish) in the two species (Shomrat et al., 2011).

It is noteworthy to remind that LTP (Long-Term Potentiation) is a form of synaptic plasticity considered to be the *in vitro* analogous for long-term memory. The combination of electrophysiological experiments including MSF-VL transection and behavioral approaches, using passive avoidance task provided further insights in the mechanisms involved in short and long-term memory in cephalopods (Shomrat et al., 2008). In the study, the effects of tetanization on learning and memory of a passive avoidance task were compared to those of transecting the same pathway (medium-superior frontal to vertical lobe tract). Tetanization accelerated and transection slowed short-term learning to avoid attacking a negatively reinforced object. However, both treatments impaired long-term recall the next day. The results suggested that the learning and memory system in the octopus, as in mammals, is separated into short- and long-term memory sites; in the octopus, the two memory sites are not independent being the VL modulating the circuitry controlling behavior and short-term learning, and the site where LTP occurs.

The analysis of molecular machinery underlying learning and memory in cephalopods is at its infancy (Zarrella et al., 2015). I will briefly mention in the following pages some of the most significant contributions, to the best of my knowledge.

Dependence of memory on protein synthesis in a specific time-window has been investigated in *S. officinalis* (Agin et al., 2003). In *Octopus vulgaris* this has been explored only for the cellular analogue of Long-Term memory, i.e. investigating LTP in octopus slice (Turchetti-Maia et al. 2018). While protein synthesis inhibition was found to impair memory recall for the "prawn-in-the-tube protocol" in *S. officinalis* (Agin, 2003), no pharmacological approach has ever investigated a possible role for *de novo* protein synthesis as an essential step to develop long-term memory *in vivo* (but see Zarrella et al., 2015). Pharmacological studies using protein synthesis inhibitors in *O. vulgaris* have been used to evaluate animal growth (Houlihan et al., 1990). A summary of some of the evidences of the effects of protein synthesis inhibitors on learning and memory is given in Table 3.

Possible involvement of nitric oxide (NO) in tactile and visual memory (Robertson, 1994; Robertson et al., 1996, 1994) as well as in LTP induction and maintenance (A.L. Turchetti-Maia et al., 2018) has been explored. In addition, the effects of cholinergic inhibitors has been also investigated (Fiorito et al., 1998).

Table 3. Pharmacological studies in invertebrates using protein synthesis inhibitors to study learning and memory. CXM= Cycloheximide; PURO=puromycin; ANI=Anisomycin.

Inhibitor	Species	Procedure	Outcome	Ref.
ANI	*Octopus vulgaris*	LTP	The VL LTP is independent of de novo protein synthesis	(Ana Luiza Turchetti-Maia, Stern-Mentch, et al., 2018)
ANI	*Aplysia*	long-term sensitization	Anisomycin blocks long-term sensitization of the gill- and siphon-withdrawal reflex of *Aplysia,* but not the short-term process.	(Castellucci, 1989)
CXM	*Sepia officinalis*	Associative learning	Cycloheximide injection between 1 and 4 h after passive avoidance training resulted in amnesia	(Agin, 2003)
CXM	*Drosophila melanogaster*	Conditioned odor avoidance	Evidence of the existence of a cycloheximide-sensitive long-term memory	(Tully et al., 1994) (De Zazzo & Tully, 1995)
CXM	*Apis mellifera*	Classical conditioning	Cycloheximide does not affect formation of long-term memory in honeybees after olfactory conditioning	(Menzel et al., 1993; Wittstock et al., 1993)
CXM	*Gryllus bimaculatus*	Classical conditioning	Cycloheximide did not affect olfactory memory retention up to 4 hr after training but significantly lowered it at 5 hr after training.	(Matsumoto et al., 2003)
CXM	*Periplaneta americana*	Spatial learning	Cycloheximide (CXM), did not impair acquisition and did not produce retention deficits up to 1 day after one-session T-maze training procedure	(Barraco, 1981)
CXM	*Helix lucorum*	Conditioned food aversion memory	Cycloheximide injection during training produced long-term memory impairment.	(Nikitin, 2017)
CXM/ANI	*Limax valentianus*.	Odor-taste associative aversive learning	Inhibitors applied to the slug 30 min prior to conditioning impaired long-term memory retention while short-term memory was not affected.	(Matsuo, 2002)
PURO	*Periplaneta americana*	Spatial learning	Puromycin (PURO), produced amnesia 5 hr after training, while acquisition was not affected	(Barraco, 1981)

Deciphering of the molecular mechanisms underlying any form of learning in cephalopods is still far to be achieved. To the best of my knowledge, the sole available study is the one of Zarrella (2011) who analyzed changes of some genes (e.g.: stathmin: Ovstm, tyrosine hydroxylase: Ov-TH, dopamine transporter: Ovdat, octopressin: Ov-OP, cephalotocin: Ov-CT) in response to fear conditioning (learned fear) and social interaction (innate fear). A differential pattern on down-regulation and up-regulation of gene expression in different regions of the octopus central nervous system resulted in these studies as a consequence of learning (review in Zarrella et al., 2015).

Apart from the above few cases, molecular approaches have been utilized to estimate the time of divergence within coleoids (Strugnell et al., 2006), polymorphism of the mitochondrial gene cytochrome oxidase III to shed light on phylogeny of the genus (Fadhlaoui-Zid et al., 2012).

Classical studies using the squid giant axon have been critical for neuroscience to understand how transporters and synaptic transmission operate. Molecular analysis of the major molecules involved in this process, the voltage-gated ion channels and ion transporters has been conducted in squid nervous system. These studies shed light especially on several characteristics of squid K(+) channels, as the formation of hetero-multimers, the editing of their mRNA (Rosenthal & Gilly, 2003).
Molecular cloning of NOS mRNA from a cephalopod was carried on in *S. officinalis* (Scheinker et al., 2005). Immunohistochemical analysis has been used to investigate the distribution or colocalization of galanin and serotonin (Suzuki et al., 2000), neuropeptide Y and FMRFamide (Di Cosmo & Di Cristo, 1998; Suzuki et al., 2002), corticotrophin releasing-factor (CRF) and neuropeptide Y (Suzuki et al., 2003), the distribution of calcitonin gene-related peptide (CGRP; Suzuki and Yamamoto, 2002), gonadotropin-releasing hormone (GnRH, Cosmo and Di Cristo 1998), acetylcholine (D'Este et al., 2008) and glutamate receptors (Piscopo et al., 2007) in the optic lobe and peduncle of *O. vulgaris.* Immunohistochemistry also allowed identification of receptors and neuromodulators in the central nervous system of octopus (e.g. NMDA receptor 2A and 2B: reviewed in Di Cosmo, Di Cristo, and Messenger (2006); oct-GnRH: Iwakoshi-Ukena et al. (2004); serotonin: Shomrat et al. (2010). *In situ* hybridization was used to study the distribution of octopressin, cephalotocin (Kanda et al., 2006) and calretinin mRNA (Altobelli & Cimini, 2007) in octopus.

Advances in studies on the molecular machinery in octopus are due to the great improvements occurred over the last five years thanks to the genome and multiple transcriptomes sequencing

available for the California two-spot octopus, *Octopus bimaculoides* (Albertin et al., 2015) and subsequently of *Octopus vulgaris* (Zarrella et al., 2019).

Genome sequencing evidenced the massive expansions in two gene families also enlarged in vertebrates: the protocadherins, neuronal development regulators, and the C2H2 superfamily of zinc-finger transcription factors.

The recent finding of massive mRNA editing in cephalopods occur. Finally, large-scale genomic rearrangements related to transposable element expansions have been also identified (Albertin et al., 2015; Petrosino, 2015; Zarrella et al., 2019).

3. Aim

The relatively simple 'brain' of the cephalopod mollusc *Octopus vulgaris,* the corresponding richness of the behavioral repertoire and the sophisticate learning and cognitive capabilities (Amodio et al., 2019; Edelman & Seth, 2009; Schnell et al., 2020) have been at the center of decades of studies aimed to explore a "model of the brain" (Young, 1964; see Marini et al., 2017 for review). These studies greatly facilitated a renewed interest for cephalopod biology, physiology and exploring 'innovations' in evolution (e.g., Albertin & Simakov, 2020; O'Brien et al., 2019, 2018; Shigeno et al., 2018), the investigation of its cognitive abilities (Edelman & Seth, 2009) and the underlying molecular machinery involved in behavioral and neuronal plasticity (Zarrella et al., 2015).

Despite the interest and the increasing number of research teams and publications over the last 5 years studying octopus and other cephalopods, the knowledge on the molecular and cellular mechanisms underlying *O. vulgaris* neural and behavioral plasticity remains limited.

In a previous study, CREB resulted to be phosphorylated following fear conditioning in *O. vulgaris* and the expression of some target genes appeared modulated by learning and early stages of memory recall (Zarrella, 2011). This work still remains the sole one available attempting to addressing this topic.

The driving hypothesis of my PhD project is that memory retrieval of a fear passive avoidance task can induce gene expression changes in *Octopus vulgaris* brain masses.

During this PhD project I contributed to shedding light on the possible involvement of protein synthesis on learning and memory through the analysis of a number of target genes known to be involved in learning and memory processes. I identified genes orthologues by fishing *O. vulgaris* transcriptome (Petrosino, 2015) and evaluated the relative expression through RT-qPCR after memory retrieval of an avoidance learning task to explore changes of gene expression linked to learning and memory recall.

This study is the first attempt to disclose – at least partially – the molecular fingerprint linked to learning in the common octopus and in any other cephalopod species. Despite the use of a biased approach in the selection of target genes, I also attempted to explore for the first time possible epigenetic modulation, suggested to contribute to learning and possibly the inter-individual variability of responses.

4. Contribution of this PhD project to the experiments and Ethical statement

For the purpose of this PhD:

i. I investigated *O. vulgaris* transcriptome (Petrosino, 2015; Petrosino et al., 2015), *O. vulgaris* genome (Zarrella et al., 2019) and other available cephalopod genomes and transcriptomes in order to identify target gene candidates' orthologues to potential 'memory-related' and epigenetic modifiers genes. I combined a throughout analysis of scientific literature, searching for gene candidates and their role and conditions into which these has been suggested to be involved in learning processes.

By adopting this biased approach, I identified 24 *O. vulgaris* orthologues of epigenetic modifiers (n = 15), epigenetically regulated memory-related (n = 4) and genes potentially related to memory formation (n = 5).

ii. I also analyzed data from previous unpublished experiments from the laboratory of Dr Graziano Fiorito at the Stazione Zoologica Anton Dohrn (see Appendix 2) in order to address the possibility that memory of an avoidance learning task maybe affected by protein synthesis. Because I have not being involved in any direct experimental activity, but only with the data analysis, I decided to include this part of my project as an annex. I hope that my effort will facilitate future possible studies encompassing a systematic analysis of protein synthesis dependence in learning and memory.

iii. I carried out a series of real time-qPCR experiments including sample treatment, RNA and cDNA isolation, and quality assessment.

iv. I carried out data analysis and interpretation and finalized the thesis with the aim to prepare few manuscripts to be submitted to peer-reviewed journals in the next future.

It is from January 2013 that the Directive 2010/63/EU, which regulates the use of animals for scientific research and educational purposes, came into force in Member States of the European Union. From hatching to death cephalopod molluscs (i.e., nautiloids, cuttlefish, squid and octopus) are the sole representatives among invertebrates that are now included in the Directive, meaning that all the procedures exceeding the threshold for induction of pain, suffering, distress or lasting

harm carried out on these animals are regulated in an identical way to any other classic vertebrate laboratory animals (Fiorito et al., 2014, 2015). The threshold for regulation is stated in Directive 2010/63/EU as ‘‘any procedure which may cause pain, suffering, distress or lasting harm equivalent to, or higher than that caused by the insertion of a hypodermic needle in accordance with good veterinary practice’’ (see also Cooke et al., 2019).
On the basis of this regulatory framework, all the experiments with live cephalopods require authorization from the National Competent Authority, following national transposition of the EU Directive (in Italy since March 2014).

All experiments that required handling and studies with live animals included in this PhD thesis have been carried out outside of the timing of my PhD project. In particular, studies on protein synthesis inhibition have been carried out between spring and autumn of 1998 and 1999 (G. Fiorito Lab: Di Dato, 2000).

Other samples utilized in Real time qPCR experiments originated from experiments carried out using octopuses caught in the Bay of Naples (Italy) between April 2004 and August 2007 (G. Fiorito Lab: Zarrella, 2011). Finally, other samples have been collected *post-mortem* from killed animals (by fishermen) for the sole purpose of obtaining tissues.
All the above cases fall outside the scope of the Directive 2010/63/EU, provided that an approved method of killing is used.
Whatever killing was required, this was performed by an adequately educated and trained person using an approved method (Andrews et al., 2013; Fiorito et al., 2015).

Thus, the experimental work of my PhD adhered to the principles stated in the Directive 2010/63/EU. As mentioned above, experiments with live octopuses included in this study were carried out before transposition of Directive 2010/63/EU in Italy (i.e. March 2014). Although no authorization was required, all procedures adopted by former fellows of Dr G. Fiorito laboratory have been performed in order to maximize animal welfare and minimize pain and distress of the octopuses included in the experiments (Andrews et al., 2013; Fiorito et al., 2015).

In order to comply to principles stated to the Directive 2010/63/EU we applied for an ethical clearance from the Stazione Zoologica Anton Dohrn Animal Welfare Body; this has been granted as

AWB-SZN case #12/2020 (see 'Gene expression and Learning and memory in *Octopus vulgaris*' at http://www.szn.it/index.php/en/who-we-are/organization/committee-for-the-animal-welfare). In the application we stated the origin of the animals, time of collection of samples, the purpose of the study and the potential publication of these data.

As mentioned above, during this PhD project I had the opportunity to have access to a dataset and samples belonging to the historical data repository relating to a large number of *O. vulgaris* collected and studied for scientific research purposes in the laboratory of Dr Graziano Fiorito at the Stazione Zoologica Anton Dohrn (Napoli, Italy). The animals were fished from the Bay of Naples (Tyrrhenian Sea).

In this sense, this PhD thesis work responds to the principle of historical data analysis, namely, collection and organization of original data, data analysis and their interpretation. In addition, I utilized "historical" samples, assessed for their quality for the purpose of the experiments I carried out.

This "type" of studies is in line with the principles of the 3Rs which are the basis of Directive 2010/63/EU - which came into force in all European countries starting from 1 January 2013 - and as mentioned, includes cephalopod molluscs such as the only representatives among invertebrates to enter the scope of the Directive and of the national legislation on the "protection" of animals for experimental purposes.

The 3R (Replacement, Reduction and Refinement) principle was developed over 50 years ago by Russell and Burch (1959) and represents an important framework for applying more "human" conditions and principles to research involving animals. The three Principles can be summarized as follows (https://www.nc3rs.org.uk/the-3rs).

Replacement = Replacement (methods that avoid or replace the use of animals), i.e. adopt approaches or solicit studies that allow to accelerate the development and use of models and tools, based on the latest sciences and technologies, to address important scientific issues without the use of animals.

Reduction = Reduction (methods that minimize the number of animals used per experiment) and that is to formulate experiments on animals using appropriate evaluations and experimental design and statistical analyses that allow robust and reproducible analyses and that lead to results that actually contribute to increasing knowledge Basic.

Refinement = Refinement (methods that minimize animal suffering and improve welfare), i.e. promoting research on animal welfare by exploiting the latest *in vivo* technologies and improving understanding of the impact of animal welfare on scientific outcomes.

The adoption of the historical data analysis approach for the purposes of this PhD Thesis responds to the 3R principle applied to cephalopod molluscs (Ponte et al., 2019) and in particular to the common octopus *Octopus vulgaris*. In analysing the original dataset and historical data and accessing to samples available at the laboratory of Dr Fiorito and Dr Ponte of the Stazione Zoologica, I certainly applied the principle of Replacement and Reduction, and more generally all three principles.

5. The search of candidate genes underlying learning and memory in octopus

During this PhD project I searched for target gene orthologues to potential 'memory-related' and epigenetic modifiers genes.
The present analysis was possible thanks to the availability of *O. vulgaris* transcriptome (Petrosino, 2015; Petrosino et al., 2015) first, and of *O. vulgaris* genome later (Zarrella et al., 2019).

Almost ten years ago, cephalopod nucleotide sequences available on GenBank were only 5926 (see Zarrella 2011). Less than 2000 of these sequences belonged to cephalopods mostly used in scientific literature, corresponding to about the 3% of the total quota of sequences known for the honeybee at that time.
Thanks to the efforts of Dr G. Fiorito Research Group at the Stazione Zoologica Anton Dohrn (SZN - Napoli, Italy), the transcriptome of *Octopus vulgaris* was already available when this PhD project started.

Using a biased approach, I combined scientific literature with Gene Ontology analysis (Ashburner et al., 2000), and fished the *O. vulgaris* transcriptome for potential gene candidates.
Table 4 provides a tabularized overview of the studies carried out in in various organisms where the involvement in learning and memory, and in the cellular analogue (LTP/LTD) of the genes considered as target for this PhD has been analyzed in great extent.

Table 4. A tabularized overview of the studies carried out in various organisms where the involvement in learning and memory, and its cellular analogue (LTP/LTD), of the genes considered as target for this PhD has been analyzed.

GENE NAME	LEARNING CONDITION	SPECIES	TIMING	EFFECT ON GENE EXPRESSION (method for evaluation)	References	Type of electrophysiological experiment	TIMING	EFFECT ON GENE EXPRESSION	SPECIES	References
CBP	Spatial learning	*Rattus norvegicus*	Immediately after testing. Testing occured 1h after training.	Increased mRNA expression (RT-qPCR)	(Bousiges et al., 2010)					
Kat2b	Spatial learning	*Rattus norvegicus*	Immediately after testing. Testing occured 1h after training.	Increased mRNA expression(RT-qPCR)	(Bousiges et al., 2010)					
Ezh2	Fear conditioning	*Rattus norvegicus*	1 h after retrieval (24h after training)	Increased protein expression	(Jarome et al., 2018)					
PP1	Fear conditioning	*Rattus norvegicus*	1 h after training	Reduced mRNA expression (RT-qPCR)	(Miller & Sweatt, 2007)					
zif268	Fear conditioning	*Rattus norvegicus*	30 minutes after testing (24 hours after training)	Increased mRNA expression (*in situ*)	(Hall et al., 2001)	Slice electrophysiology	30 min to 3 h	Increased mRNA expression (northern blot)	*Rattus norvegicus*	(Mackler et al., 1992); (French, 2001)
Notch	Fear conditioning	*Mus musculus; Rattus norvegicus*	2h after auditory fear conditioning; 10-12 h after light-dark passive avoidance recall	Decreased mRNA expression (RT-qPCR); decreased mRNA expression (RT-qPCR)	(Dias et al., 2014); (Conboy et al., 2007)	Interfering with Notch signalling impairs LTP induction		Notch antisense transgenic mice	*Mus musculus;*	(Wang et al., 2004)

GENE NAME	LEARNING CONDITION	SPECIES	TIMING	EFFECT ON GENE EXPRESSION (method for evaluation)	References	Type of electrophysiological experiment	TIMING	EFFECT ON GENE EXPRESSION	SPECIES	References
PTEN	Fear conditioning	*Rattus norvegicus*	1 h after fear conditioning retrieval (24 h after training)	Reduced protein level expression	(Jarome et al., 2018)	Slice electrophysiology		CA1 PTEN-deficient mice revealed normal LTP, while LTD is impaired	*Mus musculus;*	(Wang et al., 2006)
Htt						LTF induction on sensory-to-motor neuron co-cultures through 5-HT stimulation	incresed expression at 90 min after stimulation	Increased mRNA expression (RT-qPCR, in situ)	*Aplysia californica*	(Choi et al., 2014).
stmn	Fear conditioning	*Rattus norvegicus*	2 days after training	Increased mRNA expression (RT-qPCR)	(Federighi et al., 2013).	*In vivo* electrophysiological experiments;	between 1 and 3 h after LTP induction	Increased mRNA induction of stathmin 4 but not of stathmin (in situ)	*Mus musculus*	(Beilharz et al., 1998).
nrxn1a	Associative scent training	*Apis mellifera*	Immediately after testing. Testing took place the day after the end of a 2-days training.	Increased mRNA expression (RT-qPCR)	(Biswas, 2010)			supports LTP expression. If overexpressed pre-synaptically, along with neuroligin post-synaptic over-expression, they increase LTP synaptic strength		(Choi, 2011)
NTRK2	Spatial learning	*Rattus norvegicus*	At day 6, when highest level of performance was reached	Increased mRNA-Nuclease protection assays	(Gómez-Pinilla et al., 2001).	*In vivo* electrophysiological stimulation	2h after LTP induction	Increased mRNA expression (in situ)	*Mus musculus*	(Bramham et al., 1996)

Transcriptome fishing for gene selection

I first searched from scientific literature key molecules implicated in learning and memory in vertebrates and invertebrates. I searched candidates either by annotation or by Basic Local Alignment Search Tool (BLAST). Among the genes of interest obtained from scientific literature and Gene Ontology analysis (Ashburner et al., 2000), I selected the ones for which orthologues in *O.vulgaris* transcriptome could be found.

I investigated *O. vulgaris* transcriptome available at Stazione Zoologica Anton Dohrn (Napoli- Italy) (Petrosino, 2015; Petrosino et al., 2015) in search of the genes of potential interest.
I filtered my research on longer contigs (minimum transcript length is zif268 with 824bp) showing highest total scores, lowest E-values (inferior to 0.01) and highest percentage of identities (above 80%). As control, I blasted the coding sequence CDS derived from virtual translation of transcript selected (obtained using Virtual Ribosome http://www.cbs.dtu.dk/services/VirtualRibosome/) in search of the corresponding orthologues protein sequences in *Octopus bimaculoides, Mus musculus, Drosophila melanogaster, Caenorhabditis elegans.* Only the transcripts presenting high similarity (Query cover>80% and E-value inferior to 0.01) with model organisms were selected for the present study.

My search allowed me to identify octopus' orthologues of epigenetically regulated memory-related genes (n = 4; Table 5), genes potentially related to memory formation (n = 5, Table 6) and epigenetic modifiers (n = 15 genes; Table 7).

Short description of genes of interest

Hereunder I will provide a short description of the genes target of this study.

EPIGENETICALLY REGULATED MEMORY-RELATED GENES

I considered: memory suppressor PP1 regulating and being regulated by the epigenetic machinery; the well-studied immediate early gene, zif268, regulated by histone acetylation; Notch and Pten, both regulated by histones methylation.

Octopus orthologues of epigenetically regulated memory-related genes are listed at Table 5.

Serine/threonine-protein phosphatase PP1 (PP1)

Memory formation is associated with various epigenetic modifications triggering the transcriptional regulation that sustains its formation (Levenson & Sweatt, 2005).

Protein phosphatase 1 (PP1) acts as a suppressor of fear memory; it is a pivotal regulator of chromatin remodeling, defining histone post-translational modifications and gene transcription associated with long-term memory, at least in the mammalian brain (Genoux et al., 2002; Koshibu et al., 2011; Miller & Sweatt, 2007).

PP1 is a negative regulator of long term-memory and synaptic plasticity and its inhibition enhances LTP, strengthens associative training, and increases memory endurance (Blitzer et al., 1998; Jouvenceau et al., 2006; Genoux et al., 2002, Koshibu, 2011).

PP1 is mainly present in the nucleus, where it forms multimeric holoenzymes that regulate gene transcription (Bennett, 2005; Koshibu,2009) but its cytoplasmatic fraction is also involved in translation initiation and splicing (Bennett, 2005; Mansuy and Shenolikar, 2006; Moorhead et al., 2007).

It regulates transcription driving histones post-translational modification. It has been observed to drive histone 3 (H3) dephosphorylation and interacting with histone deacetylases and demethylases during long-term memory formation in mouse (Koshibu, 2009).

The neuron selective and reversible inhibition of PP1, in transgenic mice expressing the nuclear inhibitor of PP1 under the control of a forebrain (hippocampal and cortical structures) specific promoter, brought not only to H3 phosphorylation, but also to histone acetylation and methylation in the hippocampus (Koshibu, 2009). PP1 inhibition *in vivo* caused the increased phosphorylation of H3S10, marker induced in contextual fear learning. Furthermore, it determined an increase in H2B, H3K14, and H4K5 acetylation and H3K36 trimethylation. Changes in histone post-translational modifications (H3S10 phosphorylation, H3K14 and H4K5 acetylation, and H3K36 trimethylation) were observed at CREB promoter, a fear memory-related gene, whose mRNA expression was induced (Koshibu, 2009).

Further experiments proved the role of PP1 in memory recall after fear conditioning.

PP1 expression is finely regulated following fear conditioning in mice. The transcription rate of this gene was observed to decrease one hour after fear conditioning training. This down-regulation was associated with increased PP1 promoter DNA methylation, tested through methylation-specific

quantitative real-time PCR. Along with DNA methylation at gene promoter, several other epigenetic modifications promptly provided the gene silencing. Among these, histones acetylation and methylation. However, twenty-four hours after training PP1 expression level had returned to baseline (Koshibu et al., 2009; Miller & Sweatt, 2007).

The role in long-term memory was evidenced by the transgenic PP1 inhibition *in vivo*, which showed selectively enhanced long-term fear memory in mice (24h after fear conditioning) but had no effects in the short-term (10minutes after fear conditioning). This inhibition determined the enhancement of LTP in a transcription-dependent manner (preincubation in transcription inhibitor actinomycin D blocked the LTP increase; Koshibu,2011).

The role of PP1 in LTP seems to be specifically related to the late phase of Long Term Potentiation. Protein phosphatases activation by low-frequency stimulation before the induction of LTP on slices, selectively reduced its late phase, without affecting the early phase. This depression could be blocked by protein phosphatase inhibitors (Woo, 2002). This effect might depend on PP1 dephosphorylating action on CREB or glutamate receptors, but further studies are needed to prove this hypothesis.

In sum, PP1 represents a key molecule involved in fear memory, synaptic plasticity and chromatin remodeling. Learning abilities may depend on levels of expression of specific genes, as PP1. Animals exhibiting reduced learning abilities, and therefore tending to forget spatial learning tasks, showed a higher expression level of PP1 24h after the last spatial training trial (Haege et al., 2010).

PP1 expression is reduced during the consolidation of fear memory in rodents, but there are no data about its expression during reconsolidation process, to the best of my knowledge.

In addition to a potential role of PP1 in memory reconsolidation, spatial learning trials showed that individuals' performance could be related to PP1 expression level. This evidence induces me to wonder if this is also true for other forms of memory, such as fear memory.

Early growth response protein 1 (Egr1-b, zif268)

The early growth response protein 1 (Egr1-b) - also known as zif268, Krox24, NGFI-A, TZs8, and Zenk - belongs to the Egr family. It is an immediate early gene, upregulated in rodents during memory consolidation after fear conditioning through histone modifications (Gupta, 2010; Baumgartel, 2008). Zif268 is a transcription factor, regulating hundreds of targets, as demonstrated by the fact that its overexpression can drive the regulation of 153 genes mainly involved in signaling and synapse formation. Its expression is finely and rapidly controlled by several mechanisms that include

its own transcriptional suppression (Veyrac, 2014). Many Authors report of zif268 regulated transcription during fear memory formation (Gupta et al., 2010; Malkani & Rosen, 2000).

In Malkani & Rosen (2000) and among the members of the Egr family, only zif268 overexpression was observed in the lateral nucleus of the amygdala, but not in the hippocampus, after one-trial contextual fear conditioning in rats. The increase started after 15 minutes, peaked at 30 minutes and was still present one hour later. However, the evidence that transcriptional induction was only observed after training, but not after testing memory retrieval 24 hours later, drove scientists to suppose a role for zif268 in memory consolidation but not in memory retrieval.

This hypothesis was counteracted when a role for zif268 in fear memory retrieval has been found (Hall 2001). In this work, zif268 expression enhancement was noted in CA1 neurons of the hippocampus 30 minutes after testing phase (24 hours after training). This increase was not observed for old (28 days) contextual memories (Hall et al., 2001).

Furthermore, Maddox (2011) show zif268 involvement during memory acquisition and consolidation, but also for retrieval of auditory fear memories. Using auditory fear paradigm, training-related changes in gene expression was observed, independently from a novel context exposure.

Zif268 expression increase resulted during consolidation and appeared not to be influenced by novelty (Hall et al., 2000). Maddox and co-workers showed that zif268 knockdown by antisense oligonucleotides interfered with the reconsolidation process, 24 h after fear conditioning, whereas no effect was observed on acquisition and short-term memory (Maddox et al., 2011).

This immediate early gene is a crucial element in synaptic plasticity. Since early 1990s it was known that neuronal stimulation *in vivo* could enhance the transcription of several genes including zif268 (Abraham 1993). After 30–60 minutes from the LTP induction at mice dentate gyrus, zif268 mRNA was strongly up-regulated and this increase was NMDA-dependent. Furthermore, the greater was the zif268 increase, the longer was the synaptic potential maintained. This means that the gene up-regulation is related to LTP maintenance, rather than to LTP induction (Abraham et al., 1993; Cole et al., 1989; Wisden et al., 1990). Accordingly, zif268 mutants had no altered LTP induction or early phase, but a more rapid extinction (Jones, 2001).

However, slice preparations provided different results: when LTP was induced in slices of CA1, zif268 expression level did not show alterations (French, 2011). This result appeared in contrast with

previous experiments that showed a 2-fold zif268 up-regulation following LTP induction (Mackler et al., 1992).

The expression of the transcription factor zif268 is controlled by several epigenetic regulators that include P300/CBP complex (zif268 promoter contains CRE sites) and histone methyltransferases (Gupta et al., 2010; Veyrac et al., 2014).

Neurogenic locus Notch protein (Notch)

Notch gene was initially described in *Drosophila melanogaster;* it encodes a 300kDa polypeptide, cleaved in Golgi apparatus into the functionally active 200kDa single-pass transmembrane receptor. Notch activation (by the membrane-bound ligands Jagged and Delta) results in the cleavage of the receptor transmembrane domain and the release of the Notch intracellular signaling domain (NICD) acts as a transcription factor activating transcription of a group of transcriptional repressors that hamper neuronal differentiation. Notch contributes to stem cell pool maintenance (Artavanis-Tsakonas et al., 1999; Conboy et al., 2007).

Notch-like proteins have been observed from invertebrates to vertebrates (Artavanis-Tsakonas et al., 1999).

During development Notch signaling plays a well-documented role in establishing binary cell-fate determination, its expression in adult non-dividing cells of CNS has to be elucidated.

Several studies proved a role for Notch in long-term memory consolidation.

The blocking of the Notch signaling results in the impairment of memory consolidation without affecting acquisition process in both *Drosophila* and, similarly, in mice heterozygous for Notch null mutation (Costa et al., 2003; Ge et al., 2004; Presente et al., 2004). In the latter, mice that had just acquired a spatial learning task were able to perform the task during training but were unable to consolidate the memory and recall it during testing.

Notch signaling is also important to sustain LTP. In mammalian slice preparation from mice with antisense-reduced hippocampal Notch-1 mRNA and protein levels, paired-pulse facilitation was observed, but not LTP (Wang et al., 2004).

Mice trained for a light-dark passive avoidance learning protocol showed Notch mRNA expression downregulation 10-12 h after training, when memory was tested again, then animals were immediately sacrificed (Conboy et al., 2007). It is not known if Notch mRNA downregulation is

present 1h after testing (24 h post training). However, other studies observed the RNA downregulation 2 h after testing phase occurring 24 h after auditory fear conditioning training.

Studies shown downregulation of mRNA and protein levels of Notch receptor and ligands in the mice amygdala and dependent on micro-RNA regulation (Dias et al., 2014). However, Notch can also be regulated by histones modifications laid down by Ezh2 and Ring-1 in mice (Jarome et al., 2018; Román-Trufero et al., 2009).

The need for transcriptional blocking of the Notch pathway was further confirmed through Notch pathway activation using an anti-Notch-1 ligand, that brought to amnesia when memory was tested 10h after training (Conboy et al., 2007). As far as Notch signaling in non-dividing neurons is associated with regression of neural connections, Conboy and coworkers suggested that the pathway down-regulation during memory consolidation may favor the neurite outgrowth and stabilize new connection transiently produced after training acting on cytoskeleton rearrangements.

Phosphatidylinositol 3-4-5- tris phosphate 3-phosphatase and dual specificity protein phosphatase PTEN (PTEN)

PTEN phosphatase is a protein and lipid phosphatase and a potent tumor suppressor. As a lipid phosphatase in the cytoplasm PTEN dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3), driving the opposite reaction of the phosphatidylinositol 3-kinase (PI3K) in the PI3K/AKT/TOR signaling pathway, an essential regulator of protein transcription and translation, cell cycle progression. It is also implicated in nervous system development, learning and memory (Raught et al., 2001).

As a protein phosphatase PTEN dephosphorylates, therefore inactivates, the transcription factor CREB. The colocalization and physical association of these molecules have been experimentally proven (Gu et al., 2011).

Pten is also involved in the Notch pathway, contributing to the regulation of stem cell pool maintenance. Notch activation leads to reduced Pten expression. This downregulation is observed in adult neurogenesis and it is guided by histone modifications (Hill & Wu, 2009; Zhang et al., 2014). Furthermore, PTEN is involved in the control of chromosome stability (Shen et al., 2007) and transcriptional regulation (Freeman et al., 2003; Gu et al., 2011; Mayo et al., 2002).

During reconsolidation in mammals, PI3K/AKT/mTOR pathway is activated and ultimately brings to mTORC1-mediated protein synthesis (Jarome et al., 2018). This pathway has been widely reported as crucial in learning-dependent synaptic plasticity in neurons (Parsons et al., 2006; Gafford et al., 2011; Jobim et al., 2012; Mac Callum et al., 2014).

The pathway is highly conserved and it has been described in several invertebrates (Raught et al., 2001; Soulard et al., 2009). Similarly to what occurs in vertebrates for LTP, in *Aplysia* rapamycin inhibits LTF. TOR (Target of rapamycin) signaling is implicated in neural protein synthesis occurring in the mollusk, following stimulation (Casadio et al., 1999; Tang et al., 2002).

Pten, negatively regulates the PI3K/AKT/mTOR pathway and represents a pivotal regulator of the transcriptional and translational regulation that occurs in neurons during reconsolidation. During this period, Ezh2 lays down H3K27me3 trimethylation that switches off Pten transcription allowing the activation of the PI3K/AKT/mTOR pathway (Jarome et al., 2018).

Rats trained in a fear conditioning protocol were tested 24h after training; brains collected 1h and 24h after testing. Authors reported Enhancer of Zeste Homolog 2 (EZH2) protein levels upregulated 1h after testing along with the increase of the product of the enzyme activity, the trimethylation of histone H3 on lysine 27 (H3K27me3). Pten was downregulated 1h after fear memory retrieval in hippocampus. The expression level of both Ezh2 and Pten returned to baseline 24h after testing. The transcriptional silencing histone mark was observed along with DNA methylation at Pten promoter exclusively after retrieval of new memory, but not after training, nor after retrieval of remote memory. These events were associated with increased AKT and TOR phosphorylation (Jarome et al., 2018). However, the Authors did not exclude other possible pathways through which Pten could potentially regulate fear memory retrieval.

Pten participates to LTP and LTD and seems to have a crucial role in LTD induction. Electrophysiology experiments of CA1 PTEN-deficient mice revealed normal LTP, while LTD is impaired. During LTD Pten antagonizes PIP3 upregulation (Wang et al., 2006).

To my knowledge, no data are currently available about PTEN expression level following LTP or LTD induction.

Table 5. Epigenetically regulated memory-related genes identified in *O. vulgaris* transcriptome.

Gene name	Protein	Contig code	Uniprot HSP	Gene Ontology	Function
PP1	Serine/threonine-protein phosphatase PP1	c32984_g1_i1	P48488	phosphoprotein phosphatase activity GO:0004721	PP1 is a memory repressor and regulator of chromatin remodeling in the mammalian brain that controls histone PTMs and gene transcription associated with long-term memory. DNA methylation at the PP1 gene is enhanced following fear conditioning (Day and Sweatt 2011b).

Gene name	Protein	Contig code	Uniprot HSP	Gene Ontology	Function
zif268	Early growth response protein 1-B	c32124_g2_i2	Q6NTY6	regulation of transcription, DNA-templated GO:0006355	Increased expression level of Zif268 have been reported 24h after memory recall and this depends on DNA methylation and histone modifications (S. Gupta et al. 2010)(Bousiges et al. 2010)B
Notch	Neurogenic locus Notch protein	c30967_g5_i1	P07207	long-term memory GO:0007616, chromatin binding GO:0003682	Notch is involved in the regulation of neural ultrastructure during development, but it is also required for memory consolidation, which is believed to require remodeling of existing neurons in adults. disruption of Notch1 in CA1 of the postnatal hippocampus reveals that Notch signaling is required to maintain spine density and morphology, as well as to regulate synaptic plasticity and memory formation (Alberi et al. 2011).
PTEN	Phosphatidylinositol 3-4-5- tris phosphate 3-phosphatase and dualspecificity protein phosphatase PTEN (PTEN)	c36443_g6_i1	Q9PUT6	regulation of neuron projection development GO:0010975	Pten plays a role as a key modulator of the AKT-mTOR signaling pathway controlling adult neurogenesis, including correct synapse formation. Abnormal activation of the PI3K/AKT pathway in specific neuronal populations can underlie macrocephaly and behavioral abnormalities in rodents (Kwon et al. 2006)

GENES POTENTIALLY INVOLVED IN MEMORY FORMATION

I considered four genes, known to participate in different ways to learning and memory processes. Huntingtin is induced by neural activation and contributes to sustain long-term events; stathmin and neurexin are involved in the structural reorganization in neural cells by microtubule disassembly and new synapse formation. Finally, neurotrophins receptor supports neurons survival and might influence the animal motivational drive.

O. vulgaris orthologues of genes potentially related to memory formation are summarized at Table 6.

BDNF/NT-3 growth factors receptor (NTRK2)

Neurotrophins are evolutionarily conserved growth factors that participate during nervous system development, to the control of neuron number, and also contribute to learning and memory in adults (Minichiello, 2009).

Neurotrophins receptors (Trk receptors) are a very ancient protein family originated more than 600 million years ago, when the mollusks phylum separated from vertebrates.

Neurotrophins and their receptors have been considered a marker of higher organisms' neural systems complexity. In line with this hypothesis, it was observed that their distribution followed evolution complexity; accordingly, invertebrates such as *Caenorhabditis elegans* and *Drosophila,* with their simple nervous systems, did not show neurotrophins and their receptor homologs in their genomes.
Surprisingly, in molluscs a Trk homolog has been identified (Jaaro et al., 2001; Jaaro & Fainzilber, 2006).

In *Octopus vulgaris* transcriptome, I identified a putative BDNF/NT-3 growth factor receptor (TrkB, NTRK2).

Among neurotrophins, BDNF and NT-3 mRNA have been found to be up-regulated 4h after LTP induction in mice hippocampal slice (Patterson et al., 1992). *In vivo* electrophysiological experiments showed that not only neurotrophins but also their receptors, in particular the TrkB and TrkC mRNA expression, increased 2h after LTP induction in dentate gyrus, and returned to baseline after 6h (Bramham et al., 1996).
BDNF induction and TrKB activation have been observed in mice following fear conditioning (Rattiner, 2004), and BDNF and TrkB mRNA induction were observed in social recognition memory formation (Broad et al., 2002). The NTRK2 orthologue induction was also observed in rats following spatial learning (Gómez-Pinilla et al., 2001).
Although the activation of the neurotrophin signaling depends on the receptor phosphorylation, the evidence for the mRNA up-regulation has been observed after neural activation by LTP induction in mice (Bramham et al., 1996).

Huntingtin (Htt)

Huntingtin is a highly conserved protein, that is well-known for the syndrome that it causes when mutated, the Huntington disease. The syndrome is caused by a polyglutamine pathological expansion within Huntingtin protein that disrupts its normal biological function and its protein-protein interactions (Proskura et al., 2017). Patients with Huntington's disease show motor disturbances, but they also exhibit memory and cognitive deficits along with synaptic structure and plasticity alterations in the hippocampus many years before the former (Choi et al., 2014; Proskura et al., 2017). Huntingtin function has not been completely understood.

Literature indicates that it might be involved in microtubule-mediated transport of vesicles following LTP, functioning both as a scaffold, for the binding of other proteins, but also actively defining the transport direction and contributing to efficiency of synaptic transmission (Proskura et al., 2017).

The role of Htt in long-term maintenance of the synaptic transmission efficiency appears conserved, since contributing to the LTF in *Aplysia* and to the LTP in mice hippocampus.

Furthermore, Htt over-expression drives bdnf increase and, since Htt interacts with CBP, it could be implicated in neural-activity dependent transcription regulation (Choi et al., 2014).

The expression levels of Htt itself is dependent on neural activation. Studies in *Aplysia* reported that transcription of Htt mRNAs is induced by repeated applications of serotonin, a modulatory transmitter that is able to drive facilitation and that is released during learning. Htt showed a delayed expression, not still visible after 30minutes from serotonin treatment, but significative at 90 minutes after the end of the five pulses treatment (Choi et al., 2014).

Stathmin (stmn)

Stathmin (or Oncoprotein 18) is a neuronal growth-associated protein that regulates microtubule dynamics.

Learning and memory depend on the ability of the neural system to rearrange synaptic connections architecture, which in turn depends on cytoskeleton dynamics.

Stathmin is a cytosoluble phosphoprotein, involved in microtubule destabilization by binding tubulin dimers and inhibiting the tubulin subunits polymerization (Larsson et al., 1999).

Several studies proved that the cytoskeleton rearrangements prompted by stathmin are essential for learning and long-term memory (Hayashi et al., 2006; Martel et al., 2016; Nelson et al., 2004; Uchida & Shumyatsky, 2015).

Studies on stathmin knockout mice revealed that induction of LTP is impaired in slices derived from mice where this protein is not expressed. Furthermore, behavioral studies proved that fear memory is impaired in stathmin-KO animals (Shumyatsky et al., 2005).

A recent study focusing on post-traumatic stress disorders proved that stathmin mRNA tends to decrease following stressing events and the more intense is the stress, the lower the down-regulation as seen during first 7 days following the stressful event (Shan et al., 2020).

The consolidation of fear memory in mice triggered Stathmin-1 up-regulation when memory was tested 2 days after fear conditioning (Federighi et al., 2013).

The mRNA of the Stathmin-4, but not of Stathmin-2 has been observed to be up-regulated between 1 and 2 h following *in vivo* LTP induction in mice hippocampus (Beilharz et al., 1998).

Events that trigger neural activation, seem to induce members of Stathmin family mRNA expression.

Neurexin 1-a (nrxn1a)

Neurexins are presynaptic transmembrane proteins that control cell-adhesion and synaptic interactions on neuron surface. Their major interacting partner is represented by neuroligins. Neurexins and neuroligins are essential for long-term facilitation and learned fear in *Aplysia*. In human these molecules are mutated in some patients affected by autism, and may be implicated in emotional memory (Choi et al., 2011). Some neurexin isoforms are involved in calcium-dependent neurotransmitter release (Missler et al., 2003).

Synaptic plasticity diminishes with aging, and neurexin mRNA expression follows this decline in the hippocampus of mice(Kumar & Thakur, 2015).

Furthermore, associative scent training in the honeybees can induce neurexin expression (Biswas et al., 2010).

Sodium-dependent dopamine transporter (DAT)

Sodium-dependent dopamine transporter (DAT) is involved in dopamine re-uptake. DAT is member of the Na+/Cl- dependent neurotransmitter transporter family and is a membrane symporter that clears DA from the synaptic cleft and serves as an important regulator of signal amplitude and duration at the dopaminergic synapses (reviewed by Torres, Gainetdinov, and Caron 2003).

When DAT function is impaired, the prolonged extra-cellular lifetime of DA brings to disruption of normal locomotor activity as well as deficits in several cognitive and behavioral processes in both vertebrate and invertebrate organisms.

Mice knockout (DAT KO) or knockdown (DAT KD) for *DAT* displayed a distinct behavioral phenotype which comprises novelty induced hyperactivity, decreased habituation, locomotor activity dysregulation, lactation and maternal behavior deficits in the females, impairments in learning and memory of place preference and instrumental conditioning tasks (reviewed by Torres, Gainetdinov, and Caron 2003). Rats subjected to pharmacological treatments (*i.e.* cocaine, RTI-336) inhibiting the DAT function showed that increased inhibition of DAT activity contributed to deficits in some cognitive processes as conditional place preference tasks (Medvedev et al., 2005).

Table 6. Genes potentially related to memory, LTP and motivational drive identified in *O. vulgaris* transcriptome.

Gene name	Protein	Contig code	Uniprot HSP	Gene Ontology	Function
stmn	Stathmin	c33500_g9_i1	C6K2V8	regulation of microtubule polymerization or depolymerization GO:0031110	Stathmin is a neuronal growth-associated protein involved in the regulation of the microtubule (MT) filament system by destabilizing microtubules. Prevents assembly and promotes disassembly of microtubules. Involved in the control of the learned and innate fear
Htt	Huntingtin	c34384_g2_i1	P42859	learning or memory GO:0007611, negative regulation of neuron death GO:1901215	Huntingtin could be involved in transcriptional regulation of genes since it interacts with CBP (Choi et al. 2014). The expression of mRNAs of huntingtin is upregulated by repeated applications of serotonin, a modulatory transmitter released during learning in Aplysia (Choi et al. 2014).
NTRK2	BDNF/NT-3 growth factors receptor	c17731_g1_i1	Q91987	learning GO:0007612	NTRK2 plays a role in learning and memory by regulating both short term synaptic function and long-term potentiation. The gene is also related to depression in human (Uriguen et al. 2008).
nrxn1a	Neurexin 1-a	c35788_g1_i2	A1XQX0	cell adhesion GO:0007155	Neurexins are essential presynaptic cell-adhesion molecules.
DAT	Sodium-dependent dopamine transporter	c33231_g8_i1	Q7K4Y6	dopamine uptake involved in synaptic transmission GO:0051583	DAT is a membrane symporter that clears DA from the synaptic cleft and serves as an important regulator of signal amplitude and duration at the dopaminergic synapses. KO mice models evidenced its role in learning and behavior.

EPIGENETIC MODIFIERS

I considered several histones modifiers, i.e. histones acetyltransferases and histones methyltransferases and histones demethylase.

HATs: CREB-binding protein (CREBBP, CBP) and P300/CBP-associated factor (PCAF, Kat2b)

The three HATs mostly studied for their role on learning and memory are E1a-associated protein (p300), CREB binding protein (CBP), and p300-CBP-associated protein (PCAF). Fishing the *Octopus vulgaris* RNAseq data I identified two putative transcripts with high homology to CBP and PCAF.

CREB-binding protein is a transcription coactivator in neurons, having an essential role in long-term facilitation in *Aplysia* and in long-term fear memory formation in mice (Alarcón et al., 2004; Barrett & Wood, 2008; Bousiges et al., 2010a; Guan et al., 2002; Korzus et al., 2004).

CBP has a dual role in transcription activation; on one side, it alters directly chromatin structure through its acetyltransferase activity, while, it works as a molecular scaffold, recruiting other coactivators of the transcriptional machinery, on the other (Guan et al., 2002; Korzus et al., 1998).

It contributes to the activation of several genes essential to memory formation such as zif268, PP1, huntingtin, C/EBP (Bousiges et al., 2010a; Guan et al., 2002; J. M. Levenson & Sweatt, 2006; Veyrac et al., 2014). The first evidence of a role for CBP in memory derived from the characterization of Rubinstein-Taybi Syndrome (RTS), in which this gene is mutated. RBT patients show developmental abnormalities and mental retardation (Rubinstein and Taybi 1963);(Barrett & Wood, 2008).

Haploinsufficient mice (cbp + /-), used as a model for RBT syndrome, displayed reduced chromatin acetylation level and deficits in long-term memory. Furthermore, slice electrophysiology on cbp +/- mice hippocampi showed a deficient L-LTP. Since only L-LTP was impaired, while E-LTP was normal, CBP is probably involved in long-term synaptic plasticity (Alarcón et al., 2004).

Experiments using hippocampal slice preparation corroborated the observation that the haploinsufficient animals exhibited impaired long-term fear memory, tested 24 hours after training (Alarcón et al., 2004).

CBP is a conserved histone acetyltransferase and has an important role in synaptic plasticity in invertebrates. In the mollusk *Aplysia*, CBP binds to phosphorylated CREB and allows CREB-mediated gene transcription relaxing chromatin structure through its histone acetyltransferase activity. The CBP recruitment at gene promoter to form CREB-CBP complex occurred between 15 mins to 2 hours after serotonin stimulation (Guan et al., 2002).

In spatial memory formation, not only the activity but also CBP and PCAF mRNA expression levels increased in mice hippocampus during consolidation (Bousiges et al., 2010a).

Kat2b or PCAF, a histone acetyltransferase (HAT)

LTP is strictly dependent on HATs activity: the PCAF activity inhibition, impairs LTP induction (Wei et al., 2012); while the inhibition of HDACs that catalyze the opposite reaction enhances LTP (Jonathan M. Levenson et al., 2004; Vecsey et al., 2007).

Recent evidences shown that PCAF plays an essential role in auditory fear memory extinction, partially mediated by zif268 expression down-regulation. PCAF transcription level increased in mice during consolidation of spatial memory (Bousiges et al., 2010a).

Polycomb repressive complexes and other epigenetic modifiers

Polycomb repressive complexes (PRC), are an evolutionarily conserved family of proteins discovered in *Drosophila,* but then studied in vertebrates and invertebrates, including *Octopus vulgaris.* The PRC more extensively described in the literature are PRC1 and PRC2 that control gene expression laying down post-translational histones modifications (Imperadore et al., 2017; Liu et al., 2015).

PRC2 trimethylates histone 3 on lysine 27 (H3K27), forming a binding site that allows the recruitment of PRC1 which modifies histone H2A laying mono-ubiquitination of lysine 119. The chromatin modifications both bring to gene silencing (Cao et al., 2005).

PRC1 has been associated with the expression of a schizophrenia-related gene that mediates fear-induced anxiety-like behavior(Spadaro et al., 2015).

The role of PRC2 in neuronal specification during development has been widely recognized (Corley and Kroll, 2015), and recently recognized in prevention of neuron neurodegeneration (review in Cholewa-Waclaw et al., 2016). Along with the maintenance of adult neuron specification, PRC2 contributes to the silencing of a transcription program that impairs neuronal function and survival an is associated with neurodegenerative decline in humans (Cholewa-Waclaw et al., 2016; von Schimmelmann et al., 2016).

From *O. vulgaris* transcriptome several PRCs were identified: the polycomb group RING finger protein 1 (Ring1) and the polycomb complex protein (bmi1a), participating to the PCR1 and the histone-lysine N-methyltransferase (Ezh2), the polycomb protein (Eed) and the polycomb protein (Suz12), taking part to the PRC2.

Ezh2 and Ring-1 have been observed to regulate Notch signaling in mice (Jarome et al., 2018; Román-Trufero et al., 2009).

Furthermore, Ezh2 expression was observed to increase 1h after fear memory retrieval in mice (Jarome et al., 2018).

I selected four more methyltransferases with different roles on transcription activation:

- Kmt5aa, that monomethylates 'Lys-20' of histone H4 bringing transcriptional repression
- Prmt1 that acts on the guanidino nitrogens of arginyl residues determining monomethylation and dimethylation of histone H4 'Arg-4', activating gene transcription and

- Ash2L, a component of the Set1/Ash2 histone methyltransferase (HMT complex) and is involved in methylation and demethylation at 'Lys-4' of histone H3 reducing chromatin condensation and promoting gene transcription
- Kmt2c that methylates 'Lys-4' of histone H3 activating gene transcription.

H3K4me3 mark is an important marker of gene transcription laid down in the rat brain in response to fear conditioning and contributing to zif268 and other essential learning-related genes upregulation (Gupta et al., 2010)

I also selected a demethylase, the histone demethylase Kdm6a that acts removing methyl groups from trimethylated and dimethylated 'Lys-27' of histone H3 (Li et al., 2007; Hon et al., 2009; Zhou et al., 2011).

Table 7. Putative Epigenetic modifiers identified in *O. vulgaris* transcriptome.

Gene name	Protein	Contig code	Uniprot HSP	Gene Ontology	Function
CBP	CREB-binding protein	c36014_g6_i3	Q92793	histone acetyltransferase activity GO:0004402	Acetylates histones, giving a specific tag for transcriptional activation. Binds specifically to phosphorylated CREB and enhances its transcriptional activity toward cAMP-responsive genes. Its mRNA expression levels are increased during spatial memory consolidation in mice (Bousiges et al. 2010).
Kat2b	Ov-Kat2b/Histone acetyltransferase KAT2B	c32349_g6_i3	Q92831	chromatin remodeling GO:0006338 positive regulation of gene expression, GO:0045815	histone acetyltransferase (HAT), promotes transcriptional activation. Its mRNA expression levels are increased during spatial memory consolidation in mice (Bousiges et al. 2010).
Ring1	Ov-Ring1/Polycomb group RING finger protein 1	c27058_g1_i1	Q7ZYZ7	chromatin silencing GO:0006342	Component of a Polycomb group (PcG) multiprotein PRC1-like complex, acts via chromatin remodeling and modification of histones; it mediates monoubiquitination of histone H2A 'Lys-119', rendering chromatin heritably changed in its expressibility
Bmi1a	Ov- bmi1a/Polycomb complex protein BMI-1-A	c27499_g1_i1	Q8JIR0	chromatin silencing GO:0006342	Component of a Polycomb group (PcG) multiprotein PRC1-like complex, a complex class required to maintain the transcriptionally repressive state of many genes.
Eed	Ov-Eed/Polycomb protein EED	c36347_g1_i1	Q5ZKH3	chromatin silencing GO:0006342	Polycomb group (PcG) protein. Component of the PRC2/EED-EZH2 complex, which methylates 'Lys-9' and 'Lys-27' of histone H3, leading to transcriptional repression of the affected target gene
Suz12	Ov-Suz12/Polycomb protein suz12	c36350_g1_i1	Q0VA03	histone methylation GO:0016571	Polycomb group (PcG) protein. Component of the prc2/eed-ezh2 complex, which methylates 'Lys-9' (H3K9me) and 'Lys-27' (H3K27me) of histone H3, leading to transcriptional repression of the affected target gene.

Gene name	Protein	Contig code	Uniprot HSP	Gene Ontology	Function
Setd3	Ov-Set3/Histone-lysine N-methyltransferase setd3	c28491_g1_i1	B0VX69	positive regulation of transcription, DNA-templated GO:0045893	Protein-histidine N-methyltransferase that specifically mediates methylation of actin at 'His-73'.
Ezh2	Histone-lysine N-methyltransferase EZH2	c33426_g7_i2	Q61188	positive regulation of dendrite development GO:1900006 positive regulation of MAP kinase activity GO:0043406	Polycomb group (PcG) protein. Catalytic subunit of the PRC2/EED-EZH2 complex, which methylates (H3K9me) and 'Lys-27' (H3K27me) of histone H3, leading to transcriptional repression of the affected target gene. H3K9me2 are dynamically regulated in the rat brain in response to fear conditioning and are responsible for zif upregulation (S. Gupta et al. 2010).
Ehmt1	Ov-Ehmt1/Histone-lysine N-methyltransferase EHMT1	c32135_g11_i2	Q5DW34	negative regulation of transcription, DNA-templated GO:0045892	Histone methyltransferase that specifically mono- and dimethylates 'Lys-9' of histone H3 (H3K9me1 and H3K9me2, respectively) in euchromatin. H3K9me represents a specific tag for epigenetic transcriptional repression by recruiting HP1 proteins to methylated histones. Adult Drosophila G9a mutants (EHMT1 ortholog) have defects in learning, short- and long-term memory (Anreiter et al. 2017). Pten is downregulated by Ezh2 (J. Zhang et al. 2014)
Kmt5b	Ov-Kmt5b/Histone-lysine N-methyltransferase KMT5B /Histone-lysine N-methyltransferase SUV420H1	c29821_g13_i1	Q29RP8	histone H4-K20 trimethylation GO:0034773	Histone methyltransferase that specifically trimethylates 'Lys-20' of histone H4. H4 'Lys-20' trimethylation represents a specific tag for epigenetic transcriptional repression. Mainly functions in pericentric heterochromatin regions, thereby playing a central role in the establishment of constitutive heterochromatin in these regions. KMT5B is targeted to histone H3 via its interaction with RB1 family proteins.
Kmt5a.A	N-lysine methyltransferase SETD8-A/Ov-kmt5a.A/N-lysine methyltransferase KMT5A-A	c29821_g13_i1	Q071E0	positive regulation of gene expression GO:0010628	Protein-lysine N-methyltransferase that monomethylates both histones and non-histone proteins. Specifically monomethylates 'Lys-20' of histone H4 (H4K20me1). H4K20me1 is enriched during mitosis and represents a specific tag for epigenetic transcriptional repression. Mainly functions in euchromatin regions, thereby playing a central role in the silencing of euchromatic genes.
Kmt2c	Ov-Kmt2c/Histone-lysine N-methyltransferase 2C	c30253_g14_i1	Q8BRH4	positive regulation of transcription by RNA polymerase II GO:0045944	Histone methyltransferase. Methylates 'Lys-4' of histone H3. H3 'Lys-4' methylation represents a specific tag for epigenetic transcriptional activation. H3K4me3 are dynamically regulated in the rat brain in response to fear conditioning and are responsible for bdnf and zif upregulation (S. Gupta et al. 2010)
Ash2l	Ov-Ash2l/Set1/Ash2 histone methyltransferase complex subunit ASH2	c30280_g8_i5	Q9UBL3	positive regulation of transcription by RNA polymerase I GO:0045944	Component of the Set1/Ash2 histone methyltransferase (HMT) complex, a complex that specifically methylates 'Lys-4' of histone H3, but not if the neighboring 'Lys-9' residue is already methylated. As part of the MLL1/MLL complex it is involved in methylation and dimethylation at 'Lys-4' of histone H3. H3K4me3 are dynamically regulated in the rat brain in response to fear conditioning and are responsible for bdnf and zif upregulation (S. Gupta et al. 2010).

Gene name	Protein	Contig code	Uniprot HSP	Gene Ontology	Function
Kdm6a	Ov-Kdm6a/Lysine-specific demethylase 6A	c36159_g6_i3	O15550	chromatin remodeling GO:0006338 positive regulation of gene expression GO:0010628	Histone demethylase that specifically demethylates 'Lys-27' of histone H3, thereby playing a central role in histone code.
Prmt1	Ov-Prmt1/Protein arginine N-methyltransferase 1	c33776_g15_i2	Q28F07	Neurogenesis GO:0022008 positive regulation of transcription, DNA-templated GO:0045893	Arginine methyltransferase that methylates (mono and asymmetric dimethylation) the guanidino nitrogens of arginyl residues present in target proteins. Constitutes the main enzyme that mediates monomethylation and asymmetric dimethylation of histone H4 'Arg-4' (H4R3me1 and H4R3me2a, respectively), a specific tag for epigenetic transcriptional activation.

Transcriptome Data Analysis

For the aims of this PhD, I analyzed *O. vulgaris* transcriptome and explored the relative transcriptional profiles derived from expression data of *O. vulgaris* central and peripheral nervous system.

The target genes count - indicated as per million mapped reads (CPM) - is based on mean values of transcripts expression levels in the three biological replicates utilized for the original RNA-Seq experiment in the tissues: supra- (SEM), sub-oesophageal (SUB) masses, optic lobes (OL), and the extremity (tip) and proximal part of an arm (ARM).

The relative transcripts abundance was examined in central nervous system (SEM, SUB, OL) versus peripheral nervous system (its component represented in tip and ARM) first, and afterwards between the single parts in the brain.

Molecular fingerprint of *O. vulgaris* brain: preliminary *in silico* gene expression analysis

I attempted a preliminary *in silico* molecular characterization of the central nervous system of *O. vulgaris,* based on transcriptome data available at Stazione Zoologica Anton Dohrn (Napoli- Italy) - Dr G. Fiorito's Research Group in collaboration with Dr R. Sanges (SISSA). The transcriptome is part of another PhD thesis by Dr G. Petrosino (Petrosino et al., 2015).

The *O. vulgaris* transcriptome has been compiled from the collection and reannotation of the nucleotide sequences assembly collected from two separate RNA-seq experiments carried out on total RNAs isolated from central nervous tissues (SEM, SUB, OL), arm (tip, muscle, and isolated nerve

cord), and stellate and gastric ganglia from adults, and pre-hatching and post-hatching paralarvae of *O. vulgaris*. All samples were in biological triplicates. For quality of raw reads assessment, transcriptome assemblage strategies, and other technical details, see Petrosino (2015).

In brief, the original dataset reached 85 GB of sequence data (850 million paired-end reads). Transcripts not showing at least 0.5 reads mapping per million mapped reads (CPM) in at least two samples were discarded from the transcriptome as being expressed at too low levels and therefore likely deriving by noise or assembly artifacts. Raw sequences cleaning brought to a uniquely expressed transcript dataset of more than 64,000 transcripts, subsequently clustered in around 40,000 putative genes. Transcripts assembled presented a median length of 795 bp (average length of 1,308 bp). The completeness of the transcriptome reached 98.4% and included 32.6% of protein coding transcripts functionally annotated.

The most abundant functional class in central nervous system (2,4%) is involved in the "RNA-dependent DNA replication". As reported by Petrosino (2015; see also Petrosino et al., 2021) this is the mechanism utilized by retrotransposons to jump from one site to another in the host genome.

Furthermore, a high proportion of transcripts were ascribed to lncRNAs (7,806; 12.1%). Interestingly, the lncRNAs of the *O. vulgaris* resulted significantly higher in the central nervous system (~10%) when compared to other tissues (e.g., arm: ~7%) similarly to what occurs in mammals (Petrosino, 2015; Zarrella et al., 2019).
A highly expressed functional class of genes in central nervous system is represented by "Regulation of transcription DNA-dependent", which contains about 1,2% of transcripts that contribute to DNA-templated transcription, which currently occurs for all the physiological mechanisms that allow neural tissue survival, and is induced during memory formation (Kandel, 2001). Furthermore, the molecular function classification shows that the most abundant molecular function (8%) encodes for zinc interacting molecules, other well-represented classes are "nucleic acid binding" and "ATP binding".

As a preliminary screening, I examined the above-described *O. vulgaris* transcriptome to define the brain masses transcriptional profiles, compared to other tissues (arm and tip) also for predicting putative expression of the selected genes. The differential expression in the considered tissues is depicted as percentages of the total counts per million as deduced from transcriptome analysis.

When the expression level of the genes target included in this PhD project is compared to their expression level in other tissues as the arm and the tip, it appeared clear that the selected genes were strongly more expressed in the central nervous system than in peripheral tissues. Only PTEN and Setd3 transcripts were not enriched in the central nervous system when compared to arm and tip. The majority of the target genes showed a higher expression in SUB, SEM and OL, while expression in the ARM is reduced, showing a lower expression level than the TIP (Figure 4).

Focusing the *in silico* analysis on the central nervous system (Fig. 5) it is possible to observe a diversified relative abundance of the 24 transcripts in the octopus brain masses. The target genes count is indicated as per million mapped reads (CPM).

Eight genes (zif268, PTEN, NTRK2, Ring1, EED, ktm5aa, Ash2l and Kdm6a) resulted to be expressed with CPM values lower than 10 in all brain masses, while six genes (PP1, stmn, nrxn1a, Htt, CBP and Prmt1) appeared to be very highly expressed in all the three brain masses, with CPM counts higher than 40 in at least one mass.

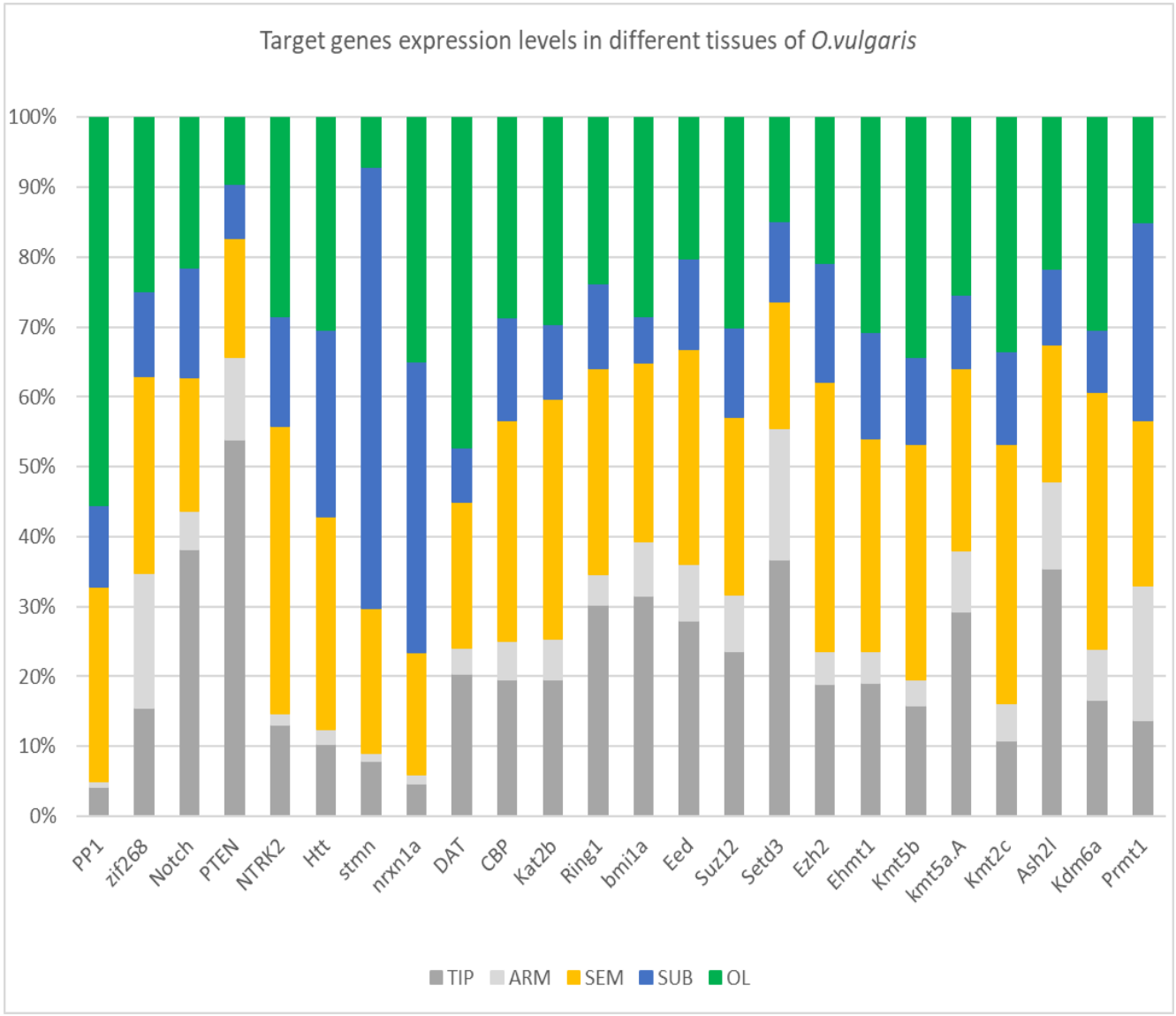


Figure 4. Relative percentage of expression of target genes in central nervous system, arm and tip.

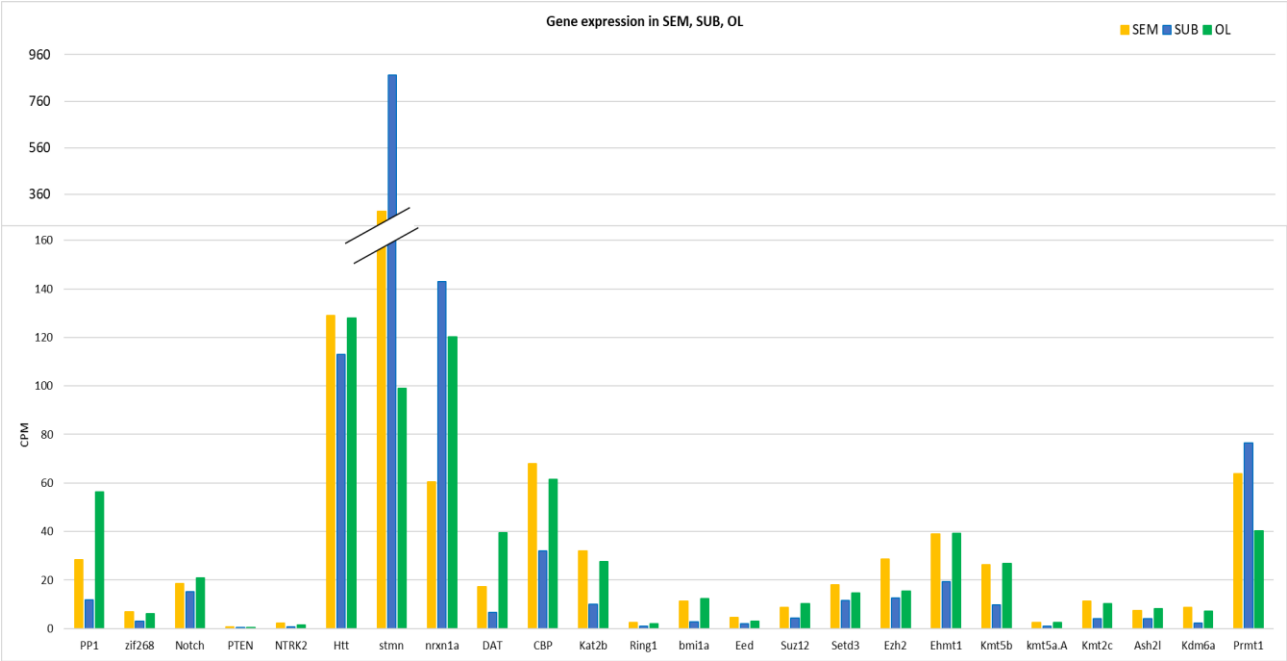


Figure 5. In *silico* expression levels of target genes in *Octopus vulgaris* brain masses.

Most of the genes appeared largely expressed in the brain than in the arm, at least in naïve animals. Future work may benefit of a larger analysis including the differential profile of all genes in relation with mass-specific transcripts; such a kind of work should include also the analysis of orphan genes (Zarrella et al., 2019).

6. Gene expression changes as a consequence of learning to avoid a stimulus in octopus

From experiments carried out on protein synthesis inhibition (see Appendix 2: Supplementary Information) and other studies (Turchetti-Maia et al., 2018), no clear evidence emerged about the dependence of *O. vulgaris* memory formation on protein synthesis.

This is a surprising finding that contrasts with the expected one considering that the cAMP response element-binding protein (CREB) activation has been evaluated in *O. vulgaris* in response to fear conditioning (Zarrella, 2011). CREB phosphorylation appeared to be induced after fear conditioning and this induction was specifically observed after testing, but not after training (Zarrella, 2011).

The activation of cAMP-response element binding protein (CREB)-dependent gene expression, participating to synaptic plasticity and modulating neuron excitability, represents a crucial step in the molecular cascade that mediates the formation of long-lasting forms of memory in various taxa (e.g., Benito & Barco, 2010; Kandel, 2001b; Radulovic & Tronson, 2010; Silva et al., 1998; Tully et al., 2003; Won & Silva, 2008).

For the aims of this PhD project, I tested target genes relative expression through RT-qPCR experiments from samples collected after memory retrieval of an avoidance learning task to explore changes of gene expression linked to learning and memory recall.

Materials and Methods

Samples from a total of 12 *O. vulgaris* of both sexes (4 females and 8 males) from 200 to 400 g were caught in the Bay of Napoli (Italy) during the summer of 2007. In the original experiments, animals were randomly assigned to control, with samples collected one hour after their arrival in laboratory ("N"aive, N = 6) and experimental group (samples collected one hour after testing session of fear conditioning "T", N = 6).

Details about the training, experimental conditions, animal care and housing are provided in Supplementary Material: see Appendix 3.

In brief, the avoidance training protocol allow to measure the octopus ability at remembering to avoid to attack a stimulus that has been associated with a negative reinforcement. During training

a cut-off latency of 60 seconds was set as maximum duration of the trial. Before the starting of the fear conditioning protocol, animals were food deprived for 24h.

Fear conditioning was tested through a three-days protocol consisting in a pre-training phase (first day), a training phase (second day) and a testing phase (third day). On the first day, animals were presented (single multi-trial-session) with a positively reinforced stimulus (reaching a white ball associated with food reward). The pre-training was performed in order to familiarize the animals with artificial stimuli, and increase motivation to attack an artificial stimulus. The pre-training phase was considered completed when octopuses attacked the stimulus within 20 seconds from its appearance in the tank for six consecutive trails. On the second day, the animal was presented with a negatively reinforced stimulus: reaching and touching a red ball was associated with a mild shock (12 V AC, duration: 2 - 3 sec). The punishment brought the animals to leaving the object abruptly and return to their den. Training was repeated until the animals stopped attacking the ball for at least 6 consecutive trials (training to criterion).

Testing phase was carried out on the third day. Twenty-four hours after training, octopuses were tested for their ability to recall the avoidance memory. Animals were presented with the red ball, but any punishment was delivered. During testing, some of the animals showed recall avoiding to touch and “freezing” at the stimulus sight.

Other octopuses, however, failed to avoid the red ball. At the end of the testing session, animals were presented with the white ball and with a live crab to control for stimulus generalization/specificity any motivational decline after the avoidance experience.

Naive controls were sacrificed one hour after their arrival.

Categorization of samples by animals’ performance and samples collection

From the dataset and data record of the original experiment, I collected brain samples already obtained from animals. The selection was made following the analysis of the animal’s learning performances. Depending on their ability to remember the task, trained (T, N=4) animals were distinguished in remembering (R, N=2) or forgetting (F, N=2). Two naive (N=2) animals were used as controls.

In the original study, animals were humanely killed and brain dissected. The brain masses OL, SEM and SUB were placed in plastic molds (Peel–A–Way Disposable Embedding Molds 22 x 22 mm

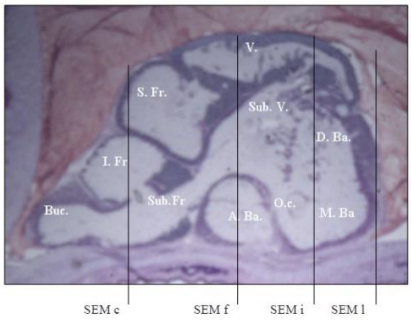
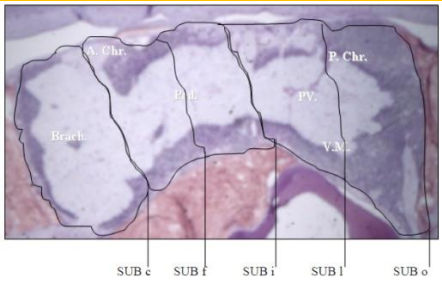
Polyscience Inc.-Warrington PA USA), each brain mass was fixed onto a sample holder using a small quantity of Tissue-Tech O.C.T. embedding compound, and immediately frozen in liquid nitrogen and stored at -80°C until processed.

Sectioning was carried out using a cryostat (Leica CM3050 S) following the anterior-posterior axis, positioning the brain parts on the holder by their foremost posterior part and collecting sections (20 µm thick). For every brain mass, a set of five slides carrying 4 sections were collected over SuperFrost Plus Microscope Slides (VWR International, Milano, Italy) for a series of experiment not considered in this PhD thesis. Along with these, one series was included in Eppendorf tubes containing twelve slices (minimal number of slices necessary to extract almost 500 ng of RNA). The quality assessment of this approach follows the same principles and quality check considered by Zarrella (2011).

Sections collected in tubes were further processed for RNA analysis, and preserved in RNAlater Solution at -80°C until further processing.

The serial sectioning allowed also to identify selected areas from each of the brain mass. Following Zarrella (2011), I considered four regions for the SEM (identified as: c, f, i, l) and five regions for the SUB (identified as: C, F, I, L, O) as illustrated in Table 8.

Table 8. Areas on brain indentified by serial sections of the octopus brain

_Brain area	Lobes	
	**SEM c**	Buccal lobe, inferior frontal lobe, sub-frontal lobe and part of superior frontal lobe
	SEM f	Superior frontal lobe, sub-frontal lobe, anterior basal lobe, vertical lobe and sub-vertical lobe
	SEM i	Anterior and median basal lobe, sub-vertical lobe, vertical lobe and optic commissure
	SEM l	Median and dorsal basal lobe, sub-vertical lobe and the posterior part of vertical lobe.
	**SUB C**	Brachial lobe
	SUB F	Anterior chromatophore lobe, pedal lobe
	SUB I	Pedal and magnocellular lobes
	SUB L	Pedal lobe, palliovisceral lobe, vasomotor lobe, posterior chromatophore lobe
	SUB O	Vasomotor lobe, posterior chromatophore lobe

Isolation and preparation of mRNAs

Filtered sterile tips, DEPC-treated sterile water and autoclaved microcentrifuge tubes were used for all subsequent steps.

Samples previously stored in RNAlater solution (-80°C ultra-low freezers) were thawed on ice, removed from solution and briefly dried on paper. They were then immersed in RNA Lysis Buffer (Promega Z3051; 500µl for samples less than 50 mg and 1000 µl for samples between 50 and 100 mg) and homogenized with Ultra-Turrax homogenizer. RNA was purified using SV Total RNA Isolation System (Promega Z3105) following manufacturer instructions. RNA was eluted in 50 µl DEPC water. Nanodrop ND-1000 UV-VIS spectrophotometer (Nanodrop Technologies) was used to read RNA optical density measurements at 230, 260 and 280 nm.

The absence of DNA contamination was verified by performing a PCR on extracted mRNA samples with β-actin primers and analyzing the sample by gel electrophoresis. In case genomic DNA contaminations were observed, the samples were treated with TURBO DNA-free™ Kit (ThermoFisher Scientific, AM1907) to remove DNA traces, following manufacturer's instruction. Final elution was performed using 50 µl of RNase, DNase free water. Samples were then stored at -80°C ultra-low freezers.

Synthesis of cDNA

cDNA was synthetized using iScript™ cDNA Synthesis Kit (BIORAD, 1708891). Appropriate amounts of mRNA (500ng) were retrotranscribed using 5x iScript Reaction Mix and iScript Reverse Transcriptase, using Nuclease free water to reach a total volume of 20µl. Tubes were then briefly centrifuged and incubated for 5mins at 25°C, 20mins at 46°C and 1min at 95°C on a Polymerase Chain Reaction (PCR) express machine. Following this, cDNA was stored at -20°C.

Primer design and efficiency

Primers were designed by Primer 3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using target sequences derived from *O. vulgaris* transcriptome (Petrosino, 2015). Primer parameters were set as follows: primer size between 18 and 27 base pairs, with optimum at 20 nucleotides length, optimal amplicon size 150-200 base pairs, melting point between 57 - 63°C (optimum at 60°C).

Primer specificities were also tested with Multiple Primer Analyzer (Thermofisher; https://www.thermofisher.com/it/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html) to estimate and possibly avoid presence of secondary structures as self-dimers, primer-dimers and hairpins.

Primers were tested for PCR on cDNA pools, analyzed on agarose gel to confirm the presence of a single band and the obtained single bands were purified using GenElute™ Gel Extraction Kit (Sigma Aldrich, NA1111) and sequenced in order to verify the identity of the amplicon with the selected sequence.

Each primer pair was subsequently tested for specificity and efficiency in order to use it for RT-qPCR experiments. The efficiency of primer pairs was evaluated according to standard curves method with the equation $E = 10^{-1/slope}$ (Pfaffl et al., 2002; Radonić et al., 2004) using five serial dilutions of a standard cDNA sample obtained by pooling cDNA samples from several individuals.

Standard curves were generated for each sample/gene combination, using the Ct value versus the logarithm of each dilution factor. To evaluate primers specificity the melting curve of each sample was controlled for the presence of a single peak and for no amplification in blank controls (Abramo et al., 2006).

Primer pairs sequences along with their amplification product size and efficiency are listed in Table 9.

Table 9. Primers used for RT-qPCR experiments. Gene identifier, protein name, Gene Ontology, primer sequence (F: forward; R: reverse), amplicon size (base pairs) and amplification efficiency (E) of reference (Ov-tuba; Ov-ub/S27A, in blue) and target genes are reported.

Gene name	Protein	Uniprot HSP	Gene Ontology	F/R	Primer sequence 5'-3'	Amplicon size (bp)	E
PP1	Serine/threonine-protein phosphatase PP1	P48488	phosphoprotein phosphatase activity GO:0004721	F	GGTGCTCTGGTAGCTGAACC	165	2
				R	AGCTGTTGCCAAAAAGAGGA		
zif268	Early growth response protein 1-B	Q6NTY6	regulation of transcription, DNA-templated GO:0006355	F	TTCTCATCACTACGGGCAAA	159	2
				R	CATGCGCATTCATACAGGTC		
Notch	Neurogenic locus Notch protein	P07207	long-term memory GO:0007616, chromatin binding GO:0003682	F	CATGCAACAACGAACACGGT	161	2
				R	GGTGTCCATCAGGGTGGTTT		

Gene name	Protein	Uniprot HSP	Gene Ontology	F/R	Primer sequence 5'-3'	Amplicon size (bp)	E
PTEN	PTEN/Phosphatidylinositol 3 4 5-trisphosphate 3-phosphatase	Q9PUT6	regulation of neuron projection development GO:0010975	F	GCAGCCAGTCTGCAGTGATA	125	1.98
				R	GGCGCCAACTTTCTTCAATGT		
NTRK2	BDNF/NT-3 growth factors receptor	Q91987	learning GO:0007612	F	TGAGCTAAGTCCCCGTGAGT	164	2
				R	GGCGATGTCTCGAACAAAGT		
Htt	Huntingtin	P42859	learning or memory GO:0007611, negative regulation of neuron death GO:1901215	F	CCACAGCATTGACCAACATC	178	2
				R	CCGCCTATCCAACGTAAGAA		
stmn	Stathmin	C6K2V8	regulation of microtubule polymerization or depolymerization GO:0031110	F	TGGAGAGAAAAGGCCAAAGA	133	2
				R	CAATAGCCTCCTGGGTGAGA		
nrxn1a	Neurexin 1-a	A1XQX0	cell adhesion GO:0007155	F	TCGGAACTGGGGTTCAAATA	180	2
				R	GCGCAAACCAATTTAGTCGT		
DAT	Sodium-dependent dopamine transporter (DAT)	Q7K4Y6	dopamine:sodium symporter activity GO:0005330	F	GCCCTAGACGGCATCAAATA	109	2
CBP	CREB-binding protein	Q92793	histone acetyltransferase activity GO:0004402	F	TGATGAAGTTTCCGTCCACA	169	2
				R	CATCCCAGCCTGTTACGAAT		
Kat2b	Ov-Kat2b/Histone acetyltransferase KAT2B	Q92831	chromatin remodeling GO:0006338 positive regulation of gene expression, GO:0045815	F	AATGCTTTCATCGCATCCGC	148	1.89
				R	TGGTTGGATGGCTGTTGGTT		
Ring1	Ov-Ring1/Polycomb group RING finger protein 1	Q7ZYZ7	chromatin silencing GO:0006342	F	CCTTTGGCAAACTTGAGGGC	193	2
				R	GGTGTCCATCAGGGTGGTTT		
bmi1a	Ov-bmi1a/Polycomb complex protein BMI-1-A	Q8JIR0	chromatin silencing GO:0006342	F	CTCCATCCTTTTTGCCGTGC	163	1.85
				R	TTCGGTAAAGGCCAGGAACC		
Eed	Ov-Eed/Polycomb protein EED	Q5ZKH3	chromatin silencing GO:0006342	F	AGCCTACGCTGACCCTTCTA	197	2
				R	ATTTGGGGTGGAATCGCAGT		
Suz12	Ov-Suz12/Polycomb protein suz12	Q0VA03	histone methylation GO:0016571	F	ATAACCAAAGCCCTGCCTCC	120	2
				R	CTGCTGGCTACCGATACCAG		
Setd3	Ov-Set3/Histone-lysine N-methyltransferase setd3	B0VX69	positive regulation of transcription, DNA-templated GO:0045893	F	AAGTGAGGTGTTAGCTCGGC	168	2
				R	GACAGCAAATCGGCAGCATT		
Ezh2	Histone-lysine N-methyltransferase EZH2	Q61188	positive regulation of dendrite development GO:1900006 positive regulation of MAP kinase activity GO:0043406	F	ACCTGTCCTTGCATAGTGGC	174	1.95
				R	AGCTCCGCATGTTTGACAGA		

Gene name	Protein	Uniprot HSP	Gene Ontology	F/R	Primer sequence 5'-3'	Amplicon size (bp)	E
Ehmt1	Ov-Ehmt1/Histone-lysine N-methyltransferase EHMT1	Q5DW34	negative regulation of transcription, DNA-templated GO:0045892	F	GTTTCTGGTGCAAGCAGGTG	140	2
				R	CCTGGACATTGACGTCAGCT		
Kmt5b	Ov-Kmt5b/Histone-lysine N-methyltransferase KMT5B /Histone-lysine N-methyltransferase SUV420H1	Q29RP8	histone H4-K20 trimethylation GO:0034773	F	TGTGATGGACAGGTTGGTGG	188	2
				R	TGCAGGGCCCAACCATAATT		
kmt5a.A	N-lysine methyltransferase SETD8-A/Ov-kmt5a.A/N-lysine methyltransferase KMT5A-A	Q071E0	positive regulation of gene expression GO:0010628	F	ATGGTAAAGGGCGAGGTGTG	105	2
				R	CTCGATCTTTGGCTGCTGGA		
Kmt2c	Ov-Kmt2c/Histone-lysine N-methyltransferase 2C	Q8BRH4	positive regulation of transcription by RNA polymerase II GO:0045944	F	TGTTGTCTGAGGGTCTTGCC	165	2
				R	GAAGAGATGGCTGCTGTGGT		
Ash2l	Ov-Ash2l/Set1/Ash2 histone methyltransferase complex subunit ASH2	Q9UBL3	positive regulation of transcription by RNA polymerase I GO:0045944	F	TCAGCCACTTGGGAATGTCC	172	2
				R	TGGATCATCTGGCTGGGGTA		
Kdm6a	Ov-Kdm6a/Lysine-specific demethylase 6A	O15550	chromatin remodeling GO:0006338 positive regulation of gene expression GO:0010628	F	CACATGTCTGCACGAGAGGT	110	2
				R	GGATATGGTGGGGATGCAGG		
Prmt1	Ov-Prmt1/Protein arginine N-methyltransferase 1	Q28F07	Neurogenesis GO:0022008 positive regulation of transcription, DNA-templated GO:0045893	F	AAGAAAGGAGGGGTGGGAGT	181	1.91
				R	ATCCTGGTCCAAGGGAAAAG		
ub/S27A	Ov Ubiquitin/ribosomal protein S27a	C0KKU3	Protein ubiquitination GO: 0016567	F	TCAAAACCGCCAACTTAACC	113	2
				R	CCTTCATTTGGTCCTTCGTC		

Reference Genes

Following Sirakov et al. (2009) and Zarrella (2011), I selected tubulin alpha chain (Ov-tubA) and Ov Ubiquitin/ribosomal protein S27a (Ov-ubi/S27A) as reference genes (Sirakov et al., 2009; Zarrella, 2011). These genes showed a stable expression in the central nervous system of *O. vulgaris*. The normalization factor was calculated from the geometric mean of reference genes and used to calculate the relative expression level of target genes.

Real-time qPCR

Optical 384-well plate were set up with 1 µl cDNA (dilution 1:5) and 2 µl 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX; Solis ByoDine), 2.9 µl of forward and reverse primers (final concentration 400 nM) and 4.1 µl of water (total volume: 10 µl). The following thermal profile was utilized: 95°C for 15 min, one cycle for cDNA denaturation; 40 times 95°C for 15 sec, 60°C for 20 sec, 72°C for 40 sec, for amplification. Then 72°C for 3 min, 65°C for 31 sec, 65°C for 5 sec +0.5°C per cycle (x 60 cycles).
The program was set to reveal the melting curve of each amplicon from 60 to 95 °C, and read every 0.5 °C. Specificity of PCR products was checked by melting curve analysis.
All RT-qPCR reactions were carried out in duplicate to capture intra-assay variability. Each assay included no-template negative controls for each primer pair and two inter-run calibrators (IRC) used to correct technical run to run variation between samples analysed in different runs.

To study expression levels for each gene of interest relative to the RGs, I applied qBASE (Hellemans et al., 2007) that allows the conversion of quantification cycle values (Cq) into calibrated normalized relative quantities (CNRQs). The removal of between-run variation due to the required multi-plate real time qPCR experiment (Ruijter et al., 2015) was not adopted as approach in the final calculation of CNRQs. CNRQs values for each sample were utilized in the following statistical analysis.

Data analysis

Normalized expression levels were obtained from Cq data output of RT-qPCR experiment following (Hellemans et al., 2007). Data were analyzed using Rstudio and JASP on global Normalized Relative Quantities of gene expression. T-test was used to evaluate differences between biological replicates. No statistically significant differences were observed (data not shown). A repeated measure ANOVA followed by Turkey post-hoc test was performed.

Statistical analysis were run according to Zar, (1999). Whenever required, one-way or repeated measure ANOVAs followed by Tukey multiple comparisons test were utilized.
For gene expression analysis, and in addition to ANOVAs, a Principal Component Analysis (PCA) followed by hierarchical clustering (Ward's method) was applied to identify patterns of changes and correlations between expression levels of genes considered.
The computational strategy adopted followed Zar (1999), Yeung & Ruzzo (2001) and Abdi & Williams (2010). Statistical differences were considered significant at $p < 0.05$.

Results: gene expression experiments

Gene expression in response to fear conditioning

As mentioned above, the driving hypothesis of my PhD project is that memory retrieval of a fear passive avoidance task can induce target genes transcription changes in *O. vulgaris* brain masses. The effect of the experience on transcriptional regulation was evaluated through real-time q-PCR by examining gene expression in different areas of the brain masses – i.e. SEM and SUB - of trained (remembering and forgetting) and control octopuses.

Due to COVID-19 pandemics I will not consider samples from OL that were expected to be analyzed during the first half of the 2020.

In the following pages I will overview the results of gene expression experiments carried on samples of *O. vulgaris* central nervous system in trained and naïve animals considering the different areas to evaluate their putative involvement in response to fear conditioning.

A first outlook is given by considering each of the 24 target genes separately.

Serine/threonine-protein phosphatase PP1 (PP1)

O. vulgaris Serine/threonine-protein phosphatase PP1 expression in SEM and SUB resulted to vary between the brain masses in each experimental condition as shown by ANOVA ($F_{(1,14)}$= 8.564, p=0.011 for naïve; $F_{(1,14)}$=7.130, p=0.018 for remembering; $F_{(1,14)}$=0.629, p=0.441 for forgetting).

PP1 expression resulted to be higher in SEM than in SUB in naïve and lower in SEM than in SUB in remembering. Gene expression in each brain mass changed as result of behavioral 'outcome' ($F_{(2,15)}$ =5.564, p= 0.016 for SEM; $F_{(2,27)}$ = 4.577, p=0.019 for SUB) and this appeared related to remembering in SEM in which PP1 resulted to be downregulated (Remembering vs Naïve p=0.019; Forgetting vs Naïve p=0.892; Forgetting vs Remembering p=0.047) and SUB in which PP1 is up-regulated (Remembering vs Naïve p=0.014; Forgetting vs Naïve p=0.312; Forgetting vs Remembering p=0.290, after *post hoc*).

To explore if the expression changes occurred in the entire masses or in some of their parts, two way ANOVA was carried out on PP1 expression level for SEM (f,i,l) and SUB (C,F,I,L,O) areas.

Two-way ANOVA in SEM (3x3 Performance*Brain areas) followed by Tukey *post hoc* comparisons revealed that PP1 expression depends on performance and brain area considered (Performance

$F_{(2,9)}$= 11.041; p=0.004; Brain area $F_{(2,9)}$=6.731, p=0.016; Performance*Bran area $F_{(4,9)}$=1.826, p=0.208). Interaction resulted not statistically significant.

In SEM PP1 was found downregulated in remembering when compared with naïve animals (Tukey post-hoc test on performance Remembering vs naïve p=0.005; Forgetting vs naïve p=0.800) and significant differences were observed between remembering and forgetting (Remembering vs Forgetting p=0.012).

PP1 does not show differential expression when behavioral performance is compared with brain areas considered. A statistically significant difference resulted only in anterior SEM, with higher expression in forgetting when compared with remembering animals (SEM f_R vs SEM f_F p=0.037).

For the sub-oesophageal mass Two-way ANOVA revealed a significant difference (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$= 21.937; p<0.001; Brain area: $F_{(4,15)}$=17.174, p<0.001; Performance*Bran area: $F_{(8,15)}$=5.712, p=0.002). In SUB, PP1 expression depended on training performance (Tukey post-hoc test on performance, Forgetting vs naïve p=0.014; Remembering vs naïve p<0.001) and significant differences were observed between remembering and forgetting octopuses (Remembering vs Forgetting, p=0.011). Tukey post-hoc comparisons on performance x brain area revealed the areas of more evident gene expression changes. PP1 induction occured in the medial SUB for remembering animals (Tukey post-hoc test, Performance*Brain area: SUB I_R vs SUB I_N p<0.001) and a statistically significant difference observed in medial SUB depending on performance (SUB I_F vs SUB I_R, p=0.003).

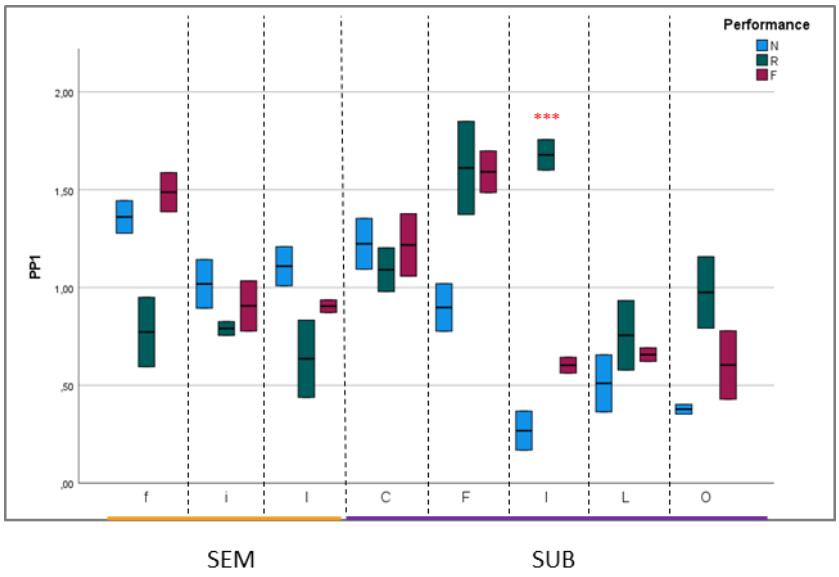


Figure 3 PP1 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Early growth response protein 1-B (zif268)

Early growth response protein 1-B in octopus central nervous system changed its expression between SEM and SUB only in "forgetting"; a higher level of expression in the SEM when compared to the SUB was observed ($F_{(1,14)}$=0.281, p=0.604 for naïve; $F_{(1,14)}$=0.010, p=0.921 for remembering; $F_{(1,14)}$=12.081, p=0.004 for forgetting). When comparing masses for behavioral outcomes, significant gene expression changes resulted in the SUB ($F_{(2,15)}$ =2.087, p=0.159 for SEM; $F_{(2,27)}$ = 7.104, p=0.003 for SUB).

Two-way ANOVA in SEM (3x3 Performance*Brain areas) followed by Tukey post hoc comparisons revealed that zif268 expression depended on performance and brain area and on the interaction (Performance $F_{(2,9)}$= 15.210; p=0.001; Brain area $F_{(2,9)}$=5.260, p=0.031; Performance*Brain area $F_{(4,9)}$=22.629, p<0.001). Significant differences were observed in anterior and posterior SEM (SEM f_F vs SEM f_N p=0.049; SEM l_F vs SEM l_N p=0.001; SEM l_R vs SEM l_N p<0.001).

As for SUB, 3x5 Performance*Brain areas Performance: $F_{(2,15)}$= 15.691; p<0.001; Brain area $F_{(4,15)}$=6.886, p=0.002; Performance*Brain area $F_{(8,15)}$=2.136, p=0.098. Tukey post hoc comparisons revealed that the posterior area of the SUB showed a statistically significant expression of zif268 (SUB O_F vs SUB O_N p=0.039).

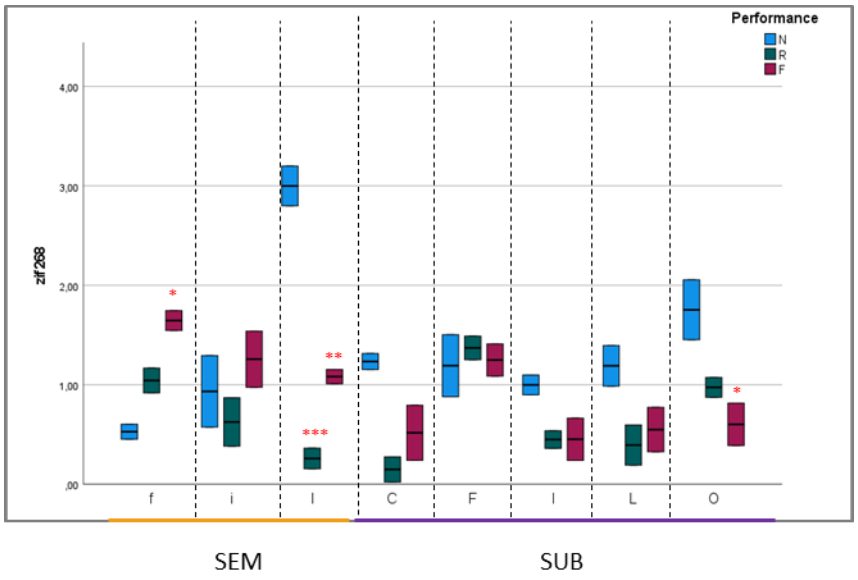


Figure 4 zif268 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Fear condition affected zif268 expression in anterior and posterior SEM and in posterior SUB.

Neurogenic locus Notch protein (Notch)

Neurogenic locus Notch protein showed significant changes in its expression depending on the brain mass considered only in forgetting group ($F_{(1,14)}$= 1.761, p=0.206 for naïve; $F_{(1,14)}$=0.570, p=0.463 for remembering; $F_{(1,14)}$=6.638, p=0.022 for forgetting), with a down-regulation in SEM compared to SUB.

Behavioural outcomes only appeared significant for this gene in the SUB ($F_{(2,15)}$ =1.285, p =0.306 for SEM; $F_{(2,27)}$ = 5.287, p=0.012 for SUB) and related to the forgetting animals in which Notch is up-regulated (Forgetting vs Naïve p=0.011).

Two-way ANOVA in SEM (3x3 Performance*Brain areas) followed by Tukey post hoc comparisons revealed no significant gene expression dependence on the factors considered (Performance $F_{(2,9)}$= 1.499; p=0.274; Brain area $F_{(2,9)}$=3.960, p=0.058; Performance*Brain area $F_{(4,9)}$=0.146, p=0.960). Two-way ANOVA in SUB (3x5 Performance*Brain areas Performance $F_{(2,15)}$=18.466; p<0.001; Brain area $F_{(4,15)}$=9.925, p<0.001; Performance*Brain area $F_{(8,15)}$=4.950, p=0.004) followed by Tukey post hoc comparisons.

In SUB the expression of Notch was significantly upregulated in forgetting when compared to naive animals (Tukey post-hoc test on performance Forgetting vs naïve p<0.001; Remembering vs naïve p=0.277) and also to remembering (Remembering vs Forgetting p=0.002). Tukey post-hoc comparisons on performance*brain area in SUB revealed a significant up-regulation in posterior SUB for forgetting group (Tukey post-hoc test on Performance*Brain area SUB L_F vs SUB L_N p=0.011).

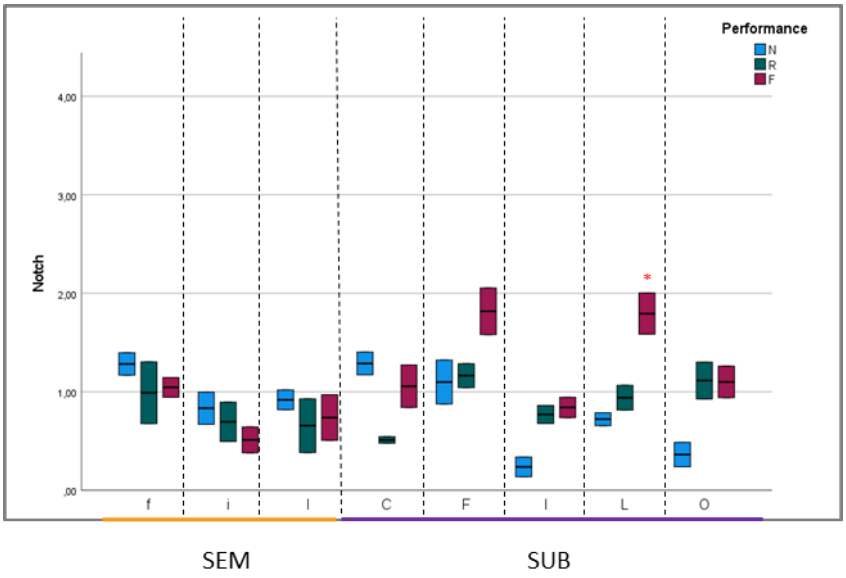


Figure 5 Notch expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Neurogenic locus Notch protein expression in *O. vulgaris* SEM was not influenced by training procedure in these experiments.

*Phosphatidylinositol 3-4-5- tris phosphate 3-phosphatase and dual specificity protein phosphatase (*PTEN*)*

I found *O. vulgaris* Phosphatidylinositol 3-4-5- tris phosphate 3-phosphatase and dual specificity protein phosphatase PTEN (*PTEN*) differently expressed between SEM and SUB in forgetting and naïve animals ($F_{(1,14)}$= 30.836, p<0.001 for naïve; $F_{(1,14)}$= 0.633, p=0.440 for remembering; $F_{(1,14)}$=19.816, p<0.001 for forgetting). PTEN expression was higher in SEM than in SUB in both naïve and forgetting animals.

In order to analyze the effect of fear conditioning on PTEN expression, I carried out an ANOVA analysis in every brain mass evaluating changes induced by the behavioral experience ($F_{(2,15)}$=5.546, p=0.016 for SEM; $F_{(2,27)}$ = 3.811, p= 0.035 for SUB). In SEM differences can be observed in remembering animals (upregulation when compared to forgetting, Forgetting vs Remembering p=0.013; Remembering vs Naïve p=0.493; Forgetting vs Naïve p=0.119) and in SUB where PTEN resulted upregulated in remembering compared to naive (Remembering vs Naïve p=0.049).

Two-way ANOVA in SEM (3x3 Performance*Brain areas) followed by Tukey post hoc comparisons revealed that PTEN expression appeared to change with performance and brain areas (Performance $F_{(2,9)}$= 8.627; p=0.008; Brain area, $F_{(2,9)}$=6.046, p=0.022; Performance*Brain area, $F_{(4,9)}$=0.561, p=0.697). Significant differences were observed between remembering and forgetting groups (Remembering vs Forgetting p=0.007), while no differences were observed in the areas considered among the three "conditions" (Two-way ANOVA for SUB, 3x5 Performance*Brain areas Performance: $F_{(2,15)}$= 5.885; p=0.013; Brain area $F_{(4,15)}$=0.879, p=0.500; Performance*Brain area $F_{(8,15)}$=2.897, p=0.036).

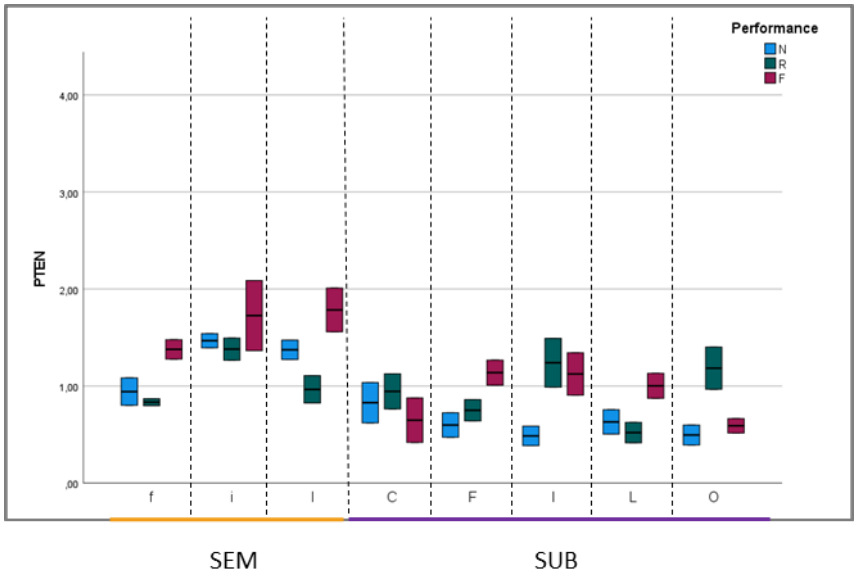


Figure 6. PTEN expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001.

In SUB PTEN expression resulted to vary with training outcomes (Tukey post-hoc test on performance Forgetting vs naïve p=0.032; Remembering vs naïve p=0.019) and significant

differences were observed between remembering and forgetting animals (Remembering vs Forgetting p=0.962). Tukey post-hoc comparisons on performance*brain area revealed no areas with significant expression changes.

These results suggested that even though PTEN expression is differently regulated in trained animals in entire masses, but no area-specific regulation of this gene appeared in my data.

*BDNF/NT-3 growth factors receptor (*NTRK2*)*

NTRK2 expression in SEM and SUB did not change between the brain masses in each experimental group as shown by ANOVA ($F_{(1,14)}$=0.016, p=0.900 for naïve; $F_{(1,14)}$=0.080, p=0.781 for remembering; $F_{(1,14)}$=0.025, p=0.877 for forgetting).

Behavioral outcomes did not significantly change the target gene expression in the brain masses ($F_{(2,15)}$ =1.072, p=0.367 for SEM; $F_{(2,27)}$ =1.850, p=0.177 for SUB). Although gene expression did not change between the masses, I verified whether a variation in the gene expression was visible between the brain areas. Two-way ANOVA for SEM show significant changes: 3x3 Performance*Brain areas, Performance: $F_{(2,9)}$= 7.428; p=0.012; Brain area: $F_{(2,9)}$=22.585, p<0.001; Performance*Brain area: $F_{(4,9)}$=12.439, p=0.001. A similar trend occurred In the SUB (3x5 Performance*Brain areas Performance $F_{(2,15)}$= 19.566; p<0.001; Brain area $F_{(4,15)}$=21.547, p<0.001; Performance*Brain area $F_{(4,9)}$=23.053, p<0.001). Thus, NTRK2 expression level depended on the brain area considered, on the performance achieved and on the interaction of both factors.

Tukey post-hoc comparisons on performance*brain area revealed the areas where expression changes occurred. NTRK2 induction was found in the anterior SEM of trained animals (Tukey post-hoc test on Performance*Brain area, SEM f_F vs SEM f_N p=0.024; SEM f_R vs SEM f_N p=0.002; SEM f_R vs SEM f_F p=0.024) and a statistically significant difference in posterior SEM depending on performance with higher expression in forgetting than remembering (SEM l_R vs SEM l_F p=0.024).

In SUB NTRK2 expression depended on training outcome (Tukey post-hoc test on performance Forgetting vs naïve p<0.001; Remembering vs naïve p<0.001) while no significant differences were observed between remembering and forgetting animals (Remembering vs Forgetting p=0.405).

NTRK2 occured with significant changes in the medial, for forgetting, and posterior SUB for remembering animals (Tukey post-hoc test on Performance*Brain area SUB F_F vs SUB F_N p<0.001; SUB O_R vs SUB O_N p<0.001) and a statistically significant difference observed in posterior SUB depending on performance (SUB F_F vs SUB F_R p=0.002).

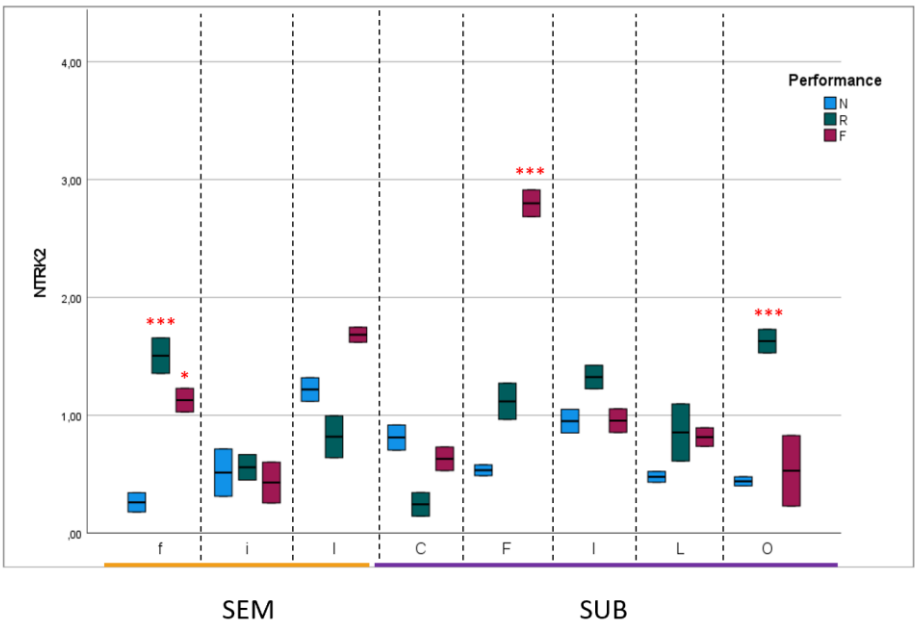


Figure 7 NTRK2 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Huntingtin (Htt)

Huntingtin regulation in *O. vulgaris* central nervous system did not change between SEM and SUB in the three conditions as revealed by ANOVA ($F_{(1,14)}$= 0.279, p=0.606 for naïve; $F_{(1,14)}$=2.094, p=0.170 for remembering; $F_{(1,14)}$=0.130, p=0.724 for forgetting) and in the single masses ($F_{(2,15)}$ =2.358, p=0.129 for SEM; $F_{(2,27)}$ = 1.287, p =0.293 for SUB).

Although the expression did not change, I verified if differences in gene expression could be evidenced at a higher spatial resolution considering performance and regional division of SEM (f,i,l) and SUB (C,F,I,L,O). In the SEM (3x3 ANOVA Performance*Brain areas) it was possible to notice that gene expression did not vary (Performance: $F_{(2,9)}$= 2.981; p=0.102; Brain area: $F_{(2,9)}$=2.192, p=0.168; Performance*Brain area: $F_{(4,9)}$=1.394, p=0.311). In SUB Two-way ANOVA was significant only when brain areas are considered (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$= 2.899; p=0.086; Brain area: $F_{(4,15)}$=10.229, p<0.001; Performance*Brain area: $F_{(8,15)}$=0.614, p=0.754) followed by Tukey post hoc comparisons.

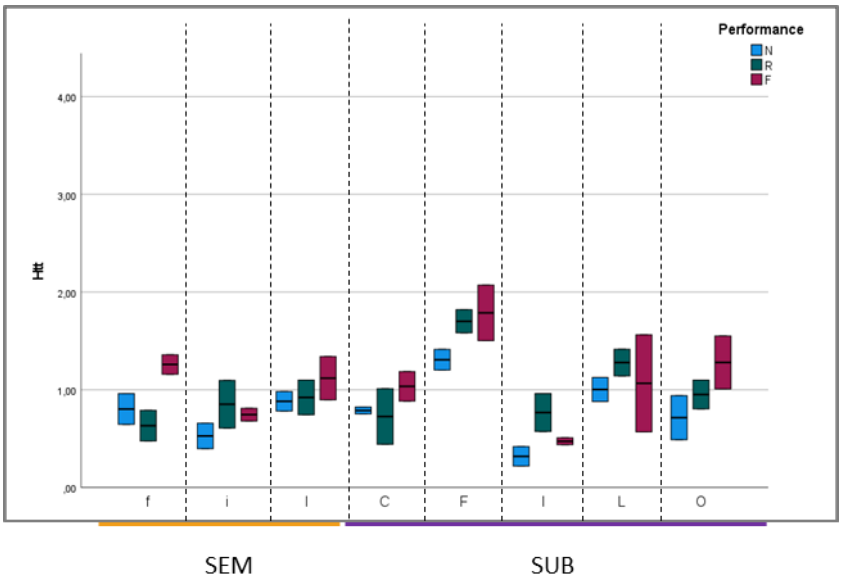


Figure 8. Htt expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, $p< 0.05$; ** highly significant, $p < 0.01$, *** very highly significant, $p < 0.001$

Huntingtin expression did not show site-specific performance-dependent variation.

Stathmin (stmn)

The expression of Stathmin in central nervous system resulted to be different between SEM and SUB in all cases, with expression in the SUB higher than in the SEM ($F_{(1,14)}$=14.720, p=0.002 for naïve; $F_{(1,14)}$=6.386, p=0.024 for remembering; $F_{(1,14)}$=9.675, p=0.008 for forgetting). Fear conditioning did not resulted in significant changes when masses were compared ($F_{(2,15)}$ =1.726, p=0.212 for SEM; $F_{(2,27)}$ = 0.649, p=0.531 for SUB). To verify if fear conditioning induced changes in gene expression in areas of brain, two way ANOVA analysis was conducted on *stmn* expression level for SEM areas (f,i,l) and for SUB areas (C,F,I,L,O). I found no significance in SEM (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$= 2.090; p=0.180; Brain area: $F_{(2,9)}$=3.558, p=0.073; Performance*Brain area $F_{(4,9)}$=0.512, p=0.729), but differences in SUB (3x5 Performance*Brain areas Performance $F_{(2,15)}$=9.024; p=0.003; Brain area $F_{(4,15)}$=68.011, p<0.001; Performance*Brain area $F_{(8,15)}$=11.063, p<0.001). Tukey post hoc comparisons revealed that the anterior area of the SUB showed a significant expression of stmn (SUB F_F vs SUB F_N p=0.002; SUB F_R vs SUB F_N p<0.001). Despite no

statistical difference, at the level of posterior SEM the expression between the different behavioral outcomes resulted to be significant (SUB O_F vs SUB O_R p=0.003).

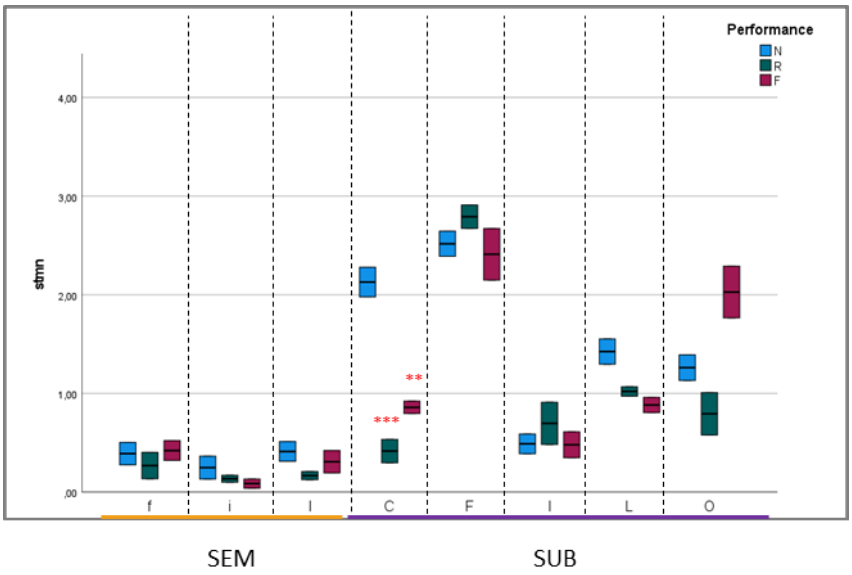


Figure 9 stmn expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

The expression profile of naïve animals have an opposite trend to RNA-seq experiment in which the expression of stmn was higher in SEM than in SUB.

Neurexin 1-a (nrxn1a)

Neurexin 1-a resulted to be differentially expressed between SEM and SUB in all cases ($F_{(1,14)}$= 6.430, p=0.024 for naïve; $F_{(1,14)}$=8.242, p=0.012 for remembering; $F_{(1,14)}$=5.311, p=0.037 for forgetting), but with expression level in SEM lower than in SUB. Fear conditioning appeared to not influence on nrxn1a expression when SEM and SUB are compared ($F_{(2,15)}$ =0.986, p=0.396 for SEM; $F_{(2,27)}$ = 0.035, p=0.966 for SUB). Two-way ANOVA (3x3 Performance*Brain areas) shown that gene expression is not related to a differential pattern when different areas of SEM are considered (Performance: $F_{(2,9)}$= 1.093; p=0.376; Brain area: $F_{(2,9)}$=2.286, p=0.157; Performance*Brain area: $F_{(4,9)}$=0.764, p=0.574). However, this appeared to occur for the SUB (Two-way ANOVA 3x5 Performance*Brain areas,

Performance: $F_{(2,15)}$= 0.268; p=0.768; Brain area: $F_{(4,15)}$=39.155, p<0.001; Performance*Brain area: $F_{(8,15)}$=4.319, p=0.007).

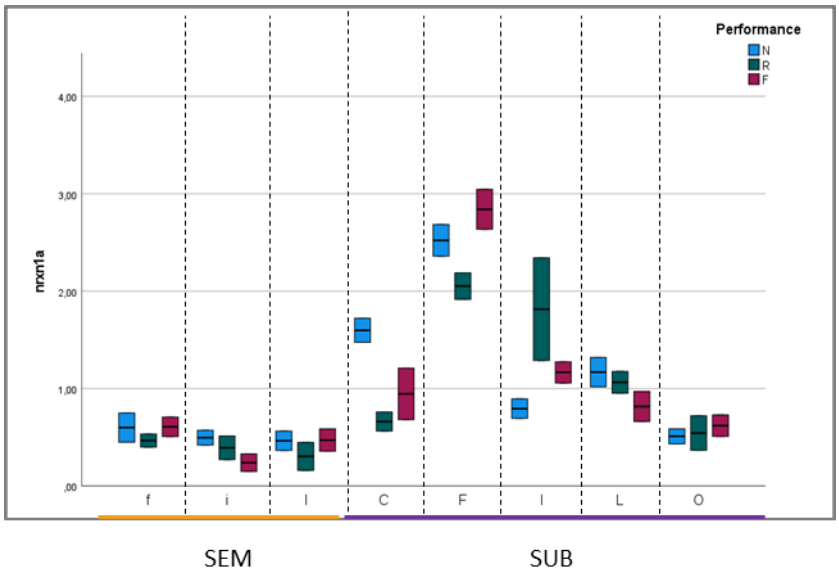


Figure 10 nrxn1a expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Thus, neurexin 1-a appeared not actively related to memory retrieval in octopus, at least considering the data available to me.

Sodium-dependent dopamine transporter (DAT)

The expression of Sodium-dependent dopamine transporter (DAT) resulted to be different between SEM and SUB (naïve: $F_{(1,14)}$=16.198, p=0.001; remembering: $F_{(1,14)}$=30.614, p<0.001; forgetting: $F_{(1,14)}$=2.214, p=0.159), depending from behavioral outcomes.

Two-way ANOVA (3x3 Performance*Brain areas) revealed that DAT relative expression changed when brain areas were considered (Performance: $F_{(2,9)}$= 20.373; p<0.001; Brain area: $F_{(2,9)}$= 90.736, p<0.001; Performance*Brain area: $F_{(4,9)}$=21.001, p<0.001). In particular, levels of DAT expression appeared to be significant when the anterior SEM of trained animals (SEM f_F vs SEM f_N p=0.048; SEM f_R vs SEM f_N p<0.001) and in median SEM areas of "forgetting" octopuses (SEM i_F vs SEM i_N

p=0.019) were considered; the difference between remembering and forgetting was also significant in these regions (SEM f_R vs SEM f_F p=0.041; SEM i_R vs SEM i_F p<0.001).

As for the suboesophageal mass, DAT expression resulted to be significant at the level of the median and posterior areas (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$= 3.079; p=0.076; Brain area: $F_{(4,15)}$=4.003, p=0.021; Performance*Brain area: $F_{(8,15)}$=8.863, p<0.001). In median regions of SUB DAT appeared up-regulated in remembering octopuses, but down-regulated in posterior parts of the SUB of trained animals (SUB I_R vs SUB I_N p=0.019; SUB O_F vs SUB O_N p=0.016 ; SUB O_R vs SUB O_N p=0.046). Thus, DAT appeared to be dynamically regulated across SEM and SUB following fear conditioning.

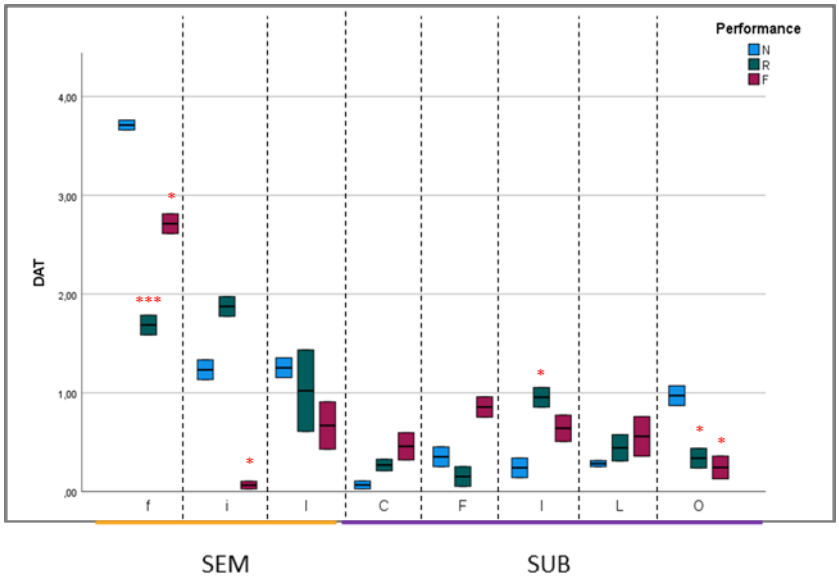


Figure 11 DAT expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Kat2b/Histone acetyltransferase KAT2B (Kat2b)

No differences between SEM and SUB in naïve and forgetting octopuses for the acetyl-transferase KAT2B was observed ($F_{(1,14)}$=3.608, p=0.078 for naïve; $F_{(1,14)}$=0.711, p=0.413 for remembering; $F_{(1,14)}$=2.567, p=0.131 for forgetting). Behavioral outcomes of fear conditioning resulted to be with no significant effect ($F_{(2,15)}$ =3.251, p=0.067 for SEM; $F_{(2,27)}$ = 1.414, p=0.261 for SUB). Two-way

ANOVA confirmed this view (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$=4.871; p=0.037; Brain area: $F_{(2,9)}$=1.263, p=0.329; Performance*Brain area: $F_{(4,9)}$=2.737, p=0.097), although downregulation of the expression of this gene was observed for remembering when compared to forgetting animals (Remembering vs Forgetting, p=0.036 after Tukey post-hoc). When the SUB is considered, the expression of Kat2B gene resulted different between different areas, but not reaching significance when behavioral performance was considered (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$= 2.965; p=0.082; Brain area: $F_{(4,15)}$=7.338, p=0.002; Performance*Brain area: $F_{(8,15)}$=1.530, p=0.227).

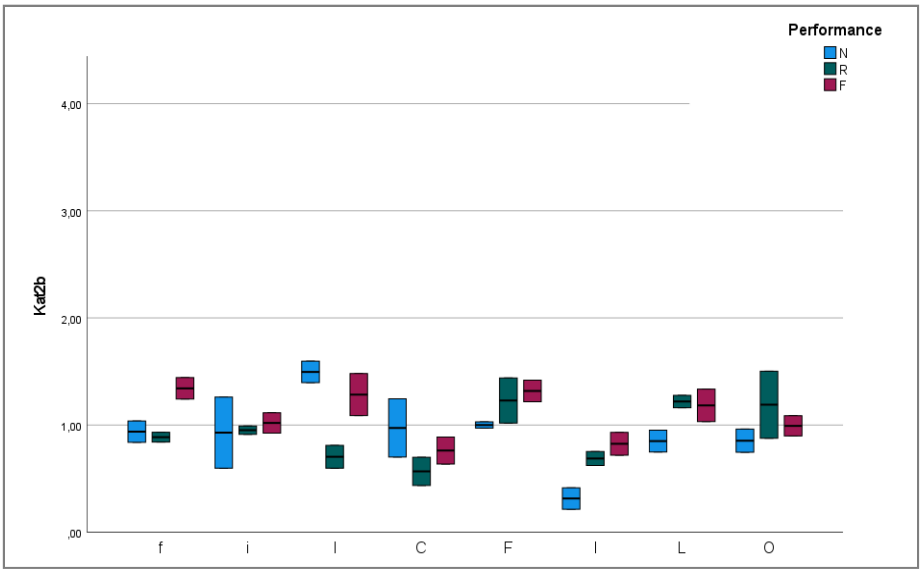


Figure 12 Kat2b expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

CREB-binding protein (CBP)

The acetyl-transferase CREB-binding protein expression resulted to be in general higher in SEM compared to SUB in naïve and forgetting octopuses ($F_{(1,14)}$=6.831, p=0.020 for naïve; $F_{(1,14)}$=0.536, p=0.476 for remembering; $F_{(1,14)}$=8.211, p=0.012 for forgetting), and behavioral performance resulted to induce significant differences in the SEM ($F_{(2,15)}$ =7.152, p=0.007 for SEM; $F_{(2,27)}$ = 0.399, p=0.675 for SUB), but largely due to remembering animals where a down-regulation was observed (Remembering vs Naïve, p=0.005), with CBP expression changing significantly in the posterior areas

of SEM (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$= 11.026; p=0.004; Brain area: $F_{(2,9)}$=1.720, p=0.233; Performance*Brain area $F_{(4,9)}$=2.671, p=0.102) of remembering animals compared to naïve (Fig. 15). As for the SUB (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$= 1.004; p=0.390; Brain area: $F_{(4,15)}$=6.650, p=0.003; Performance*Brain area: $F_{(8,15)}$=3.285, p=0.023) I observed marginal significant down-regulation in remembering animals when compared to naïve in anterior areas of the SUB (Tukey post-hoc test, Performance*Brain area: SUB C_R vs SUB C_N p=0.043).

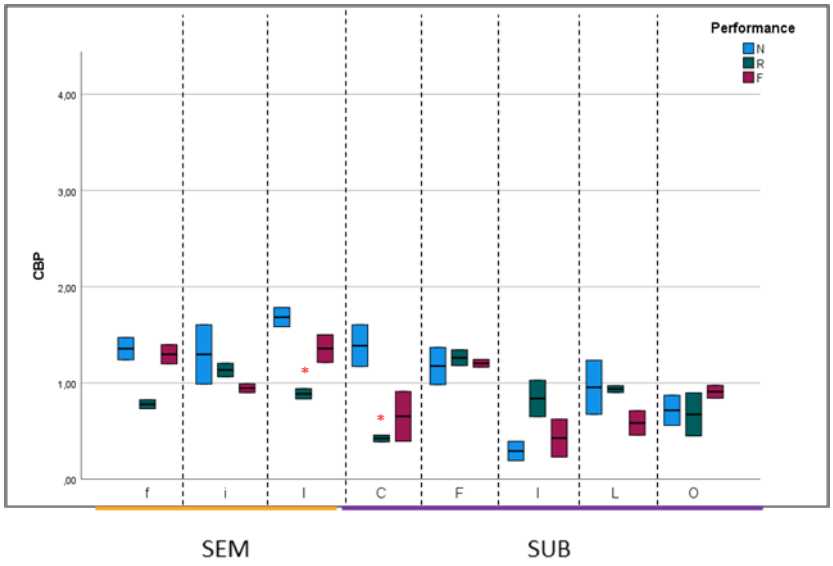


Figure 13 CBP expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Kdm6a/Lysine-specific demethylase 6A (Kdm6a)

The demethylase Kdm6a showed a significantly higher expression level in SEM than in SUB in the three conditions observed ($F_{(1,14)}$=39.077, p<0.001 for naïve; $F_{(1,14)}$=9.459, p=0.008 for remembering; $F_{(1,14)}$=7.426, p=0.016 for forgetting). The expression of the gene appeared affected by behavioral outcomes in SEM ($F_{(2,15)}$ =9.890, p=0.002 for SEM; $F_{(2,27)}$ =0.071, p=0.932 for SUB) with a significant downregulation in the trained groups (SEM, Remembering vs naïve: p=0.007; SEM Forgetting vs naïve: p=0.003). Two-way ANOVA revealed that Kdm6a expression was significantly influenced by the behavioral outcomes in the SEM (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$=46.839, p<0.001; Brain area: $F_{(2,9)}$=0.384, p=0.692; Performance*Brain area: $F_{(4,9)}$=15.318, p<0.001). Tukey

post-hoc comparisons on performance*brain area revealed significant down-regulation of remembering and forgetting in anterior SEM and in forgetting in median areas of the SEM (SEM f_R vs SEM f_N $p<0.001$; SEM f_F vs SEM f_N $p<0.001$; SEM i_F vs SEM i_N $p=0.018$). A similar analysis for the SUB (3x5 Performance*Brain areas, Performance: $F_{(2,15)}=0.112$; $p=0.895$; Brain area: $F_{(4,15)}=2.002$, $p=0.146$; Performance*Brain area: $F_{(8,15)}=2.478$, $p=0.062$) did not evidence any significant variations of gene expression.

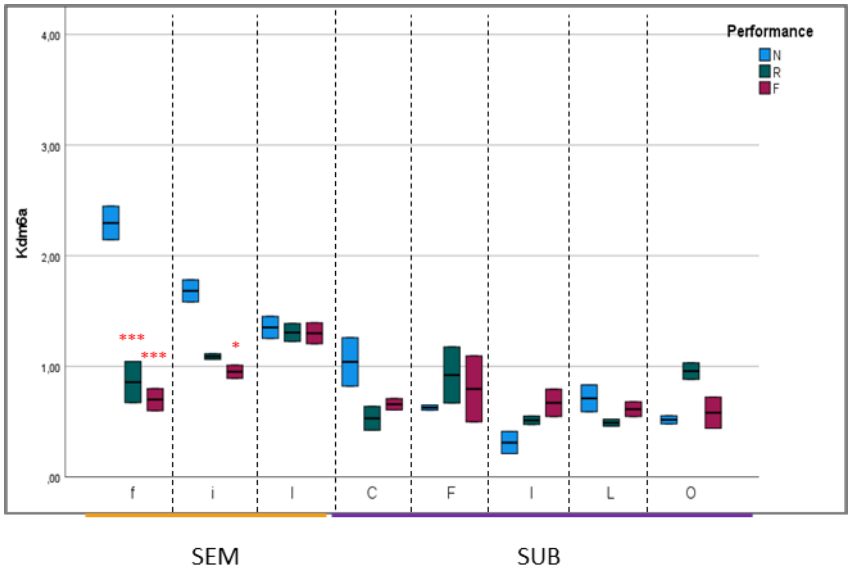


Figure 14 Kdm6a expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, $p< 0.05$; ** highly significant, $p < 0.01$, *** very highly significant, $p < 0.001$

Ov-Prmt1/Protein arginine N-methyltransferase 1 (Prmt1)

The expression of gene Protein arginine N-methyltransferase 1 resulted with a significant higher expression level in the SUB when compared to the SEM in octopus ($F_{(1,14)}=16.978$, $p<0.001$ for naïve; $F_{(1,14)}=14.965$, $p=0.002$ for remembering; $F_{(1,14)}=8.790$, $p=0.010$ for forgetting). Prmt1 expression was not affected by behavioral experience in SEM or SUB ($F_{(2,15)}=1.610$, $p=0.233$; $F_{(2,27)}=2.871$, $p=0.074$, respectively). A two-way ANOVA (3x3 Performance*Brain areas, Performance: $F_{(2,9)}=1.990$; $p=0.193$; Brain area: $F_{(2,9)}=0.675$, $p=0.533$; Performance*Brain area: $F_{(4,9)}=2.046$, $p=0.171$) revealed that Prmt1 expression was not significantly linked to behavioral performances in the supraoesophageal mass. However, a similar pattern was not confirmed in the suboesophageal mass

(3x5 Performance*Brain areas, Performance: $F_{(2,15)}$=12.091; p<0.001; Brain area: $F_{(4,15)}$=16.865, p<0.001; Performance*Brain area: $F_{(8,15)}$=3.097, p=0.011) with Prmt1 expression with levels increased in median part of the SUB in subjects that fully remembered the fear conditioning task (Fig. 17).

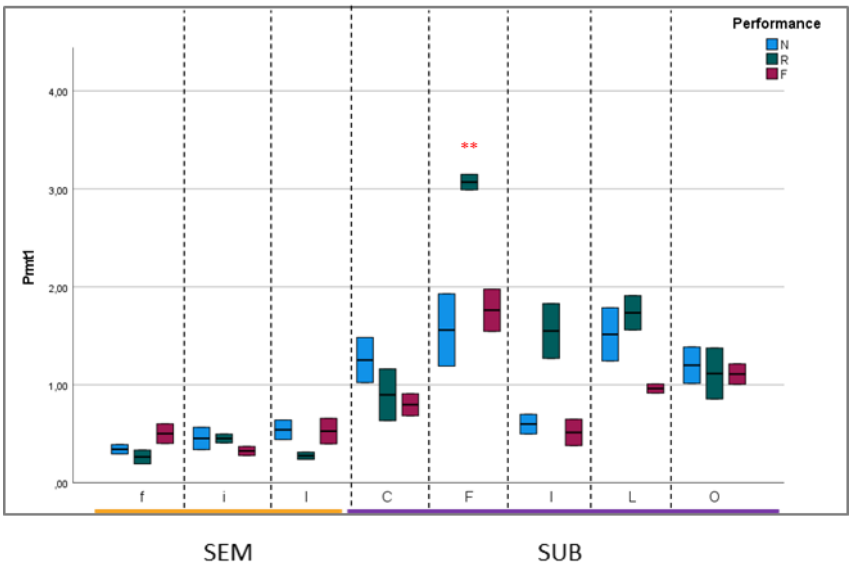


Figure 15 Prmt1 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Kmt2c/Histone-lysine N-methyltransferase 2C (Kmt2c)

In the supraoesophageal mass, the expression of Histone-lysine N-methyltransferase Kmt2c resulted to be significantly higher than in SUB only for forgetting *O. vulgaris* ($F_{(1,14)}$=0.912, p=0.356 for naïve; $F_{(1,14)}$=3.518, p=0.082 for remembering; $F_{(1,14)}$=7.851, p=0.014 for forgetting). It appeared linked to behavioral outcomes after fear conditioning ($F_{(2,15)}$ =2.829, p=0.091 for SEM; $F_{(2,27)}$ = 6.249, p=0.006 for SUB) with the gene being up-regulated in remembering octopuses as shown by post hoc Tukey test (SUB Remembering vs naïve p=0.011). Furthermore, Kmt2c was significantly upregulated in anterior area of the SEM of forgetting octopuses (SEM f_F vs SEM f_N p=0.049). In the SUB I did not notice any significant variation of gene expression (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$=8.910; p=0.003; Brain area: $F_{(4,15)}$=2.382, p=0.098; Performance*Brain area: $F_{(8,15)}$=1.746, p=0.168).

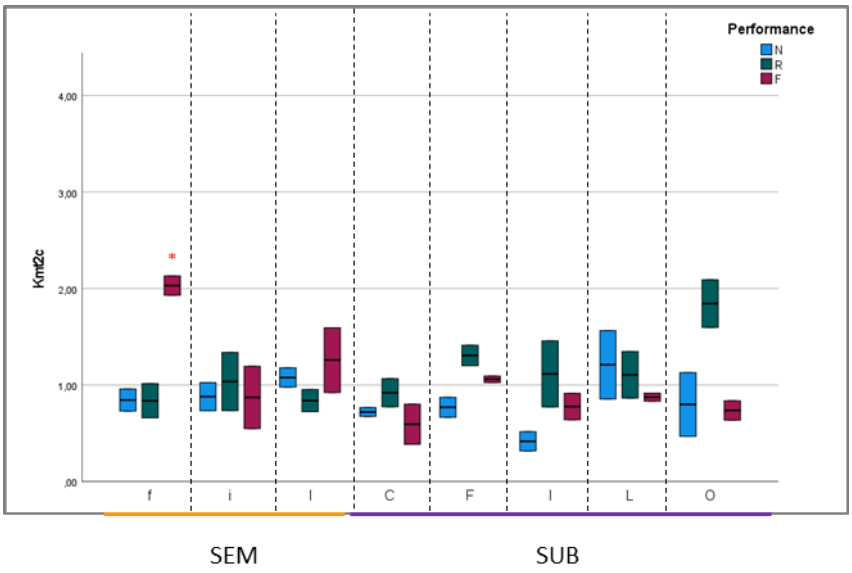


Figure 16 Kmt2c expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Ash2l/Set1/Ash2 histone methyltransferase complex subunit ASH2 (Ash2l)

The Histone-lysine N-methyltransferase Ash2l expression did not show significant variations between SEM and SUB in octopus in any of the conditions considered ($F_{(1,14)}$=0.0003, p=0.987 for naïve; $F_{(1,14)}$=0.055, p=0.818 for remembering; $F_{(1,14)}$=0.004, p=0.953 for forgetting). In the suboesophageal mass it resulted to be marginally significant, when behavioral performance was considered ($F_{(2,15)}$ =1.428, p=0.271 for SEM; $F_{(2,27)}$ = 3.579, p=0.042 for SUB) with significant differences observed comparing remembering and forgetting octopuses (SUB, Remembering vs Forgetting: p=0.044), but again only marginally. The two-way ANOVA (SEM - 3x3 Performance*Brain areas, Performance: $F_{(2,9)}$=3.082; p=0.096; Brain area: $F_{(2,9)}$=1.715, p=0.234; Performance*Brain area: $F_{(4,9)}$=4.986, p=0.021; SUB - 3x5 Performance*Brain areas, Performance: $F_{(2,15)}$=5.399; p=0.017; Brain area: $F_{(4,15)}$=0.559, p=0.696; Performance*Brain area: $F_{(8,15)}$=2.937, p=0.034) confirmed this view (Fig. 19).

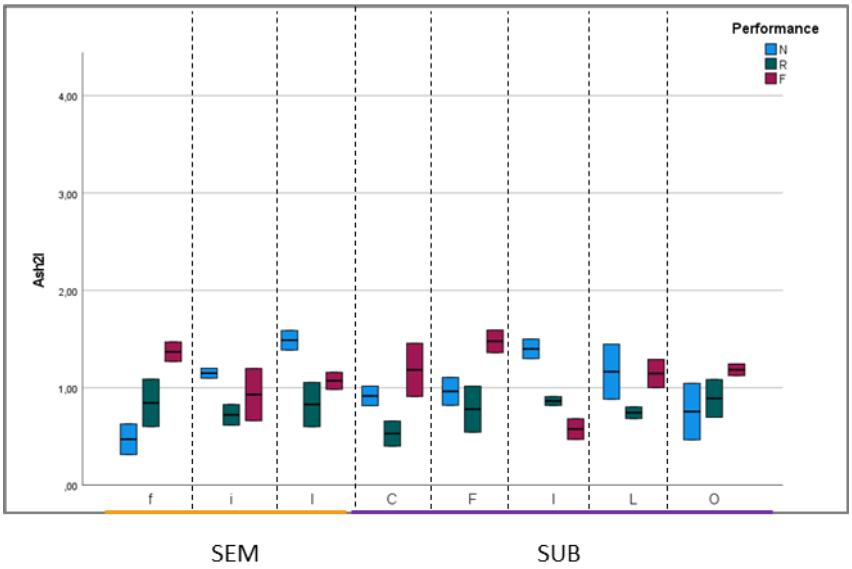


Figure 17 Ash2l expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Set3/Histone-lysine N-methyltransferase setd3 (Setd3)

Comparison of expression level for the Histone-lysine N-methyltransferase setd3 gene in the two brain masses in *O. vulgaris* revealed that no difference in naïve, lower expression in SEM compared with the SUB in remembering, and an opposite trend in forgetting animals ($F_{(1,14)}$= 0.032, p=0.860 for naïve; $F_{(1,14)}$=12.558, p=0.003 for remembering; $F_{(1,14)}$=6.571, p=0.023 for forgetting). Behavioral outcomes appeared linked to behavioural outcomes ($F_{(2,15)}$ =15.506, p<0.001 for SEM; $F_{(2,27)}$ = 5.421, p=0.010 for SUB) and remembering animals with lower expression compared to naïve (SEM: Remembering vs naïve p<0.001; SUB: Remembering vs naïve p=0.004) also in the case of forgetting octopus (SEM: Forgetting vs Remembering p=0.002). This has been confirmed by two-way ANOVA (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$=69.989; p<0.001; Brain area: $F_{(2,9)}$=8.229, p=0.009; Performance*Brain area: $F_{(4,9)}$=10.562, p=0.002) with attention to brain areas. Setd3 resulted to be downregulated in anterior and posterior SEM of remembering animals (SEM f_R vs SEM f_N p=0.002; SEM l_R vs SEM l_N p<0.001) and in the posterior areas of SEM of forgetting octopus (SEM l_F vs SEM l_N p=0.004). A similar pattern resulted for the SUB after two-way ANOVA (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$= 24.873; p<0.001; Brain area: $F_{(4,15)}$=11.003, p<0.001; Performance*Brain area: $F_{(8,15)}$=8.109, p<0.001), showing a significant drop of Setd3 expression in

anterior areas of the SUB in remembering and forgetting animals (SUB F_R vs SUB F_N p<0.001; SUB F_F vs SUB F_N p=0.003, after Tukey post hoc tests).

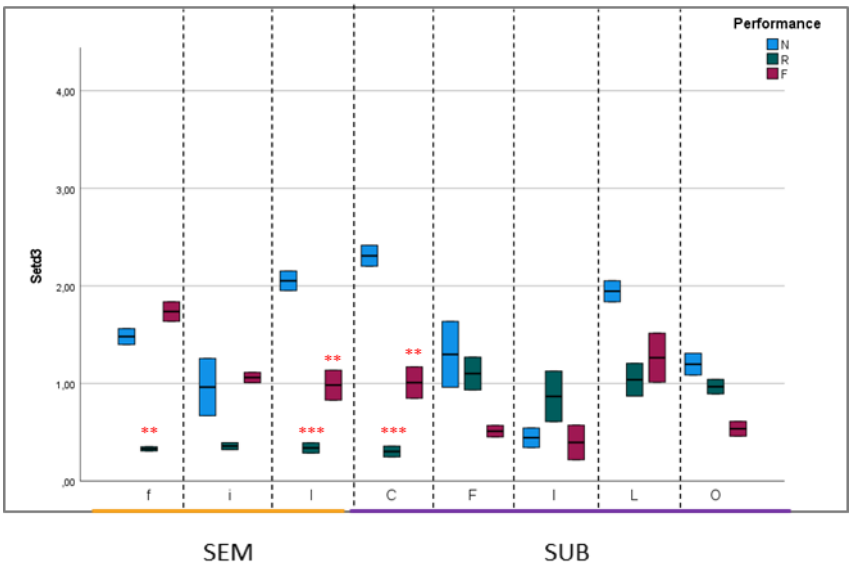


Figure 18 Setd3 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Kmt5b/Histone-lysine N-methyltransferase KMT5B /Histone-lysine N-methyltransferase SUV420H1 (Kmt5b)

I found no significant difference of the expression of the Histone-lysine N-methyltransferase KMT5B between SEM and SUB ($F_{(1,14)}$= 1.638, p=0.221 for naïve; $F_{(1,14)}$=0.001, p=0.970 for remembering; $F_{(1,14)}$=0.008, p=0.931 for forgetting). Kmt5b expression was linked to behavioral outcomes ($F_{(2,15)}$ =9.002, p=0.003 for SEM; $F_{(2,27)}$ = 2.911, p=0.072 for SUB) and the variation observed in the SEM resulted to be related to fear conditioning with a Kmt5b downregulation (Forgetting vs naïve p=0.008; Remembering vs naïve p=0.005). The pattern of expression of the gene in different areas of the octopus brain appeared linked to a downregulation in the median and posterior parts of the supraesophageal mass (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$=81.321; p<0.001; Brain area: $F_{(2,9)}$=21.625, p<0.001; Performance*Brain area: $F_{(4,9)}$=20.815, p<0.001; SEM i_F vs SEM i_N p=0.006; SEM i_R vs SEM i_N p=0.024; SEM l_F vs SEM l_N p<0.001; SEM l_R vs SEM l_N p<0.001, after Tukey post hoc tests).

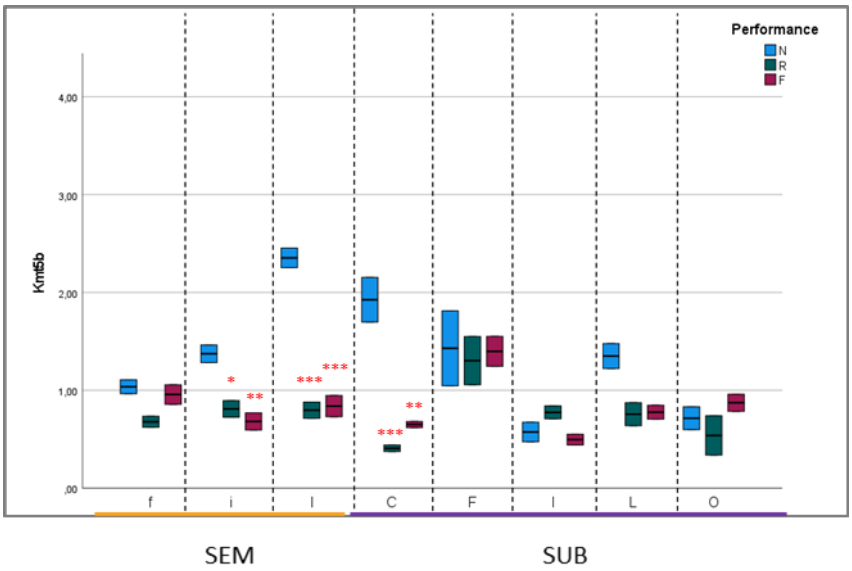


Figure 19 Kmt5b expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

A similar pattern emerged for the subesophageal mass with a significant downregulation in the anterior part of the SUB (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$=10.400; p=0.001; Brain area: $F_{(4,15)}$=9.953, p<0.001; Performance*Brain area: $F_{(8,15)}$=5.207, p=0.003; Tukey post hoc: SUB C_F vs SUB C_N p=0.003; SUB C_R vs SUB C_N p<0.001).

N-lysine methyltransferase SETD8-A/Ov-kmt5a.A/N-lysine methyltransferase KMT5A-A (kmt5a.A)

I found higher expression of the Histone-lysine N-methyltransferase kmt5a.A in the SEM of *O. vulgaris* compared to the SUB in naïve animals ($F_{(1,14)}$=4.906, p=0.044 for naïve; $F_{(1,14)}$=0.100, p=0.756 for remembering; $F_{(1,14)}$=0.055, p=0.818 for forgetting). Kmt5b expression was not affected by behavioral performance after fear condition ($F_{(2,15)}$ =3.175, p=0.071 for SEM; $F_{(2,27)}$ = 0.042, p=0.959 for SUB). Taking into account the relative expression of the gene in different areas of the brain linked to the behavioral outcome, my results show no significant difference in the SEM (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$=5.289; p=0.030; Brain area: $F_{(2,9)}$=1.925, p=0.201; Performance*Brain area: $F_{(4,9)}$=3.034, p=0.077) and a marginal difference (downregulation) in median areas of the SEM for forgetting octopuses (SEM i_F vs SEM i_N p=0.046), and a similar pattern

in the SUB (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$=0.075; p=0.928; Brain area: $F_{(4,15)}$=3.560, p=0.031; Performance*Brain area: $F_{(8,15)}$=2.357, p=0.073).

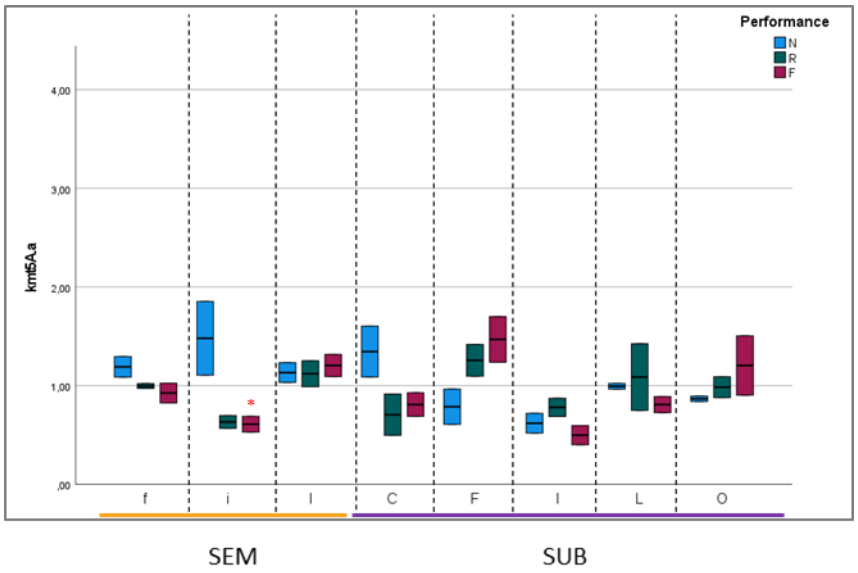


Figure 20 kmt5A.a expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Ring1/Polycomb group RING finger protein 1 (Ring1)

In *O. vulgaris* the Ring1/Polycomb group RING finger protein 1 expression changed between SEM and SUB only in animals 'forgetting' where a higher level of expression in the SEM compared to the SUB was observed ($F_{(1,14)}$=0.029, p=0.996 for naïve; $F_{(1,14)}$=2.119, p=0.168 for remembering; $F_{(1,14)}$=19.755, p<0.001 for forgetting; $F_{(2,15)}$ =4.834, p=0.024 for SEM; $F_{(2,27)}$ = 5.952, p=0.007 for SUB). In the supraesophageal mass I observed a downregulation relative to naïve animals (Tukey post-hoc test on performance, Forgetting vs naïve p= 0.029), that corresponded to an up-regulation of Ring 1 in remembering compared to naïve in the SUB (Remembering vs naïve p= 0.029). In the SUB a differential expression between remembering (up-regulated) and forgetting (down-regulated) was also significant (Remembering vs Forgetting, p=0.010). A two-way ANOVA carried out to reveal differences in the pattern of gene expression in different brain areas linked to the behavioral outcomes for the SEM (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$= 7.357; p=0.013; Brain

area: $F_{(2,9)}$=1.378, p=0.300; Performance*Brain area: $F_{(4,9)}$=2.768, p=0.094) revealed that fear conditioning induced Ring 1 expression in anterior SEM (SEM f_F vs SEM f_N p=0.022). For the suboesophageal mass (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$= 6.033; p=0.012; Brain area: $F_{(4,15)}$=0.042, p=0.996; Performance*Brain area: $F_{(8,15)}$=1.525, p=0.229) Ring 1 expression appeared linked to the animals performance (Remembering vs naïve, p=0.038; Remembering vs Forgetting p=0.015). No significant regional difference in gene expression was observed in the areas of the SUB.

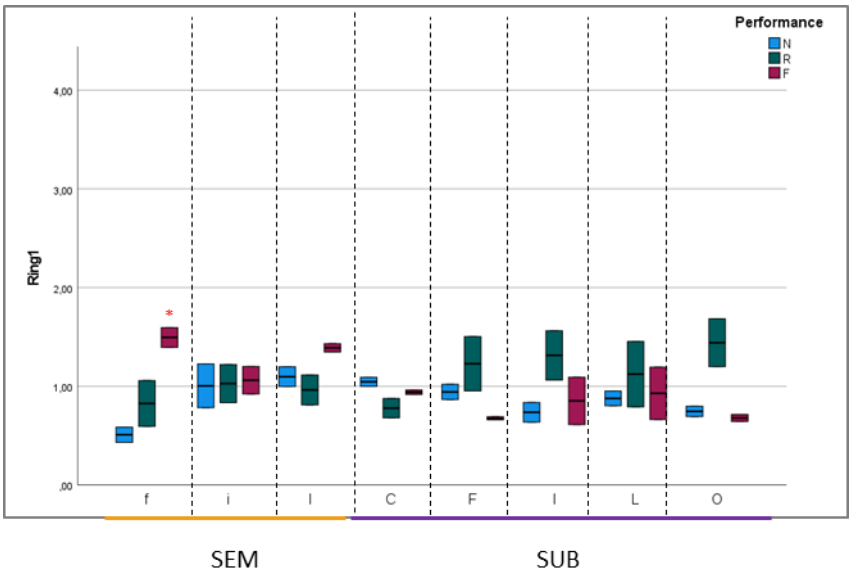


Figure 21 Ring1 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Ehmt1/Histone-lysine N-methyltransferase EHMT1 (Ehmt1)

The Histone-lysine N-methyltransferase EHMT1 showed a higher expression in *O. vulgaris* SEM than in SUB in naïve and remembering groups ($F_{(1,14)}$= 10.055, p=0.007 for naïve; $F_{(1,14)}$=7.793, p=0.014 for remembering; $F_{(1,14)}$=0.018, p=0.895 for forgetting). Ehmt1 expression resulted linked to behavioral outcome in both SEM and SUB ($F_{(2,15)}$ =5.327, p=0.018 for SEM; $F_{(2,27)}$ = 3.648, p=0.040 for SUB), and in the SEM remembering and forgetting animals showed lower expression than naïve (SEM: Remembering vs naïve p=0.036; Forgetting vs naïve p=0.029), but marginally in the SUB (Forgetting vs Remembering p=0.049). Ehmt1 expression increased in anterior part of the supraesophageal mass (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$=13.222; p=0.002; Brain

area: $F_{(2,9)}$=10.396, p=0.005; Performance*Brain area: $F_{(4,9)}$=1.860, p=0.202; SEM f_F vs SEM f_N p=0.026, after Tukey post hoc test). A differential pattern of expression of the gene resulted in different areas of the SUB, but no significant difference emerged when the performance of animals after fear conditioning was considered (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$=6.953; p=0.007; Brain area: $F_{(4,15)}$=4.874, p=0.010; Performance*Brain area: $F_{(8,15)}$=2.121, p=0.100).

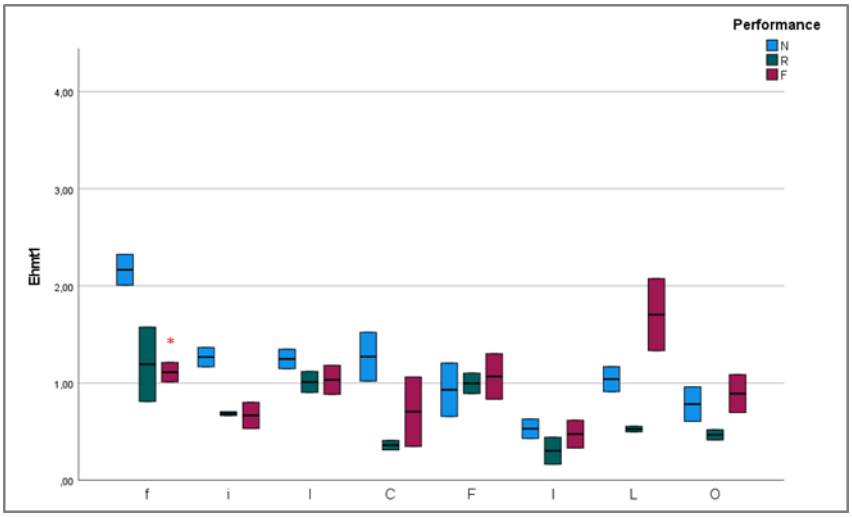


Figure 22 Ehmt1 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-bmi1a/Polycomb complex protein BMI-1-A (bmi1a)

Polycomb complex protein BMI-1-A resulted significantly higher in SEM compared to the SUB in all the conditions ($F_{(1,14)}$=6.388, p=0.024 for naïve; $F_{(1,14)}$=4.738, p=0.047 for remembering; $F_{(1,14)}$=10.779, p=0.005 for forgetting), but marginal for remembering octopuses. Behavioral outcomes appeared linked to gene expression ($F_{(2,15)}$ =4.306, p=0.033 for SEM; $F_{(2,27)}$ = 6.436, p=0.005 for SUB) with changes that appeared related to a down-regulation in SEM (Remembering vs Naïve p=0.027) and SUB (Remembering vs Naïve p=0.004). When the pattern of expression between different regions of the brain was considered (3x3 Performance*Brain areas, Performance: $F_{(2,9)}$= 67.253; p<0.001; Brain area: $F_{(2,9)}$=2.452, p=0.141; Performance*Brain area: $F_{(4,9)}$=55.097 p<0.001) I found significant changes in the anterior part of the SEM for forgetting and in median and

posterior areas of the same mass (SEM f_F vs SEM f_N p<0.001; SEM i_F vs SEM i_N p<0.001; SEM i_F vs SEM i_N p<0.001; SEM l_F vs SEM l_N p=0.001; SEM l_R vs SEM l_N p<0.001), that corresponded to changes in the anterior part of the SUB (3x5 Performance*Brain areas, Performance: $F_{(2,15)}$=13.205; p<0.001; Brain area: $F_{(4,15)}$=1.674, p=0.208; Performance*Brain area: $F_{(8,15)}$=4.212, p=0.008; SUB F_F vs SUB F_N p=0.017; SUB F_R vs SUB F_N p=0.003, after Tukey post hoc).

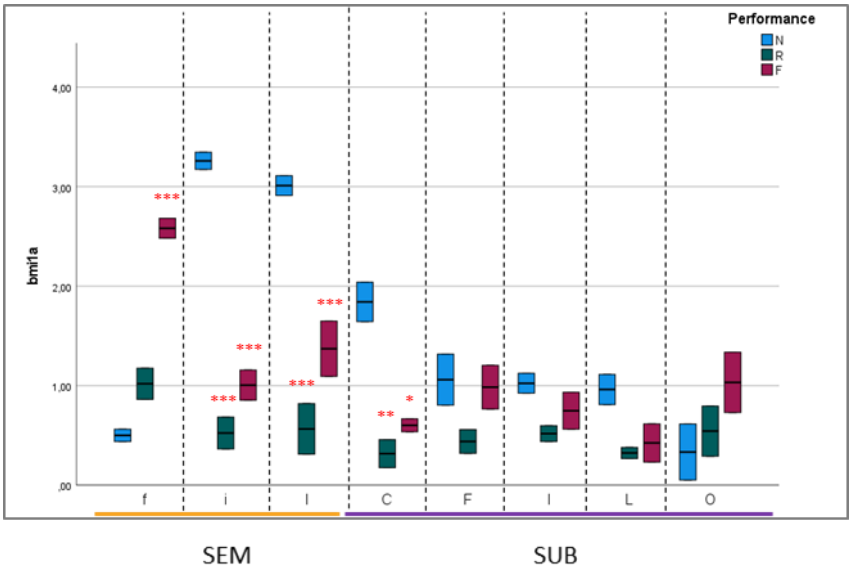


Figure 23 bmi1a expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Ov-Suz12/Polycomb protein suz12 (Suz12)

Octopus vulgaris Polycomb protein Suz12 was expressed at higher level in SEM than in SUB in naïve group as reported from ANOVA analysis ($F_{(1,14)}$= 16.443, p=0.001 for naïve; $F_{(1,14)}$=0.470, p=0.0893 for remembering; $F_{(1,14)}$=2.873, p=0.112 for forgetting).

Suz-12 expression was affected by behavioral experience only in the SEM ($F_{(2,15)}$ =18.192, p<0.001 for SEM; $F_{(2,27)}$ = 2.123, p=0.139 for SUB) where remembering exhibited lower expression than naïve (Remembering vs naïve p<0.001) and the reduction was also significant when compared to forgetting expression level (Forgetting vs Remembering p=0.005).

Two-way ANOVA in SEM (3x3 Performance*Brain areas: Performance $F_{(2,9)}$= 14.902; p=0.001; Brain area $F_{(2,9)}$=0.813, p=0.474; Performance*Brain area $F_{(4,9)}$=0.415, p=0.794) revealed that the gene was differently expressed depending on animals' performance.

In SEM Suz12 was found highly downregulated in remembering when compared with naïve and forgetting animals, (Tukey post-hoc test on performance Remembering vs naïve p=0.001; Forgetting vs Remembering p=0.018).

No statistically significant differences were observed in the single regions considered of the SEM.

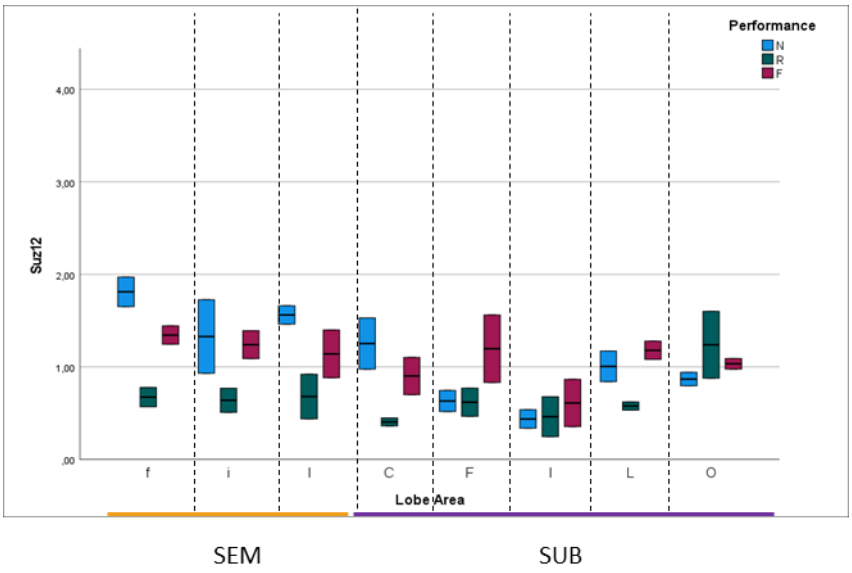


Figure 24 Suz12 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Two-way ANOVA in SUB (3x5 Performance*Brain areas; Performance $F_{(2,15)}$=3.349; p=0.063; Brain area $F_{(4,15)}$=3.114, p=0.047; Performance*Brain area $F_{(8,15)}$=1.894, p=0.136) followed by Tukey post hoc comparisons revealed that gene expression depended on the area considered but no statistical significance was found between the experimental groups for the brain areas considered.

Ov-Eed/Polycomb protein EED (Eed)

The expression of Polycomb protein EED expression resulted to change between SEM and SUB, but resulted to be mainly due to octopuses coded as remembering ($F_{(1,14)}$=1.398, p=0.257 for naïve; $F_{(1,14)}$=5.753 p=0.031 for remembering; $F_{(1,14)}$=1.211, p=0.290 for forgetting; $F_{(2,15)}$=4.646, p=0.027

for SEM; $F_{(2,27)}$ = 3.095, p=0.062 for SUB; Forgetting vs Remembering, p=0.022). A two-way confirmed that Eed expression was related to behavioral performance and brain areas considered (Performance: $F_{(2,9)}$= 15.500; p=0.001; Brain area: $F_{(2,9)}$=6-997, p=0.015; Performance*Brain area: $F_{(4,9)}$=6.763, p=0.008).

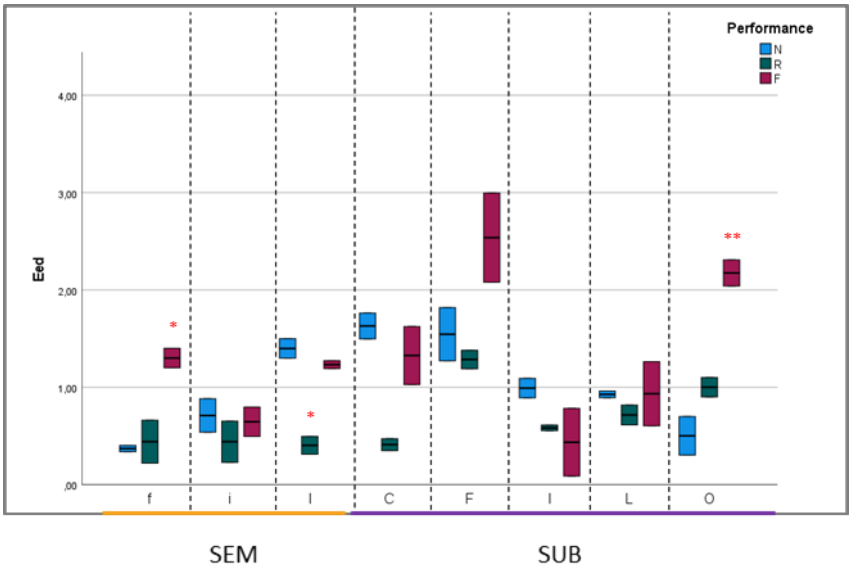


Figure 25 Eed expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Significant differences in relative gene expression were observed at the level of the anterior SEM for 'forgetting' and posterior part of SEM for 'remembering' animals (SEM f_F vs SEM f_N p=0.019; SEM l_R vs SEM l_N p=0.012). As for the sub-oesophageal mass, a two-way ANOVA showed that the posterior area of the SUB was interested to a significant Eed up-regulation in forgetting animals(3x5 Performance*Brain areas; Performance: $F_{(2,15)}$= 12.103; p<0.001; Brain area: $F_{(4,15)}$=11.320, p<0.001; Performance*Brain area: $F_{(8,15)}$=5.663, p=0.002; SUB O_F vs SUB O_N p=0.004).

Histone-lysine N-methyltransferase EZH2 (Ezh2)

The Histone-lysine N-methyltransferase Ezh2 expression in *O. vulgaris* resulted elevanted in the SEM than in SUB in naïve and forgetting animals ($F_{(1,14)}$= 6.514, p=0.023 for naïve; $F_{(1,14)}$=0.090, p=0.768 for remembering; $F_{(1,14)}$=4.850, p=0.045 for forgetting). Ezh2 expression was affected by behavioral

outcome only in the SEM ($F_{(2,15)}$ =5.979, p=0.012 for SEM; $F_{(2,27)}$ = 1.004, p=0.380 for SUB). Furthermore, animals coded as remembering had a lower expression of this gene when compared to naïve (Remembering vs naïve p=0.01, for SEM). Similarly gene expression was influenced by behavioral outcome and linked to the octopus brain area (3x3 Performance*Brain areas; Performance: $F_{(2,9)}$=19.160; p<0.001; Brain area: $F_{(2,9)}$=10.087, p=0.005; Performance*Brain area: $F_{(4,9)}$=4.723, p=0.025). Tukey post-hoc comparisons confirmed that areas of interested to significant changes in the expression of Ezh2 resulted to be the posterior part of the supra-oesophageal mass (SEM l_R vs SEM l_N p=0.003, for remembering). At the level of the sub-oesophageal mass the expression of gene at different parts of the SUB did not resulted linked to the learning experience (3x5 Performance*Brain areas; Performance: $F_{(2,15)}$=1.872; p=0.188; Brain area: $F_{(4,15)}$=6.451, p=0.003; Performance*Brain area: $F_{(8,15)}$=1.188, p=0.368).

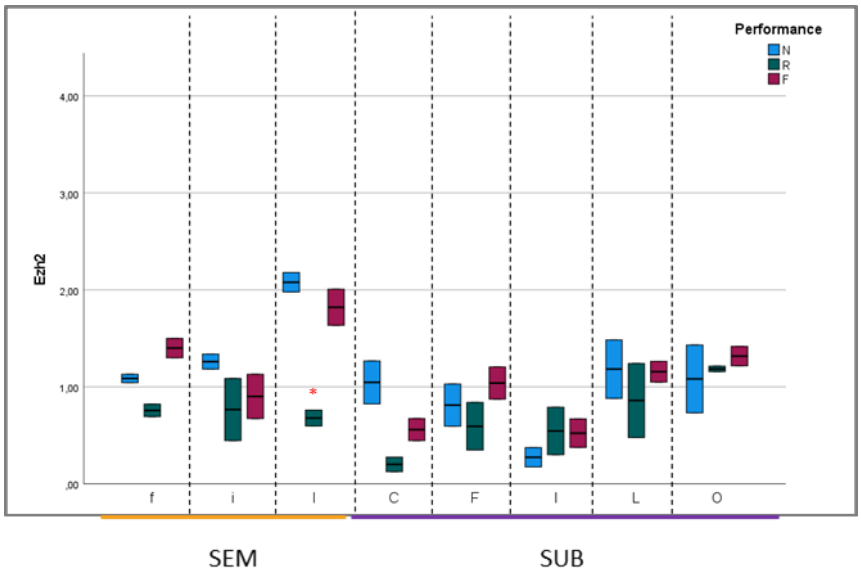


Figure 26 Ezh2 expression level in different brain areas considered SEM (f,i,l) and SUB (C,F,I,L,O). The distribution is shown by light blue (naïve group), green (remembering group) and red (forgetting group). Vertical box plot as median (lines), 25th and 75th percentiles (boxes). The differences between the groups that resulted significant by two-way ANOVA analysis are indicate in red as follows: * significant, p< 0.05; ** highly significant, p < 0.01, *** very highly significant, p < 0.001

Gene expression changes as a consequence of behavioral outcome after learning

I considered the $\log_2$ fold change (fc) between remembering and naïve (plotted on Y axis) and fc between forgetting and naïve (X axis), to show differential expression of genes after fear conditioning. The expression of genes upregulated in *O. vulgaris* trained to the task (both forgetting

and remembering) appear in the first quadrant; the down regulated genes after training appear in the third quadrant, and the genes differently regulated between remembering and forgetting animals will appear in the second and forth quadrants. The values closer to the I-III quadrant bisector represent the genes whose expression is similar among remembering and forgetting groups.

The main variation in gene expression for the genes considered in this Thesis and potentially involved in memory formation occurred in the anterior SEM $_f$ area of the octopus brain accounting for: superior frontal lobe, sub-frontal lobe, anterior basal lobe, part of the vertical and sub-vertical lobes (Fig. 29). The neurotrophin receptor BDNF/NT-3 growth factors receptor (NTRK2) resulted as upregulated in SEM $_f$ of trained animals, while the early growth response protein 1-B (zif268) resulted up-regulated in the same region only in forgetting animals. In the median part of the supra-oesopfageal mass (SEM i; including anterior and median basal lobe, part of the sub-vertical and vertical lobe and optic commissure) only sodium-dependent dopamine transporter (DAT) appeared down-regulated in forgetting animals. Finally, at the level of the posterior part of the SEM (SEM l; including median and dorsal basal lobe, posterior part of the sub-vertical and vertical lobes) early growth response protein 1-B (zif268) appeared down-regulated in both remembering and forgetting animals.

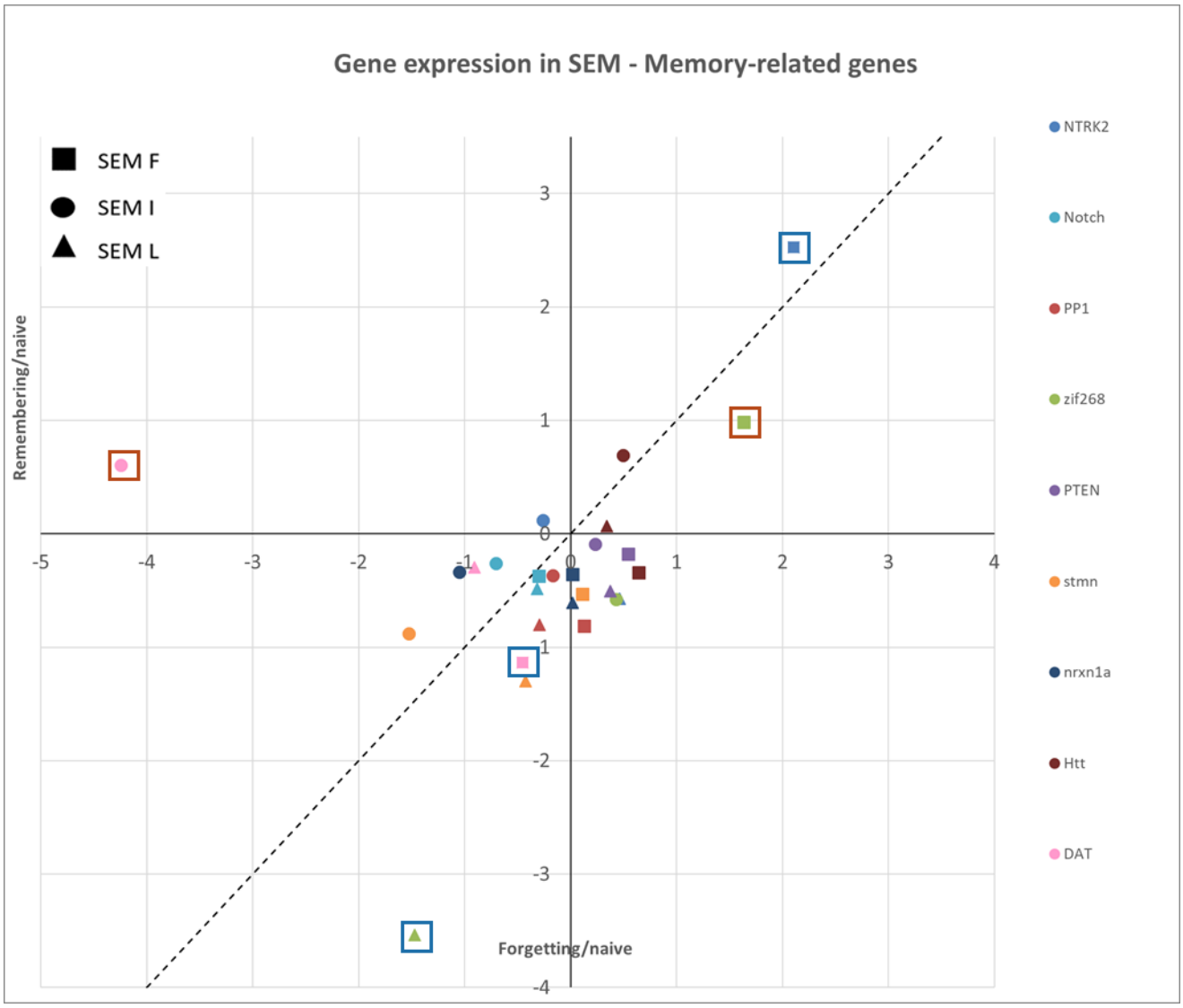


Figure 27 Regional gene expression in the SEM. Squares evidence the statistically significant variation in gene expression- remembering vs naïve (blue square); forgetting vs naïve (red square); both forgetting vs naïve and remembering vs naïve.

Memory-related gene expression variation occurring in SUB (Fig. 30) accounted for a downregulation in trained animals of Stathmin (stmn; SUB C, including brachial lobe). In SUB F (anterior chromatophore lobe and pedal lobe) BDNF/NT-3 growth factors receptor (NTRK2) was up-regulated in forgetting animals. At the level of region SUB I (including pedal and magnocellular lobes) I observed an up-regulation of serine/threonine-protein phosphatase PP1 and sodium-dependent dopamine transporter (DAT) in remembering animals. Gene expression analysis at the level of SUB L (pedal lobe, palliovisceral lobe, vasomotor lobe, posterior chromatophore lobe) revealed up-regulation of Neurogenic locus Notch protein (Notch). In SUB O (vasomotor lobe, posterior chromatophore lobe) BDNF/NT-3 growth factors receptor (NTRK2) resulted up-regulated in

remembering animals, while sodium-dependent dopamine transporter (DAT) was down-regulated in trained animals.

Figure 28 Regional gene expression in the SUB. Squares evidence the statistically significant variation in gene expression- remembering vs naïve (blue square); forgetting vs naïve (red square); both forgetting vs naïve and remembering vs naïve.

As for the genes involved in epigenetic modifications, at the level of the supra-oesophageal mass (SEM $_F$)[1] Kdm6a demethylase resulted to be downregulated in trained animals, Histone-lysine N-methyltransferase setd3 downregulated in remembering octopus, and histone-lysine N-methyltransferase 2C kmt2c increased its expression only in remembering animals. At the level of SEM i[2] Kdm6a demethylase appeared downregulated in animals coded as forgetting, and for SEM l[3]

[1] Including: superior frontal, sub-frontal, anterior basal, part of the vertical and sub-vertical lobes
[2] Including: anterior and median basal lobe, part of the sub-vertical and vertical lobes and optic commissure
[3] Including: median and dorsal basal lobe, posterior part of sub-vertical and vertical lobes

CREB-binding protein CBP (in remembering) and the histone-lysine N-methyltransferase setd3 in both remembering and forgetting octopuses (Fig. 31).

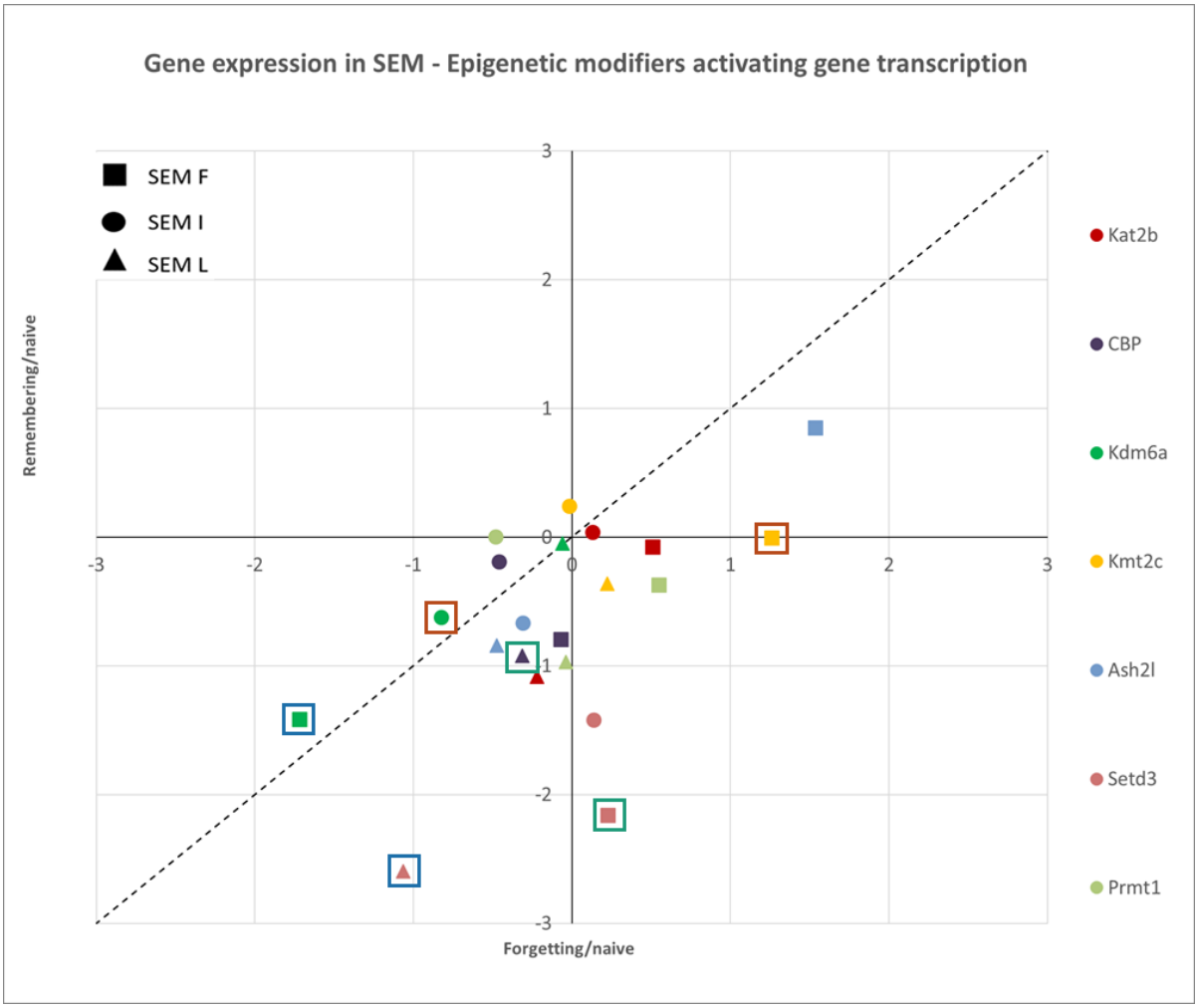


Figure 29 Regional expression of epigenetic regulators involved in chromatin decondensation in SEM. Squares evidence the statistically significant variation in gene expression- remembering vs naïve (blue square); forgetting vs naïve (red square); both forgetting vs naïve and remembering vs naïve

Epigenetic modifiers increasing chromatin condensation changed their expression level as an effect of fear conditioning and in relation to animals' performance.

In the superior frontal, sub-frontal, anterior basal, vertical and sub-vertical lobes (SEM f) I observed a variation in gene expression only for forgetting animals. In this region Polycomb group RING finger protein 1 (Ring1), Polycomb complex protein BMI-1-A (bmi1a) and Polycomb protein EED (Eed) resulted up-regulated, while histone-lysine N-methyltransferase EHMT1 (Ehmt1) downregulated. In anterior and median basal lobe, part of the sub-vertical-, vertical lobe and optic commissure (SEM

i) a decrease in histone-lysine N-methyltransferase KMT5B (Kmt5b) and Polycomb complex protein BMI-1-A (bmi1a) expression was observed.

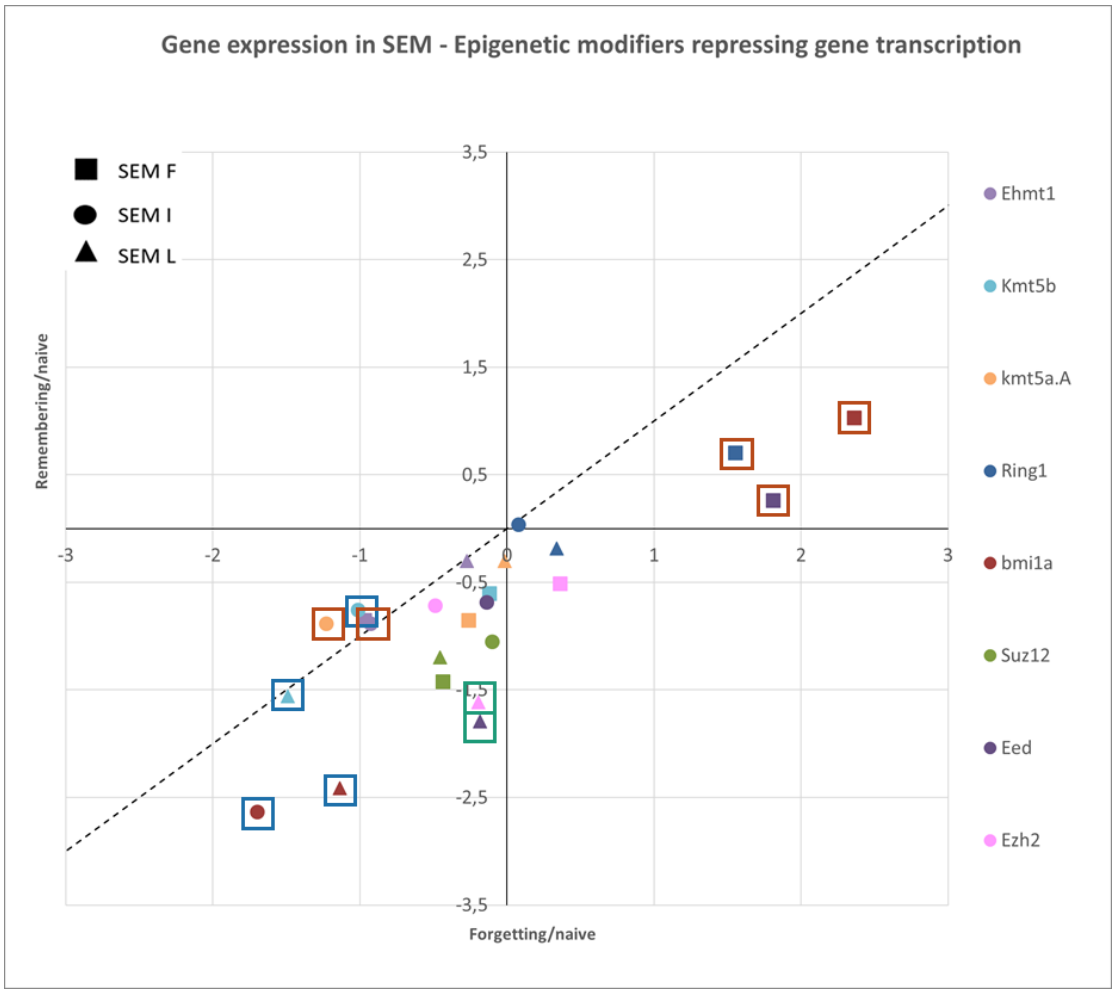


Figure 30 Regional expression of epigenetic regulators involved in chromatin condensation in SEM. Squares evidence the statistically significant variation in gene expression- remembering vs naïve (blue square); forgetting vs naïve (red square); both forgetting vs naïve and remembering vs naïve

Finally, in median and dorsal basal lobe, and posterior part of sub-vertical lobe and vertical lobe (SEM l) histone-lysine N-methyltransferase KMT5B (Kmt5b), Polycomb complex protein BMI-1-A (Bmi1a) resulted downregulated in trained animals; remembering octopuses shown downregulation of Polycomb protein EED (Eed) and histone-lysine N-methyltransferase Ezh2.

Several epigenetic modifiers contributing to chromatin relaxation changed their expression level in anterior SUB (Fig. 33).

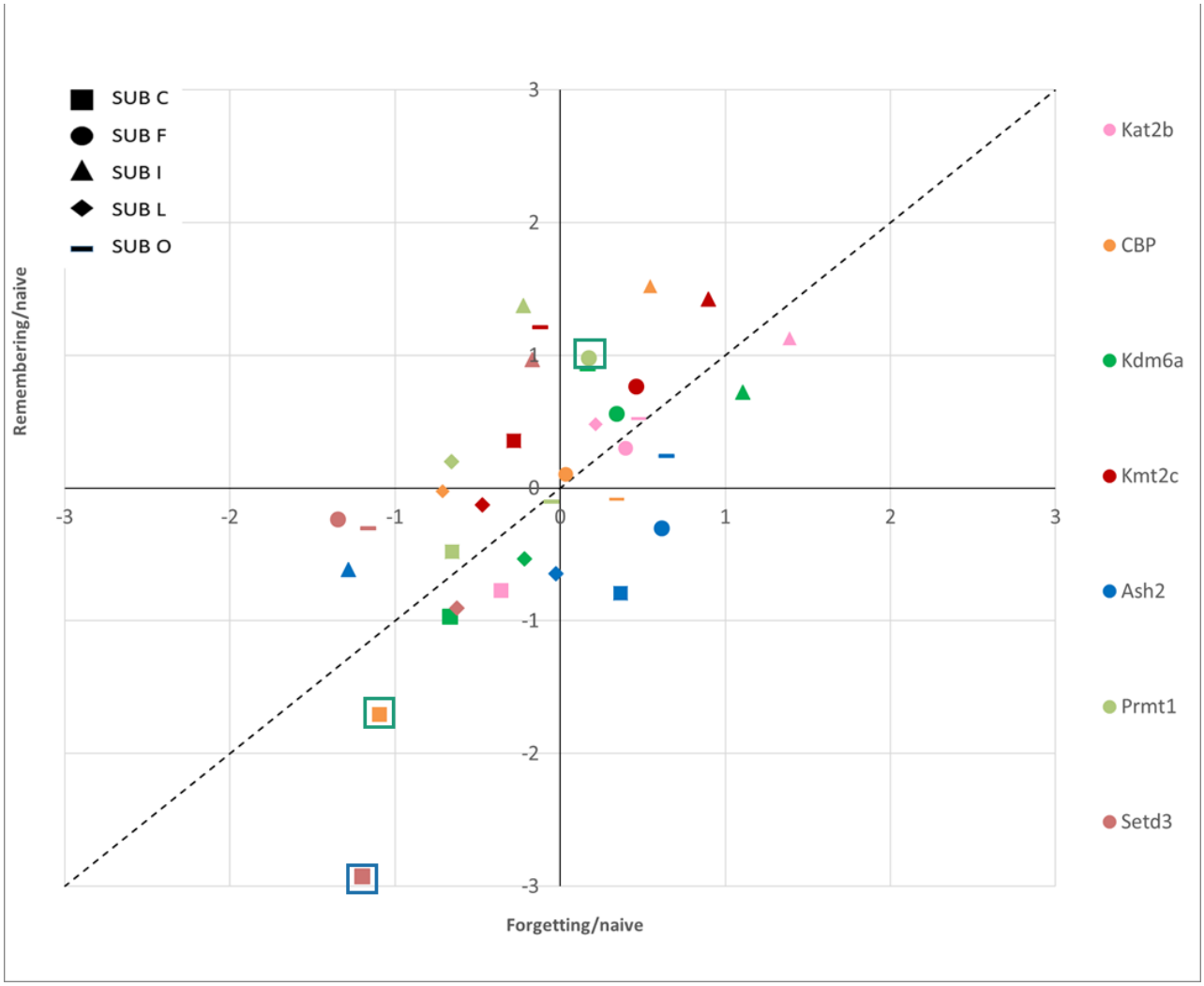


Figure 31 Regional expression of epigenetic regulators involved in chromatin decondensation in SUB. Squares evidence the statistically significant variation in gene expression- remembering vs naïve (blue square); forgetting vs naïve (red square); both forgetting vs naïve and remembering vs naïve

In SUB C (brachial lobe) CREB-binding protein CBP (remembering) and histone-lysine N-methyltransferase setd3 (Setd3) resulted down-regulated. Prmt1 appeared up-regulated in remembering octopuses in region SUB F.

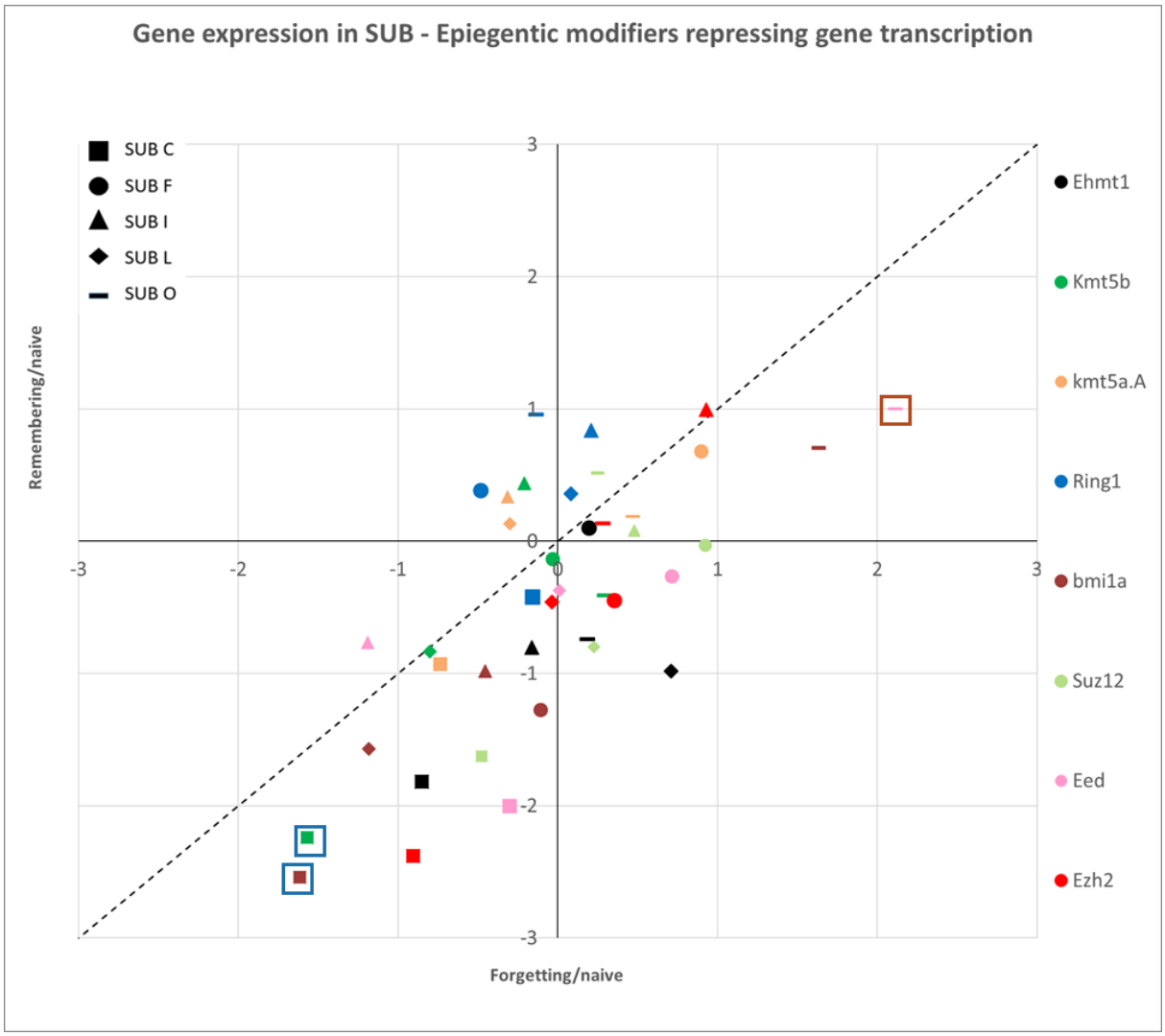


Figure 32 Regional expression of epigenetic regulators involved in chromatin condensation in SUB**.** Squares evidence the statistically significant variation in gene expression- remembering vs naïve (blue square); forgetting vs naïve (red square); both forgetting vs naïve and remembering vs naïve.

When I considered the relative expression of genes considered as epigenetic modifiers contributing to chromatin condensation I found in anterior SUB C (brachial lobe) a decrease of the expression of Polycomb complex protein BMI-1-A (Bmi1a) and histone-lysine N-methyltransferase KMT5B (kmt5b); at the level of SUB O (including vasomotor and the posterior chromatophore lobes) Polycomb protein EED resulteddownregulated.

Summary of results and overall gene expression changes in *O. vulgaris*

To summarize my results, I prepared a tabularized overview (Table 10) of the observed relative changes in gene expression in *Octopus vulgaris* supra- and subesophageal mass after training of a fear conditioning protocol.

Table 10 A tabularized overview of the genes that change their expression level in *Octopus vulgaris* SEM and SUB. Gene expression significant log_2fc variation in single areas of the octopus brain (SEM f,i,l and SUB C,F,I,L,O) is highlighted taking into account octopus performance after training of a fear conditioning paradigm.

	Gene	Brain area	Remembering	Forgetting
Memory-related genes	NTRK2	SEM f	↑	↑
	zif268	SEM f		↑
	DAT	SEM f	↓	↓
	DAT	SEM i		↓
	zif268	SEM l	↓	↓
Epigenetic modifiers contributing to chromatin relaxation	Kdm6a	SEM f	↓	↓
	Kmt2c	SEM f		↑
	Setd3	SEM f	↓	
	Kdm6a	SEM i		↓
	CBP	SEM l	↓	
	Setd3	SEM l	↓	↓
Epigenetic modifiers contributing to chromatin condensation	Ring1	SEM f		↑
	Ehmt1	SEM f		↓
	bmi1a	SEM f		↑
	Eed	SEM f		↑
	Kmt5b	SEM i	↓	↓
	kmt5a.A	SEM i		↓
	bmi1a	SEM i	↓	↓
	Kmt5b	SEM l	↓	↓
	Eed	SEM l	↓	
	Ezh2	SEM l	↓	
	bmi1a	SEM l	↓	↓
Memory-related genes	stmn	SUB C	↓	↓
	NTRK2	SUB F		↑
	PP1	SUB I	↑	
	DAT	SUB I	↑	
	Notch	SUB L		↑
	NTRK2	SUB O	↑	
	zif268	SUB O		↓
	DAT	SUB O	↓	↓
Epigenetic modifiers contributing to chromatin relaxation	CBP	SUB C	↓	
	Setd3	SUB C	↓	↓
	Prmt1	SUB F	↑	
Epigenetic modifiers contributing to chromatin condensation	Kmt5b	SUB C	↓	↓
	bmi1a	SUB C	↓	↓
	Eed	SUB O		↑

The general emerging picture is of a dynamic pattern of gene expression and of possible interplay between various gene families. It is also interesting that within the same brain mass a differential display of gene expression occurs, suggesting the dynamic neural control based on gene expression as a consequence of training experience.

Finally, and in order to provide an overview of the possible correlations between changes in the expression levels of the genes considered in this study, I utilized a Principal Component Analysis (PCA) approach followed by a clustering strategy (Yeung and Ruzzo, 2001).
To search for possible descriptors of the specific changes due to learning and memory phenomena, I ran a single PCA considering relative gene expression in naïve and animals after training of a fear conditioning paradigm. This approach allows to explore the relative abundance of gene expression values between the two responses (R/F) we observed independently from the relative low expression of the genes considered. A Varimax rotation with Kaiser normalization was applied to obtain a Rotated Component Matrix and the relative regressed values for each of the resulting components. An explanation of the strategy adopted for this analysis maybe found in Jolliffe (2002) and Abdi & Williams (2010). A tabularized overview of the results after PCAs is presented in Table 11.
I identified six components accounting for a total of 77.4% of variance. The following allocation of genes within components resulted after PCA:
Component 1 (29.0% of the total variance) - Htt, nrxn1a, PTEN, stmn, Prmt1, Eed
Component 2 (18.6% of the total variance) - DAT, Notch, CBP, Kdm6a, Ehmt1, kmt5a.A, Suz12
Component 3 (10% of the total variance) - zif268, Setd3, Kmt5b, bmi1a, Ezh2
Component 4 (8.9% of the total variance) - Kat2b, Kmt2c, Ring1
Component 5 (6.5% of the total variance) - NTRK2, Ash2l
Component 6 (4.5% of the total variance) - PP1

This strategy allowed to identify inter-relationships in the expression of genes involved in structural synaptic changes (Htt, nrxn1a, PTEN, stmn) but also genes coding for epigenetic modifiers associated to chromatin remodeling occurring during neural development (Prmt1, Eed), attributed to Component 1.

Table 11 Rotated Component matrices calculated after PCA analysis (Rotation Method: Varimax with Kaiser Normalization; rotation converged in 5 iterations) of gene expression levels for the genes considered and resulting from samples belonging to SEM and SUB of remembering, forgetting and naive octopuses. Data following real-time qPCR experiments. Marked in boldface are statistically significant correlation values and their attribution to PCA components.

Rotated Component Matrix[a]

	Component					
	1	2	3	4	5	6
NTRK2	.161	.031	-.103	.342	**.732**	.228
Htt	**.688**	.203	-.050	.325	.359	-.069
zif268	-.008	-.011	**.824**	.181	.164	-.044
nrxn1a	**.798**	-.092	.049	-.094	.175	.377
stmn	**.928**	-.048	.163	-.094	.139	.003
PTEN	**-.542**	.171	.102	.458	.239	.388
PP1	.286	.292	.057	.319	.092	**.733**
DAT	-.348	**.652**	-.038	.098	-.145	.182
Notch	.498	**.566**	-.112	.073	.409	.020
Kat2b	.223	.293	.329	**.701**	.179	-.128
CBP	.160	**.559**	.542	.319	.036	.276
Kdm6a	-.275	**.793**	.151	.042	-.125	.251
Kmt2c	.049	.088	.068	**.858**	-.008	.089
Ash2l	-.016	-.089	.460	-.036	**.746**	-.090
Setd3	.241	.249	**.731**	.203	-.265	-.093
Prmt1	**.898**	-.198	.007	.194	-.064	.087
Ehmt1	.047	**.867**	.203	-.134	.003	-.105
Kmt5b	.307	.284	**.805**	-.057	.128	.149
kmt5a.A	.270	**.561**	.202	-.024	.367	.144
Ring1	-.090	-.256	.144	**.726**	.084	.222
bmi1a	-.306	.170	**.756**	.064	.273	.179
Suz12	-.116	**.679**	.431	.186	.101	-.097
Eed	**.520**	.057	.294	.025	.678	-.021
Ezh2	-.063	.444	**.557**	.422	.175	-.336

All the genes involved in "dopamine-modulated" responses (DAT, Notch, CBP) and epigenetic modifiers associated with histones methylation (Kdm6a, Ehmt1, kmt5a.A, Suz12) were attributed to component 2. The immediate-early gene (zif268), genes involved in epigenetic modifications, (Setd3, Kmt5b, bmi1a, Ezh2) resulted to belong to component 3. Genes involved in epigenetic modifications (Kat2b, Kmt2c, Ring1) were attributed to component 4, and NTRK2 and Ash2l - genes

involved in neurotrophins response - to component 5. Finally, the sixth component include one gene, the memory suppressor protein phosphatase PP1.

Despite being preliminary and based on a simplified descriptive analysis, these results are intriguing in my view, since they show for the first time the involvement of several important molecular machineries in the regulation and establishment in response to learning in the octopus brain.

Following PCA, I carried out a hierarchical cluster analysis based on the regression scores obtained after PCAs. The discrete groupings (Figure 35) revealed that samples belonging to SEM and SUB clustered significantly separate from the remaining ones. The discrete groupings reveal that samples belonging to the SEM and SUB are not segregated in different clusters, with different areas here considered of each mass being attributed to a given cluster. The identification of samples attribution to experimental outcomes (naïve, forgetting and remembering *O. vulgaris*), also provide further support of the hypothesis that an active orchestration in the modulation of behavioral response after training and learning occurs in the animal.
This require further analysis, and I hope that my data will serve as the basis for future experiments.

Overall, the topology resulting from the hierarchical cluster analysis from data derived from regression scores after the PCA indicate a clear pattern of gene expression considering epigenetic modifiers and memory related genes in octopus in samples here considered.

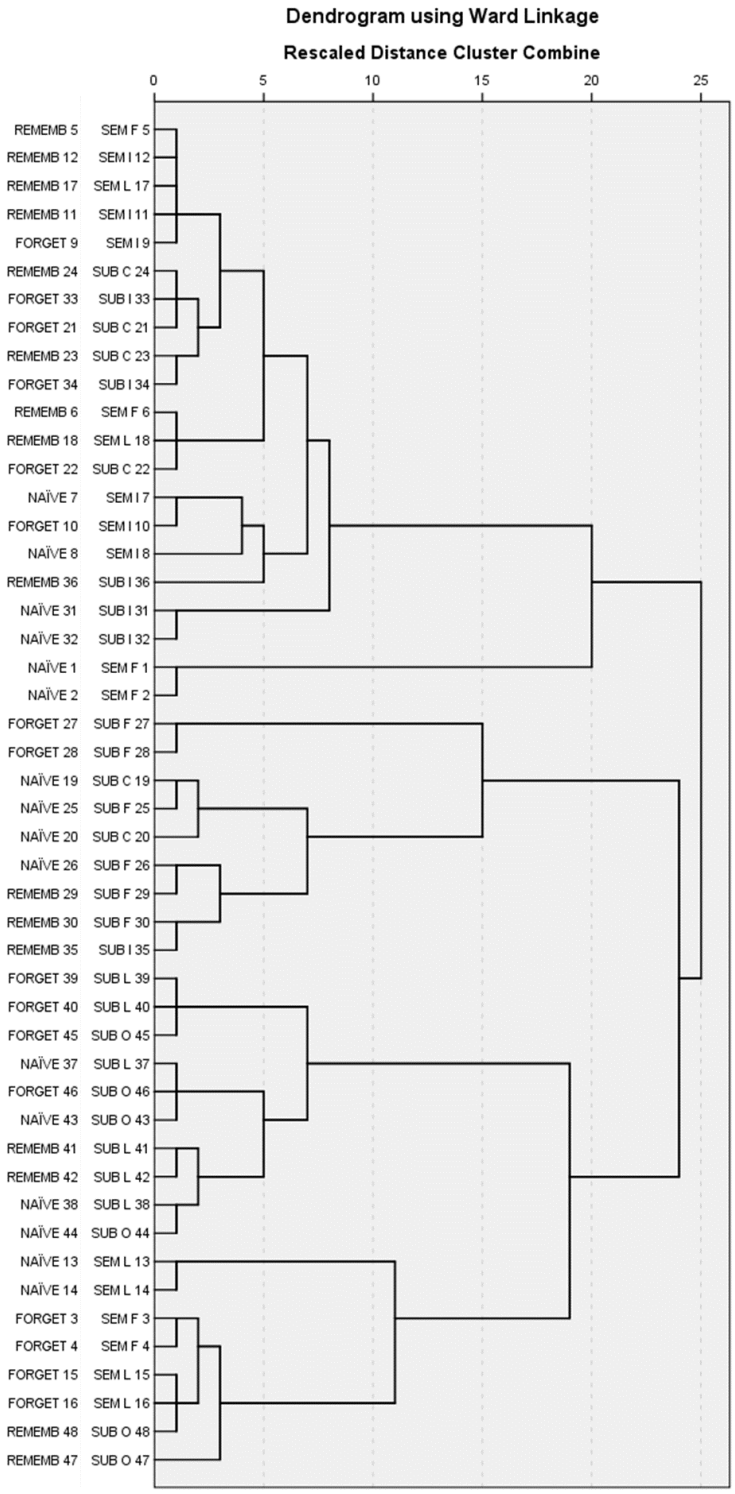


Figure 33. Hierarchical cluster analysis of the samples considered for this study allocated on the basis of the relative gene expression.

Overall, these results are novel and unprecedented.

7. Discussion

Protein synthesis dependent learning in octopus

Even though the biochemical nature of memory trace is still unknown, numerous studies evidenced the role of gene transcription and translation as key steps toward long-term memory formation (e.g., Agin et al., 2003; Davis & Squire, 1984; Lefer et al., 2013).

The structural changes occurring during LTM are supported by the synthesis of proteins that allow the neuronal reorganization. Furthermore, a plethora of memory-related genes (e.g., Cavallaro et al., 2002; Guan et al., 2002; E. R. Kandel, 2001) are induced when long-term memory is established and those appear to be ultimately regulated by epigenetic modifications (Guan et al., 2002; Gupta et al., 2010; Miller et al., 2008).

Despite the central role of *Octopus vulgaris* in behavioral neuroscience and the increasing number of scientific publications over the last decade studying octopus and other cephalopods at molecular resolution, our knowledge on the molecular and cellular mechanisms underlying *O. vulgaris* neural and behavioral plasticity is still limited.

Among cephalopods, *Sepia officinalis* is the unique for which a well-defined time-window for protein synthesis occurrence was defined (Agin et al., 2003). In *O. vulgaris, in vitro* attempts to verify the memory dependance on protein synthesis using LTP (Turchetti-Maia et al. 2018) did not manage to provide a well-defined framework.

However, CREB activation as a result of fear memory consolidation (Zarrella, 2011) suggests that molecular mechanisms of memory formation are conserved in *O. vulgaris*. This work still remains the sole one available attempting to addressing this topic.

The results obtained from protein synthesis inhibition experiments analyzed for the purposes of this PhD project (see Appendix 2) revealed that cycloheximide could induce prolonged protein synthesis inhibition (around 80% 1h after injection; around 65%, 4 h after injection). Protein synthesis inhibition did not alter octopuses' ability to learn an avoidance learning task, as proved by the fact that acquisition was not influenced by the drug injection. However, cycloheximide impaired octopuses' ability to retain and recall the memory trace. However, memory dependance on protein synthesis (since drug administration before training was able to erase memory formation) has been challenged by further experiments suggesting that cycloheximide induced a state-dependent effect. When injections occurred before training, a memory impairment at the testing phase was observed, but when the drug was administered just after the training, the memory recall was not affected.

When administration of the drug or of the control solution occurred before training and before The suggested state-dependent effect induced by cycloheximide in octopus did not allow to rule out its effects in consolidation by a reduced protein-synthesis.

The present data represent to our knowledge, one of the few cases of state-dependent learning described among invertebrates (see: Pompilio et al., 2006; Rankin, 2004).
Protein synthesis inhibitors have already been shown to cause state-dependent effects in learned task (Bradley & Galal, 1988; Lee et al., 1989). However, these are linked to reported cycloheximide side effects in vertebrates including the alteration of adrenocortical hormones resulting in amnesia of avoidance response (Nakajima, 1975).
I cannot exclude that our findings are linked to the training protocol utilized. The massed training protocols applied for induce fear conditioning in *O. vulgaris* involve a single training session and relatively short inter-trial intervals. These conditions are ascribable to cases in which *de novo* protein synthesis is not involved in memory formation. Thus, it is possible that massed avoidance learning could have brought the formation of a type of memory which may not result to be protein-synthesis dependent, at least at the timing tested in the experiments with octopus included in this thesis.
However, previous evidences applying similar experimental behavioral paradigms in octopus found robust memory recall lasting for weeks or months (for review see Borrelli & Fiorito, 2008).

Forms of memory which are protein synthesis independent have been observed to be dependent on the persistent activation of kinases (Wittstock et al., 1993); this seems not to be the case, since CREB is activated in this form of learning in *O. vulgaris* (Zarrella, 2011). One other hypothesis is that in the case of massed training in octopus remaining levels of protein synthesis in the presence of the inhibitor are sufficient for memory establishment. Future experiments are needed to elucidate the biological mechanisms underlying long-term avoidance learning in *Octopus* and to explore the consolidation dependency on protein synthesis in this animal and in different learning paradigms.

Gene expression and fear conditioning in octopus

Despite the fact that data available to me did not allow to prove whether memory formation is a protein-synthesis dependent process in *O. vulgaris*, intriguing results derived from the analysis of gene expression in the central nervous system of animals trained through fear conditioning.

In recent years cephalopods research has been boosted by a series of independent approaches based on the study of the molecular and gene machinery in these invertebrates.

In a time-window of less than five years, five cephalopod genomes have been published for *Octopus bimaculoides* (Albertin et al., 2015), *Callistoctopus minor* (Kim et al. 2018a, 2018b)*, Euprymna scolopes* (Belcaid et al., 2019)*, Architeuthis dux* (Da Fonseca et al., 2020) and *O. vulgaris* (Zarrella et al., 2019). The knowledge on the underlying molecular and cellular machinery is increasing for some cephalopod species. However, a scarce number of studies adopted molecular biology approaches to investigate the molecular mechanisms of long term memory formation (Zarrella, 2011).

This PhD represents the first attempt after Zarrella (2011) where an analysis of the effects of learning and memory recall in *O. vulgaris* is carried out at the level of gene expression.

From the evaluation of gene expression in animals trained through fear conditioning, significant changes in gene expression in defined areas of the octopus's brain were observed. The pattern of changes, for most of the genes, resulted to be two-folds between the conditions considered, i.e. trained versus naïve; forgetting versus naïve; remembering versus naïve; forgetting versus remembering.

Summary of findings

The following notes are listed considering supra-oesophageal (SEM) and sub-oesophageal (SUB) masses as separate entities.

The **NTRK2** orthologue has been reported in rodents up-regulates following both, electrophysiological and behavioural experiments. In particular, up-regulation was observed following *in vivo* LTP induction in dentate gyrus (Bramham et al., 1996) as well as following social recognition memory formation (Broad et al., 2002) and spatial learning (Gómez-Pinilla et al., 2001). I found a marked up-regulation of Ov-NTRK2 in a region of SEM (SEM f) that includes the superior frontal lobe system, a center of regulation of behavioral response and the vertical and sub-vertical lobe system, centers involved in visual and tactile memory (review in Sanders, 1975). Furthermore, the up-regulation was significantly higher for remembering when compared to forgetting animals. It is possible to suppose that NTRK2 expression is induced after training and the differential level of

induction observed between remembering and forgetting octopuses contributes to the different memory abilities observed.

The immediate early gene **zif268** shows very sharp up-regulation following fear conditioning and LTP induction following learning in other species (peak after 30 minutes, but still visible one hour after testing; Hall et al., 2001; Veyrac et al., 2014). In my experiments the gene resulted to be up-regulated, as expected, but only in "forgetting" animals and in the SEM region including the superior frontal lobe system, the anterior basal lobe and part of the vertical and sub-vertical lobes.
Since memory recall is evaluated 1h after testing phase it is possible to hypothesize that zif268 up-regulation in remembering animals reached the peak of its expression faster than in forgetting for which the gene induction is observed. The down-regulation is observed in the basal lobe system and may be related to a specific role in motor decision-making control.

I observed a down-regulation of ***DAT*** occuring mainly in the regions of SEM including the superior frontal system, the vertical and sub-vertical lobe thus suggesting that these are the main regions where modulation of reward occurs both for remembering and forgetting animals.
Dopamine transporter increased expression in the SUB has been observed in pedal and magnocellular lobes of remembering animals. While a downregulation of DAT resulted in the posterior areas of the SUB of forgetting animals. **zif268** resulted down-regulated in the posterior part of the SEM.
In mice, **PP1** overexpression in hippocampus has been associated with poor performances. In my experiments I found PP1 induction in remembering animals, but in a region known to be related to motor control (pedal lobe, SUB) and to defence and fast escape reaction (magnocellular lobes, SUB), thus let us to hypothesize that PP1 induction in these areas may be related to the "silencing" of the animal's attack response.

Since CREB had been found activated following fear conditioning in SEM (Zarrella, 2011), it would have been expected that an up-regulation of the CREB-binging protein and other acetyltransferases (Kat2b) could occur. Their induction is reported following spatial learning in mice (Bousiges et al., 2010b). No significant up-regulation was observed in supra-esophageal mass in octopus. CBP appeared downregulated in basal lobes in SEM and in brachial lobe in the SUB in *O. vulgaris*.

I found Histone methyltransferase (Kmt5b, kmt5A.a) downregulated in SEM I, i.e. the anterior and median basal lobe and in vertical and sub-vertical lobes of the octopus supra-oesophageal mass. Ring1 and bmi1a – belonging to the Polycomb repressing complex 1 – appeared up-regulated in SEM f (including the superior frontal lobe and sub-frontal) of forgetting animals. Eed and Ezh2 (part of the Polycomb repressing complex 2) resulted downregulated only in the median and dorsal basal lobe (SEM l) in "remembering" octopus. Eed appeared up-regulated in superior frontal, sub-frontal and vertical and sub-vertical lobes (SEM f). The differential expression of genes of Polycomb complexes in octopuses with different performances after fear conditioning suggests a potential role for these genes on memory consolidation.

At the level of the sub-oesophageal mass CBP appeared downregulated. The histone methyltransferase Prmt1 has a high basal expression level in the SUB, as derived from RNA-seq data analysis, and is the only "opening chromatin" epigenetic modifier appeared to be induced in the SUB after fear conditioning, mainly at the level of the brachial lobe of remembering animals, thus suggesting its contribution to the animal locomotion control. Several histone methyltransferases (bmi1a, Kmt5b) have been downregulated after training in brachial lobe.

Table 12 A tabularized overview of the genes that change their expression level in *Octopus vulgaris* SEM and SUB. Gene expression significant log_2fc variation in single areas (SEM f,i,l and SUB C,F,I,L,O) is highlighted in relation with animals performance after fear conditioning - R: remembering; F: forgetting.

	Gene	R	F	Brain area	Brain area
Memory-related genes	NTRK2	↑	↑	SEM f	Superior frontal lobe, sub-frontal lobe, anterior basal lobe, vertical lobe and sub-vertical lobe
	zif268		↑	SEM f	
	DAT	↓	↓	SEM f	
	DAT		↓	SEM i	Anterior and median basal lobe, sub-vertical lobe, vertical lobe and optic commissure
	zif268	↓	↓	SEM l	Median and dorsal basal lobe, sub-vertical lobe and the posterior part of vertical lobe.
Epigenetic modifiers contribuing to chromatin relaxation	Kdm6a	↓	↓	SEM f	Superior frontal lobe, sub-frontal lobe, anterior basal lobe, vertical lobe and sub-vertical lobe
	Kmt2c		↑	SEM f	
	Setd3	↓		SEM f	
	Kdm6a		↓	SEM i	Anterior and median basal lobe, sub-vertical lobe, vertical lobe and optic commissure
	CBP	↓		SEM l	Median and dorsal basal lobe, sub-vertical lobe and the posterior part of vertical lobe.
	Setd3	↓	↓	SEM l	
Epigenetic modifiers contribuing to	Ring1		↑	SEM f	Superior frontal lobe, sub-frontal lobe, anterior basal lobe, vertical lobe and sub-vertical lobe
	Ehmt1		↓	SEM f	
	bmi1a		↑	SEM f	

	Gene	R	F	Brain area	Brain area
chromatin condensation	Eed		↑	SEM f	
	Kmt5b	↓	↓	SEM i	Anterior and median basal lobe, sub-vertical lobe, vertical lobe and optic commissure
	kmt5a.A		↓	SEM i	
	bmi1a	↓	↓	SEM i	
	Kmt5b	↓	↓	SEM l	Median and dorsal basal lobe, sub-vertical lobe and the posterior part of vertical lobe.
	Eed	↓		SEM l	
	Ezh2	↓		SEM l	
	bmi1a	↓	↓	SEM l	
Memory-related genes	stmn	↓	↓	SUB C	Brachial lobe
	NTRK2		↑	SUB F	Anterior chromatophore lobe, pedal lobe
	PP1	↑		SUB I	Pedal and magnocellular lobes
	DAT	↑		SUB I	
	Notch		↑	SUB L	Pedal lobe, palliovisceral lobe, vasomotor lobe, posterior chromatophore lobe
	NTRK2	↑		SUB O	Vasomotor lobe, posterior chromatophore lobe
	zif268		↓	SUB O	
	DAT	↓	↓	SUB O	
Epigenetic modifiers contribuing to chromatin relaxation	CBP	↓		SUB C	Brachial lobe
	Setd3	↓	↓	SUB C	
	Prmt1	↑		SUB F	Anterior chromatophore lobe, pedal lobe
Epigenetic modifiers contribuing to chromatin condensation	Kmt5b	↓	↓	SUB C	Brachial lobe
	bmi1a	↓	↓	SUB C	
	Eed		↑	SUB O	Vasomotor lobe, posterior chromatophore lobe

I found a marked up-regulation of neurotrophine receptor *NTRK2* in a region of SEM that includes the superior frontal lobe system and the vertical and sub-vertical lobe system. Also, the immediate early gene zif268 resulted to be up-regulated, as expected, but only in "forgetting" animals. I found down-regulation in posterior SEM (essentially constituted by the basal lobe system) and down-regulation of *DAT* in the same area. The relative expression changes of some of the genes in the SUB also suggests a potential involvement of this neural structure in the control of the behavioral response. The fact that a dynamic expression of genes occur in this structure let us to suggest that that neural plasticity occur also in this area, where no indication about neural plasticity in this mass has been previously suggested (Young, 1991; Zarrella, 2011).

My data also provide preliminary exploration of possible involvement of genes acting as epigenetic modifiers during learning in the octopus; this is also un-precedented.

In my data their expression changes as a function of correctness of memory formation, i.e. in a different way in remembering and forgetting octopus. Although the variation of their expression is

not informative about the presence of the mark that these enzymes lay down, they represent an indicator for the potential involvement of epigenetic machinery in the learning process.

I found histone methyltransferase (Kmt5b, kmt5A.a) downregulated in SEM, histone acetyltransferases CBP and Kat2b with no significant variation, apart from a downregulation of CBP in remembering animals. Ring1 and bmi1a – belonging to the Polycomb repressing complex 1 – appeared up-regulated in SEM of forgetting animals. Eed and Ezh2 - part of the Polycomb repressing complex 2 - downregulated in SEM of remembering animals.

Closing remarks

The PCA allowed to explore some correlations between genes. I identified six components including genes involved in neural ultrastructure modifications, genes related to dopamine and neurotrophin responsiveness and epigenetic regulation in neural development, epigenetic modifiers and the memory suppressor. My data suggested that the relative expression of genes involved in structural synaptic changes (Htt, nrxn1a, PTEN, stmn) and those coding for epigenetic modifiers associated to chromatin remodeling occurring during neural development (Prmt1, Eed) are inter-related (Component 1). Similarly, the genes involved in "dopamine-modulated" responses (DAT, Notch, CBP) and epigenetic modifiers associated with histones methylation (Kdm6a, Ehmt1, kmt5a.A, Suz12) were attributed to the same component (2). Their relative expression accounted for more than 50% of the overall variance.

The immediate-early gene (zif268), genes involved in epigenetic modifications (Setd3, Kmt5b, bmi1a, Ezh2) also appeared inter-related (Component 3) as for the case of genes involved in epigenetic modifications (Kat2b, Kmt2c, Ring1; Component 4), and NTRK2 and Ash2l - genes involved in neurotrophins response (Component 5). Remarkably, 4.5% of variation resulted to be attributed to a single gene, the memory suppressor protein phosphatase PP1.

The application of hierarchical cluster approach shown a discrete groupings revealing that samples belonging to the SEM and SUB are not segregated in different clusters, with different areas here considered of each mass being attributed to a given cluster. The identification of samples attribution to experimental outcomes (naïve, forgetting and remembering *O. vulgaris*) provided further support to the hypothesis that an active orchestration in the modulation of behavioral response after training and learning occurs in the animal at gene level.

In conclusion, although it was not possible to establish protein-synthesis dependence of fear memory in *O. vulgaris*, due to potential state-dependent effects (a finding that merits future studies in other context and with other substances), the gene expression changes observed following fear conditioning memory recall let me to support the hypothesis that in *O. vulgaris* a transcription-dependent memory formation occurs.

Furthermore, a significant change in the relative expression profile of genes was observed in different behavioral outcomes after learning in *O. vulgaris*. The active variation of the expression of selected genes provides – for the first time - insights about their involvement in memory formation and their potential role in defining the learning performances.

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Addenda

Summary of the Contents

Appendix 1. A tabularized overview of *O. vulgaris* central nervous system, its lobes and main functions

Data about cell number and size distribution are derived from Young (1963) and Zarrella (2011). SEM: supra-oesophageal mass; SUB: sub-oesophageal mass; OL: optic lobes. For reference see also Young, (1971).

	LOBE	FUNCTION	N OF CELLS 10^3	CELL DIMENSION (µm diameter)
SEM	**Buccal lobe**	Controlling eating, motor control of feeding	>1235	5≤10
	Inferior frontal system	Elaboration of chemotactile information from arms	>5308	<5 5≤20 Sub-frontal <3
	Superior frontal system	Regulates behavioural responses	1854	Lateral 5-10 Medial <5
	Vertical lobe system	Regulation of exploratory behavior, visual and tactile learning and memory	25066	Amacrine cells ≈3 5-10
	Sub-vertical lobe	Regulation of exploratory behavior, learning and memory	810	5-25
	Basal lobes system	Higher center for motor control Regulate posture, head and eyes movement, and feeding (anterior b.l.); controls action of defence and avoidance (dorsal b.l.); Movements of funnel and mantle (median b.l.)	2605	<5 5≤15
	Pre-commissural lobe	"meeting-point" for many fibre systems; main output channel of vertical lobe system	78	<5 10-15
OL	**Optic lobe**	centres for visual processing and integrative responses	64470	<5-10
	Olfactory lobe	chemoreceptor function	136	<5-10
	Peduncle	Control of movement, including attack		<5-10
	Optic gland	endocrine function	142	5-15
SUB	**Brachial lobe**	Arms and suckers motor coordination	341	5 (pre-brachial) 25(post-brachial)
	Anterior chromatophore lobe	Arms, head and eyes movement; controls chromatophores of the anterior part of the body	217	<5-20

	LOBE	FUNCTION	N OF CELLS 10^3	CELL DIMENSION (µm diameter)
SUB	**Pedal lobe**	Arm, head and eyes movement; controls chromatophores of the anterior part of the body Other functions (see Young, 1971)	243	<5-20
	Palliovisceral lobe	Control of the mantle and the viscera	108	<5-25
	Vasomotor lobe	Control of blood vessels	1307	<5-15
	Posterior chromatophore lobe	controls chromatophores of the posterior part of the body	309	5-20
	Magnocellular lobe	Intermediate motor center between SEM and SUB. Controlling defense and escape behavior	581	5≤20

Appendix 2. Protein synthesis inhibition and its effects on memory recall in octopus – data analysis from previous experiments

As mentioned in the Introductory sections of this Thesis, it is largely acknowledged in scientific literature that memory - in its long form – is protein synthesis dependent. The evidence is consistent from a large number of studies in both vertebrates and invertebrates (for refs see Main Thesis: e.g., Barraco and Stettner 1976; Davis and Squire 1984; Vasquez 1976). Several experiments shown that pharmacological treatments (protein synthesis inhibitors) are effective if administrated in a specific time-window in which memory is dependent on protein synthesis (for refs see Main Thesis: e.g., Davis and Squire 1984; Goelet et al. 1986). Additionally, the "intensity" of training it has been correlated to protein synthesis: inhibitors are less effective at impairing memory when the training strength increases (for refs see Main Thesis: e.g., Meiri and Rosenblum 1998). Reconsolidation or extinction also are known to depend on protein synthesis, and the duration of re-exposure to a learning context can guide the memory toward one or the other outcome (Pedreira & Maldonado, 2003). I will not cover reconsolidation as phenomenon in this Thesis.

Although learning and memory have been widely studied in octopus, little is known about the molecular basis and the time course of consolidation.

In *Aplysia* the long-term facilitation is impaired by both transcriptional and translational inhibitors (for refs see Main Thesis: e.g., Goelet et al. 1986), and in the cuttlefish the protein inhibitor cycloheximide revealed a specific time-window to protein inhibition sensitivity (for refs see Main Thesis: Agin et al. 2003). Anisomycin was found to have no effect on octopus LTP induction or maintenance (for refs see Main Thesis: Turchetti-Maia et al. 2018).

However, the formation of long-term memory of fear conditioning in *O. vulgaris* is associated with an increased phosphorylation of CREB (Zarrella, 2011). The activation of CREB is considered a molecular switch from labile, short memory trace to a long-lasting form, in both invertebrates and vertebrates; it initiates a transcription cascade bringing to the protein synthesis-dependent memory phase (for refs see Main Thesis: e.g., Kandel 2001; Silva et al. 1998; Tully et al. 2003).

Although several pharmacological studies have been carried on in *O. vulgaris* (for refs see Main Thesis: Fiorito et al. 1998; Robertson 1994; Robertson et al. 1996; Robertson et al. 1994), none of them investigated the role of *de novo* protein synthesis in long-term memory.

Here I analysed and report a series of experiments carried out to study the effect of a significant, selective and reversible protein synthesis inhibition on octopus learning and retention abilities in an

avoidance learning task. The experiments investigated *O. vulgaris* long-term avoidance memory and the need for *de novo* protein synthesis including the evaluation of a possible state-dependency effect of the protein inhibitor.

As mentioned in the main Thesis, I accessed data from experiments carried out before 2000 and that have never been published. Accessing to these data is compliance with Open Data principles and address 3Rs principles as stated in Directive 2010/63/EU.

Data I accessed are based on five different experiments.

In experiment 1, several inhibitors were tested and the most efficient (cycloheximide, CXM) was chosen for the following steps. In experiment 2 cycloheximide kinetics was investigated to evaluate inhibition effectiveness over time. As a consequence (experiment 3) it was verified any effect of the inhibitor injection on the motivational drive of octopus. Next (fourth experiment) it was evaluated the effect of cycloheximide on training performance. Finally, in the last experiment addressed whether memory impairment observed in animals injected before training, was caused by true amnesia or by drug state-dependency effect.

As mentioned, data included in this section received an ethical clearance (AWB-SZN case #12/2020).

For my contribution to this part see 'Contribution of this PhD project to various sections and experiments'

Description about animals, their care and related information are available as Appendix 3.

Evaluation of efficiency of inhibition of several protein synthesis inhibitors in *Octopus vulgaris* brain.

A total of 24 naïve *O. vulgaris* were randomly divided into four groups: (ANI) anisomycin injected (12,5 mg/ml), (CXM) cycloheximide injected (10mg/ml), (EME) emetine injected (0,1 mg/ml); as control (SW) animals received sea water injection. All the experiments followed protocols developed by Agnisola and coworkers (Agnisola et al., 1996; G. Fiorito et al., 1998).

Thirty minutes after the first injection all animals were injected again with 100μl of a mixture of [3H]aa tritiated amino acids. Again, 30 minutes after the second injection, the animals were anesthetized in 2% ethanol in sea water (in the attempt to limit other chemical interference with protein synthesis) for 10 minutes and sacrificed. Experiments have been carried out between 1998 and 1999.

Supraoesophageal mass, optic lobes and hepatopancreas (used as control tissue) were collected and processed.

Anisomycin, Cycloheximide and Emetine utilized in these experiments were purchased from SIGMA. All these drugs block protein synthesis at translational level, with different mechanisms (Davis & Squire, 1984). The powder compounds were dissolved 30 minutes before use. Anisomycin was dissolved in filtered (0.45μm filter) sea water, and the pH was adjusted to 7.8 using 3M HCl. Emetine was prepared in distilled water and pH was adjusted to 6.4 (at higher pH the solute forms flocculates). Cycloheximide was dissolved in filtered sea water.
Experiments performed by Dr. G. Fiorito's Research Group at Stazione Zoologica Anton Dohrn in previous years allowed to establish the correlation between the optimal protein inhibitor dose and the level of inhibition on *in vitro* brain preparations (Di Dato, 2000). From these data, the dose of inhibitor was chosed. Cycloheximide dose was determined by analogous *in vitro* experiments performed by previous studies in octopus.

Tritiated leucine injection was used to evaluate amino acids incorporation into proteins in the first and the second experiment. Thirty minutes after the first injection, all animals were injected again with 100μl of a mixture of [3H]aa tritiated amino acids (Amersham Pharmacia Biotech: an aqueous sterile solution containing 2% of ethanol, and a mixture of Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val; 37MBq/ml, 1mCi/ml).
The 2% ethanol contained in the Amersham solution did not have an anesthetic effect. This was proved through the administration of 100 μl of a 2% ethanol in sea water solution to naïve animals. Until one hour after the injection, the animals maintained their respiratory frequency (Agnisola et al., 1996) and their motivational drive to attack crabs (Fiorito, unpublished). Anesthesia was performed in 2% ethanol in sea water for 10 minutes, starting 30 minutes after the [3H]aa tritiated amino acids injection.
All procedures of tissue homogenization, protein assay, and radioactivity measurement were carried on ice. Samples were sonicated in NaOH 0,1N (Branson Sonifier). A 2 ml aliquot of homogenates was precipitated in TCA 12% for 30 minutes on ice. Samples were centrifuged 16000 x g, 20 minutes, 4°C. The supernatant was stored while the pellet was resuspended in TCA 12% for 20 minutes on ice and then centrifuged 16000 x g, 20 minutes, 4°C. The pellet was resuspended in TCA 12% for 20 minutes on ice and then centrifuged 16000 x g, 20 minutes, 4°C. The pellet was

precipitated in 2 ml NaOH 0,1N. The protein quantification of the precipitate was performed using the Bradford method, using BSA (Sigma) for the standard curve.

Radioactivity (measured in dpm) incorporated in the tissues was evaluated using the scintillation counter Beckman (LS1701). The pellet radioactivity was compared to the total radioactivity in the first supernatant, giving information on the incorporation level, calculated according to the formula:

$$\text{Incorporation} = \frac{\text{dpm}_{\text{pellet}}}{\text{dpm}_{\text{pellet}} + \text{dpm}_{\text{super}}}$$

The percentage of inhibition (% I) was calculated according to the formula (Flood et al., 1972):

$$\%\,\text{I} = \left(1 - \frac{\text{incorporation\_sample}}{\text{incorporation\_control}}\right) * 100$$

Through tritiated leucine ([^{3}H]leu) injection into the brachial heart (Prozzo & Giuditta, 1973)(first studied protein synthesis in *Octopus vulgaris* central nervous system. The amino acids uptake into proteins in *O. vulgaris* nervous system proceeds similarly in the three main brain masses, SEM, SUB and OLs. The uptake reached a maximum 20-30 minutes after the injection, then exponentially decreases, and it stops within 2 hours. Other organs, especially the hepatopancreas, showed lower radioactivity than the brain.

Several protein synthesis inhibitors (cycloheximide, puromycin, and chloramphenicol) were able to interfere with protein synthesis in the optic lobe (Prozzo & Giuditta, 1973).

This experiment utilized three widely tested protein synthesis inhibitors, cycloheximide, anisomycin and emetine, on the central nervous system masses of *Octopus vulgaris.* First is was evaluated the effect of different protein synthesis inhibitors injections on protein synthesis inhibition in two tissues, brain and hepatopancreas.

The results are expressed as percentages of inhibition and radioactivity incorporation in the tissues and are described in Table S1.

Table S1. Percentage of inhibition and radioactivity incorporation levels (dmp/mg) in the brain and the hepatopancreas of *Octopus vulgaris.* SW=sea water, CXM= cycloheximide; ANI= anisomycin; EME= emetine

	EXPERIMENTAL GROUP	% INHIBITION	dpm/mg PROTEINS
BRAIN	SW	-	991.9
	CXM	79	184
	ANI	66	190.25
	EME	65.3	134.3
HEPATOPANCREAS	SW	-	451.93
	CXM	53	221
	ANI	55	86
	EME	51	63

All inhibitors induced inhibition higher than 50% in both the brain and the hepatopancreas, however protein synthesis inhibition was higher in the brain than in the hepatopancreas. Radioactive amino acids incorporation into the tissue resulted to be inversely proportional to the inhibition effectiveness. The amino acids incorporation level in the brain resulted to be from five to seven times higher in the control than in the inhibitors groups, while it was from two to seven times increased in control versus "inhibited" groups in the hepatopancreas.

The comparison with amino acids incorporation (dpm/mg) of controls allowed to note that higher levels of incorporation resulted in the brain, as described by Prozzo and Giuditta (1973). Amongst the inhibitors utilized, cycloheximide showed the highest inhibition percentage ($\%I_{CXM}$ = 79%; $\%I_{ANI}$ = 66%; $\%I_{EME}$ = 65.3% in the brain), despite its reversibility (Stanton & Sarvey, 1984).

Considering the relative incorporation percentages (see figure S1), CXM showed the lowest [^{3}H]aa incorporation level.

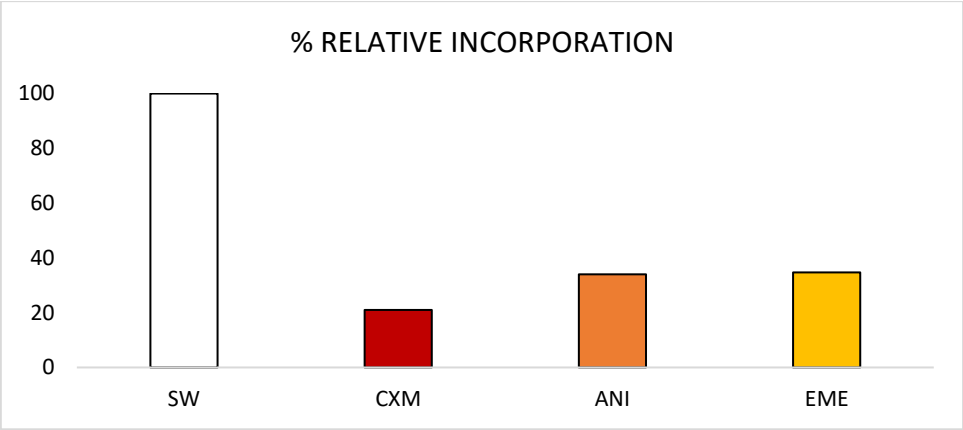

Figure S1. Average relative [3H]aa incorporation percentages in the brain. 100% is the percentage of the control group.

These data show that inhibition of protein synthesis affect brain more than other tissues (at least those here considered) and the concentration of inhibitors utilized resulted to be sufficient to induce a significant inhibition of protein synthesis. These first findings allowed the starting of a subsequent behavioral pharmacological study.

Cycloheximide inhibition kinetics in *Octopus vulgaris*

Cycloheximide was selected as the protein synthesis inhibitor because this reversible drug was shown to induce a percentage of protein synthesis inhibition close to 80% in octopus' brain. The kinetics of this inhibition was therefore investigated.

Based on literature data on memory formation in *O. vulgaris* (G. D. Sanders, 1970b; G. D. Sanders & Barlow, 1971; Young, 1961) and in other species (e.g., Alberini et al., 1994; C. D. Beck & Rankin, 1995; Fulton et al., 2008) was possible to hypothesize that memory consolidation in octopus lasts at least four hours from the end of the training.

In this second experiment the kinetics of cycloheximide-induced inhibition was evaluated.

Treatments and sampling procedures were the same as described for experiment 1.

Tissue radioactivity as an effect of tritiated [^{3}H]aa mixture incorporation during protein synthesis was measured at different time points from inhibitor injection to evaluate the inhibition effectiveness over time.

A total of 12 animals were randomly assigned to 4 groups: a control group and three groups injected with cycloheximide at time zero and with the tritiated [^{3}H]aa mixture at different time points (1h, 2h and 4h) in order to evaluate the inhibition effect on *de novo* protein synthesis rate at different time-points from cycloheximide administration.

Each group consisted of three *O. vulgaris*; samples were then processed in triplicates.

Figure S2 show the reduction of cycloheximide inhibitory effect with time: inhibition percentage of protein synthesis resulted to be 79% at 1 h after injection, 74.68% 2h later and reaches 67.56% after 4h.

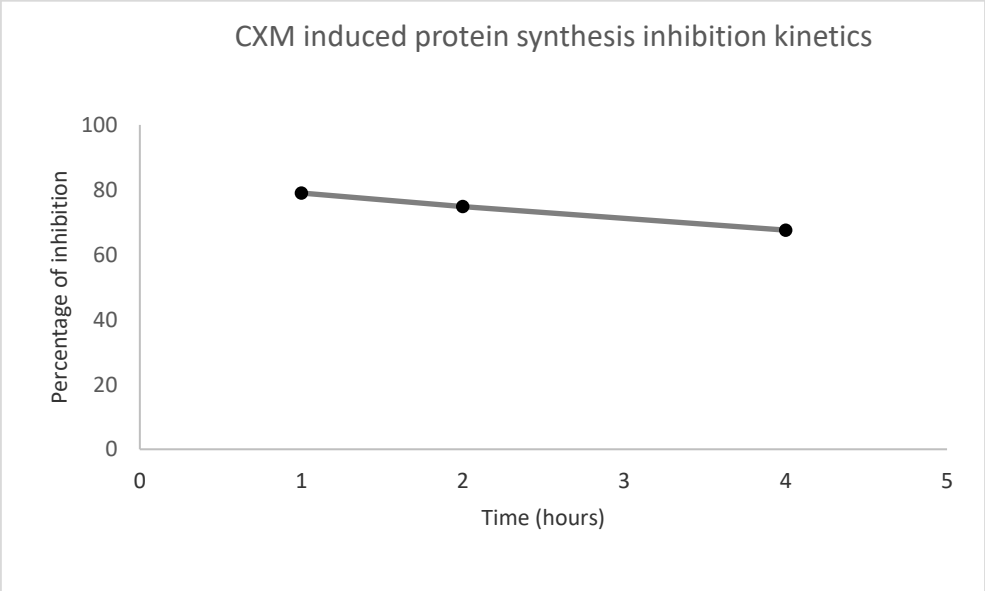

Figure S2. Inhibition kinetic of protein synthesis induced by cycloheximide injections.

According to the available knowledge, to impair protein-synthesis dependent memory recall it is necessary that inhibitor action lasts longer than the acquisition period. Since after four hours inhibition resulted to be still high, the drug effect was considered to cover the period need for consolidation to occur.

Testing the effect of inhibitor injection on the motivational drive of *Octopus vulgaris*

To study the effect of protein synthesis inhibition on long-term memory any possible side effect on the behavioral performance of the animal have to be excluded. For this purpose, *O. vulgaris* were

injected in the brachial heart with sea water or cycloheximide and tested for their readiness to attack in order to identify any nonspecific side-effect and/or effects on the motivational drive related to the drug administration (following Agnisola et al., 1996).

A total of 16 naïve, well-acclimatized animals were randomly attributed to two conditions and injected in the brachial heart with sea water or cycloheximide. The volume of the injections was proportional to the weight of the animal (10mg/ml/kg for cycloheximide solution). Readiness to attack was assessed at regular intervals from the injection (i.e., 10, 20, 40 and 60 minutes after injection) by presenting the animals with a crab tied to a thread (Amodio et al., 2014). Four and 24 hours after the injection each octopus was fed with a live crab. The response was considered to be "positive" if animal responded to the tethered crab within 20 seconds (a score of 100 was assigned for each positive response).

Figure S3 summarizes the outcome of these experiments.

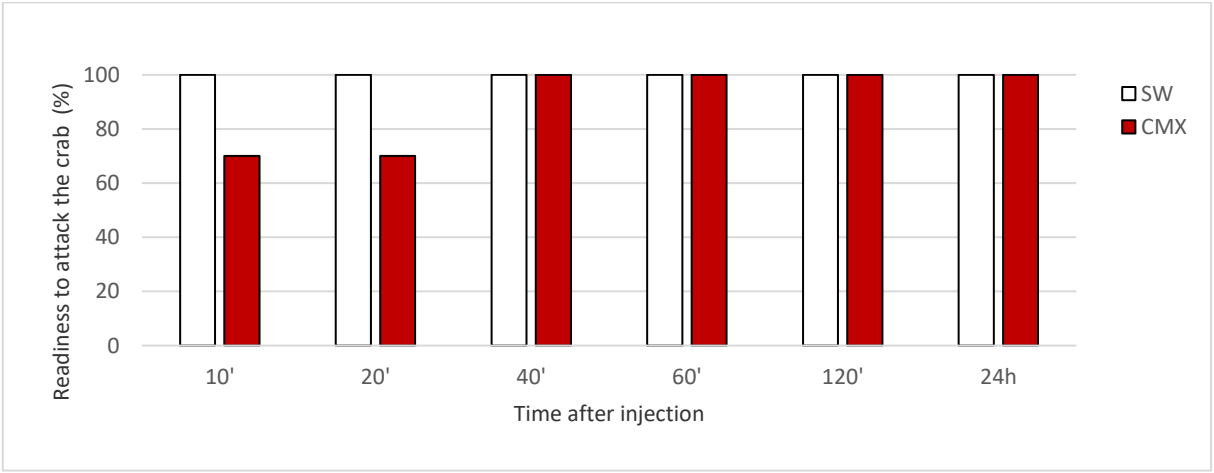

Figure S3. Readiness to attack response after injection by *O. vulgaris*. Percentage at different time points after injection for the control (SW) and the inhibitor (CXM).

A slight motivational drop of about 30% was observed after cycloheximide injection, which lasted until 20 minutes later. Afterwards, and starting from 40 minutes after injection no differences were observed among the cycloheximide and control injection: all animals showed a normal predatory behavior independently from the treatment.

These data support the hypothesis that no effects on the motivational drive can be ascribed to cycloheximide protein synthesis inhibition, although protein synthesis is inhibited.

Effects of protein synthesis inhibition on avoidance learning and memory in octopus

The experimental approach was followed by focusing on the effects of protein synthesis inhibition on avoidance learning and memory retention. A total of 28 *O. vulgaris* were utilized in these experiments.

A massed fear conditioning training protocol was utilized. The protocol included pre-training, training and testing phases. The stimulus presentation was organized into a single session of trials with very short inter-trial intervals; the series of trials ended when the training criterion was reached, i.e. five consecutive avoidance (no-attacks) responses to the stimulus.

The reliability of this training protocol has been assessed in a series of studies carried out by the G.Fiorito Lab and known to allow all animals to reach the training criterion rapidly, a robust memory retention and stimulus-specific learning (for reviews see Zarrella, 2011).

During pre-training, each animal was presented with a white bead associated with a reward (a piece of anchovy); a red plastic ball was utilized as stimulus for training (training against preference) as reviewed by Borrelli (see: Borrelli & Fiorito, 2008; Borrelli, 2007; Zarrella et al., 2005). The pre-training phase allowed the animal to familiarize with the artificial stimulus. Each trial lasted one minute and the interval training time was fixed to two minutes. Pre-training ended when the animal attacked the stimulus within 20 sec for five consecutive trials. The training session started twenty-four hours after pre-training and lasted less than one hour. It consisted in the repeated presentation of the stimulus. Any attack to the stimulus was punished with a mild shock (12V). Each trial lasted one minute and the inter-training intervals was fixed to two minutes. Training was concluded when the animal reached the training criterion consisting of no attacks to the stimulus for five consecutive trials.

The animals were divided into two groups. In the first group, animals were injected with sea water (N=7, control animals) or with 10 mg/ml cycloheximide (N=9) thirty minutes before training. The testing phase, to verify memory retention, started 24 hours after training and involved five trials of one minute each (inter-trial interval of 2 min).

During testing, no reinforcement (negative or positive) was adopted.

For the second part of the experiment other individual octopuses trained as the previously described, were injected 30 minutes after the end of the training session with sea water or with 10 mg/ml cycloheximide (Figure S4 schematize the experiments.

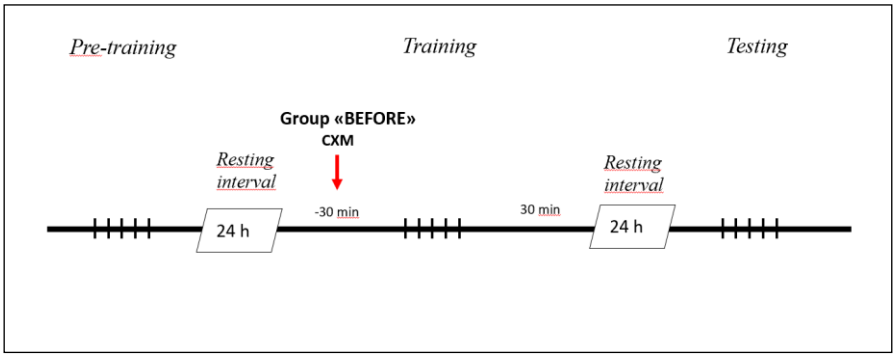

Figure S4. A schematic summary of experimental procedures. Animals underwent pre-training, training and testing. The first group of animals was injected 30 minutes before the training start.

All *O. vulgaris* learned to avoid the stimulus (Fig. S5). They associated the stimulus with the punishment and modified their tendency to attack succeeding in avoiding the stimulus.

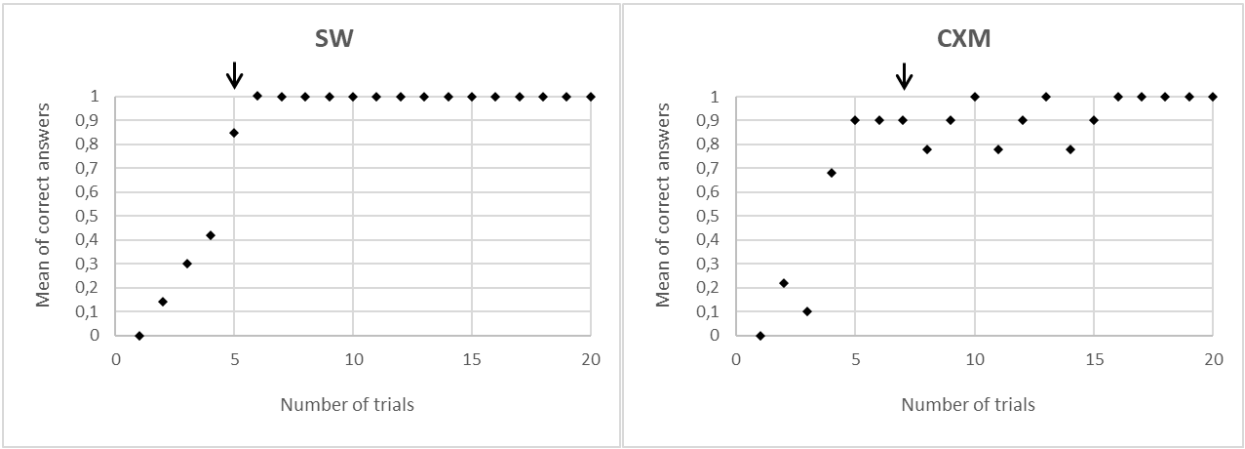

Figure S5. Acquisition curves. SW=sea water; CXM=cycloheximide, 10mg/ml. Injections were performed 30 minutes before the training start. The arrow points the average trial at which the animals reach the training criterion.

As shown by the acquisition curves, there was no difference between the acquisition capacity of the control and the cycloheximide group injected 30 minutes before the starting of the training. The CXM group reached the training criterion at the seventh trial (control after 5 trials, Figure S5). However, this difference is not statistically significant (N=7,9; Mean ± SE = 4.57±0.97, 6.77 ± 5.01; t-student=1.28, p=0.23, NS).

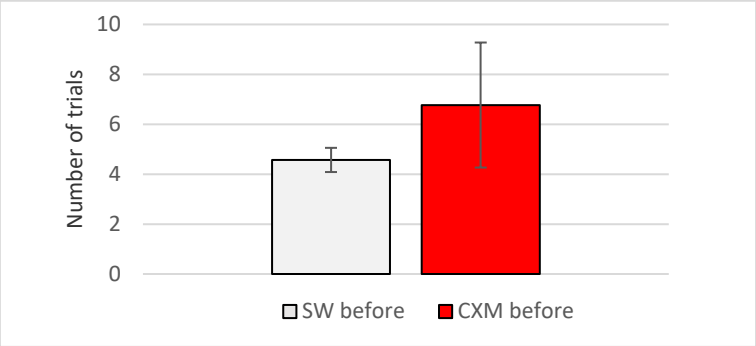

Figure S6. Number of trials needed to reach learning criterion in presence and absence of the drug, (SW=sea water; CXM=cycloheximide, 10mg/ml). Injections were performed 30 minutes before the training start.

All the octopuses learned the task in about 15 trials and the learning curve of SW-treated resulted to overlap to the one calculated after the performance of the CXM-treated animals and was further comparable to not-injected animals (data not shown).

Data shown that acquisition was not affected by the procedure: animals learned the task independently from the injection and also were not affected by protein synthesis inhibition.

During testing phase (retention, Figure S7) CXM and SW injected animals revealed a significant difference between; the CXM injected octopuses' responses were not consistent when compared to controls. Heterogeneity in the retention curve is suggested to be attributed to possible memory recall impairment. Overall the retention levels (Fig. S8) observed, considered as cumulative responses to the five trials during the testing phase, shows a statistically significant difference ($t_{(12,8)}$=2.73, p = 0.017, after correction for heterogeneity of variances).

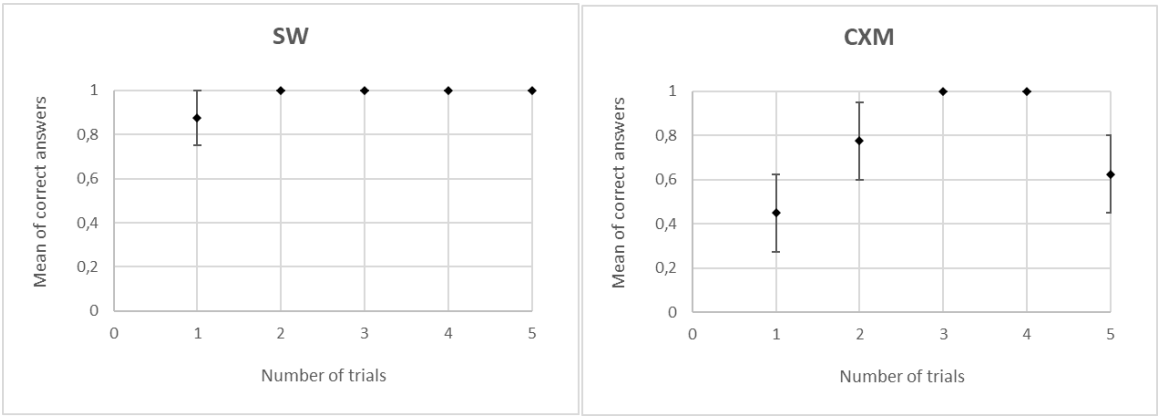

Figure S7. Retention curves. The mean of the correct answers to the test (24h after training), are reported trial by trial. The correct answer of no attack is scored 1; the wrong attack response is scored 0.

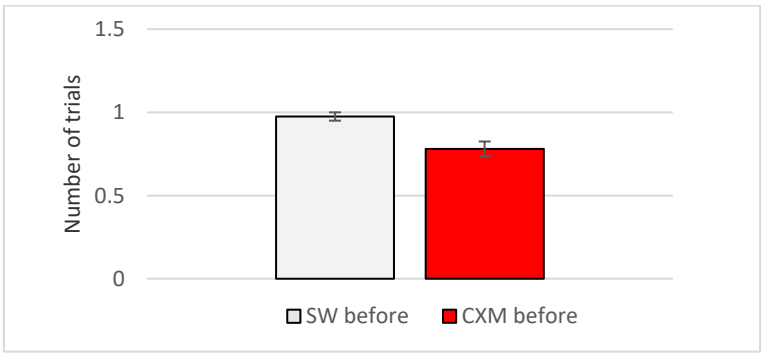

Figure S8. Number of correct trials during testing phase (SW=sea water; CXM=cycloheximide, 10mg/ml). Injections were performed 30 minutes before the training started.

These data suggest that long-term memory formation of an avoidance task appears to be protein-synthesis dependent in the octopus.

Since protein synthesis was inhibited for a long period following the injection of the inhibitor (see experiment 2) data in my hands from these experiments support the hypothesis that memory formation resulted to be sensitive to *de novo* protein-synthesis disruption.

In order to test whether the deficit in memory recall observed in the animals injected before the training was related to protein synthesis inhibition and not to other factors, another group of octopuses was injected after the end of the training session with cycloheximide (sea water injections were used as controls; Figure S9).

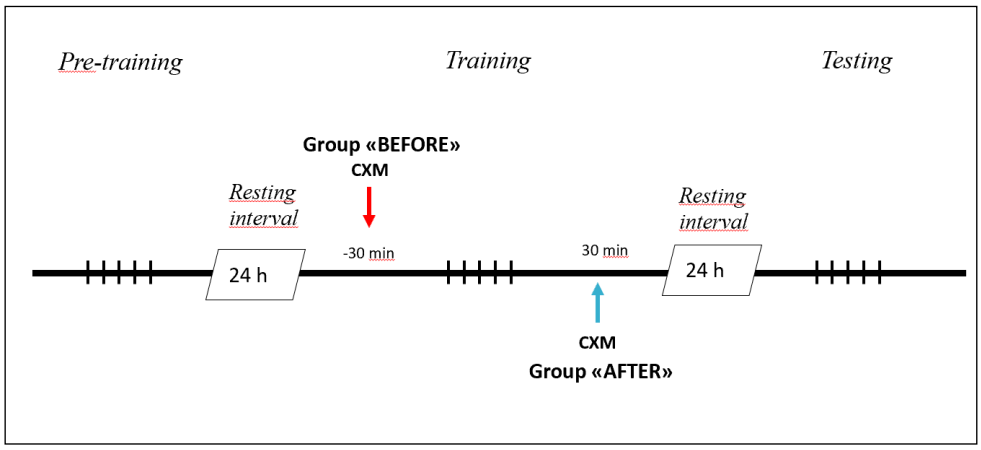

Figure S9. A schematic view of the two experimental groups used to test the effect of cycloheximide on memory retention. The first group, named Group "before" was injected with CXM 30 minutes before the start of the training; the second group, named Group "after" was injected 30 minutes after the end of the training.

Animals injected 30 minutes after the end of the training did not show any deficit during the testing phase (Figure S10).

When the injection followed training phase, *O. vulgaris* always performed with an accurate memory recall of the task, independently from the type of injection (cycloheximide or sea water).

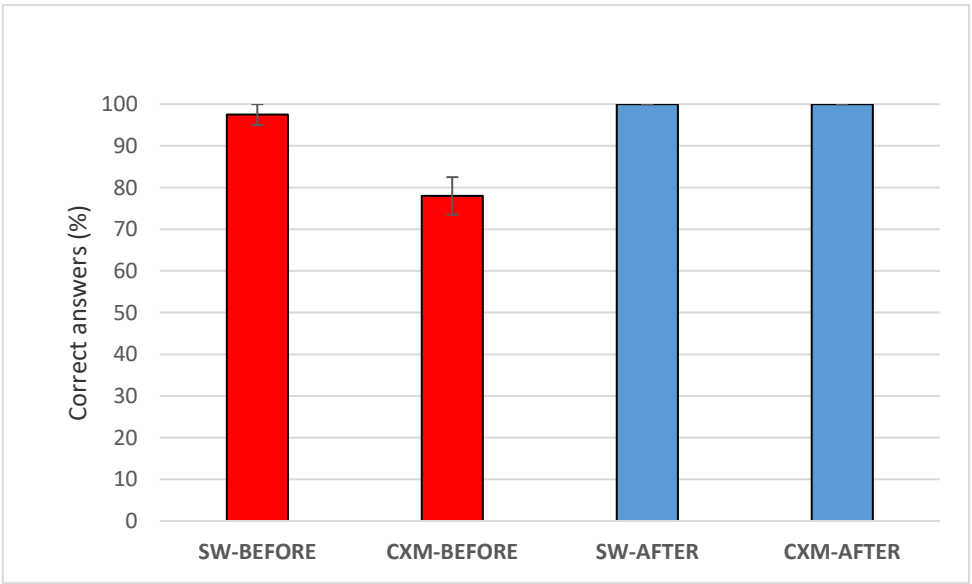

Figure S10. Percentage means of correct answers to the test. Animals were injected with sea water (SW) or cycloheximide (CXM) 30 minutes before the training start or 30 minutes after the training end.

Evaluation of possible CXM-dependent effect

To further evaluate whether the reduction in memory recall observed in animals injected before the training was linked to the inhibition of protein synthesis and not to other possibly drug-induced state in octopus a further experiment was carried out.

State-dependent learning occurs when a task is learned under certain drug-induced states and consequently the retrieval is effectively achieved when the subject is tested under the same drug effect (e.g., Overton, 1984). Under such circumstances, memory recall is impaired when the tests are conducted without drug administration. From previous experiments we observed a poor recall when the drug was administrated before the training, thus suggesting the effects of protein synthesis inhibition on memory consolidation. The impairment at memory testing could be induced by a true amnesia or by a state-dependency effect. In the former case, memory trace is weakened or abolished, in the latter, a latent form of memory is present and can be observed through the resetting of the conditions established during training (e.g., Overton, 1984). In order to verify whether in these experimental conditions state dependency occurred an experiment was designed to address this issue.

We expected that, if state dependency was present, the animals injected with the same solution, the drug or the sea water before training and testing, would be the ones remembering the task.

A total of 20 naïve and well acclimatized *O. vulgaris* were attributed randomly to four experimental groups. The training protocol used was the same described for previous experiment. In this case all animals were injected twice, 30 minutes before training and 30 minutes before testing (see Fig. S11). Five animals were injected twice with sea water (SW-SW); five animals received two injections of cycloheximide (CXM-CXM); five octopuses were injected with cycloheximide before training and sea water before testing (CXM-SW); five animals were injected with sea water before training and cycloheximide before testing (SW-CXM).

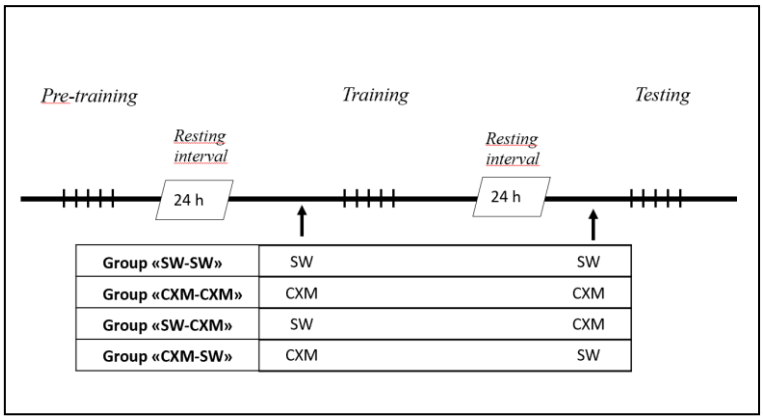

Figure S11. Experimental design to test state-dependence hypothesis. The arrows indicate the injections. The rows indicate the experimental groups (SW-SW; CXM-CXM; CXM-SW; SW-CXM)

Figure S12 show the average correct responses of the animals during testing phase. Data shown that there are no significant differences between the CXM-CXM and SW-SW groups, but also between the CXM-SW and SW-CXM groups.

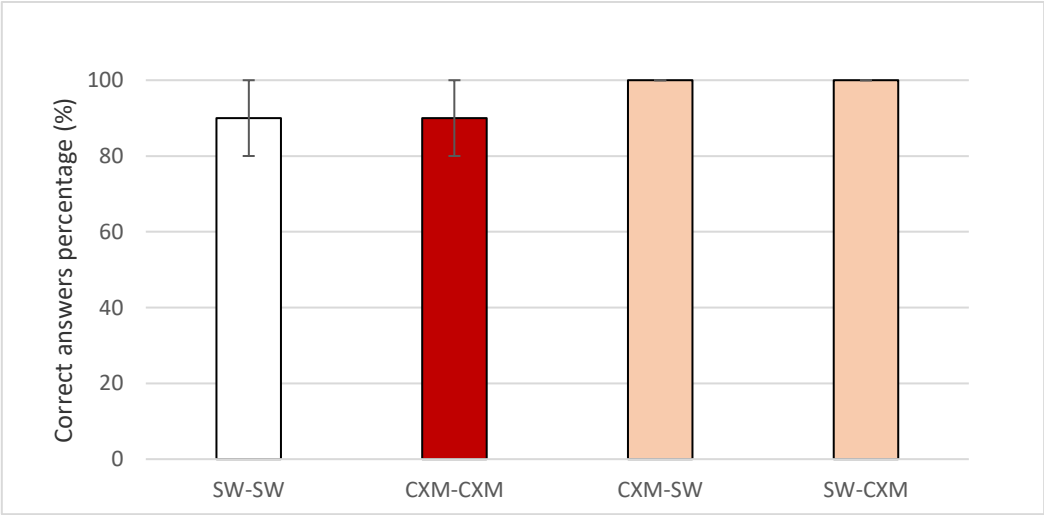

Figure S12. Percentage means of correct answers to the test. Animals were injected twice, with sea water (SW) or cycloheximide (CXM) in the following combination (SW-SW; CXM-CXM; CXM-SW; SW-CXM). First injection was performed 30 minutes before the training start; second injection was performed 30 minutes before testing.

The analysis of the variance (on arc-sin transformed values) is not significant ($F_{(3.16)}$=0.67, p = 0.581) nor are significant the post-hoc comparisons.

A slight difference is observed between the groups. This difference is however not significant.

In conclusion, animals that are trained in the presence of the drug are able to remember when tested in the presence of the drug, as opposed to animals just injected before testing.

State-dependency is probably the answer to this finding. However, alternative explanations to state-dependency might be ascribed.

First of all, it could be claimed that the experimental design for state-dependency is more stressing than the ones of previous experiments. The double injection might induce a non-specific motivational drop, which the animals associate to the task, causing a conditioning by treatment, that however has never been observed in octopus until now.

If this hypothesis is the correct one, the suppression of the conditioned response to attack the red ball, which has means that the animal performs a correct response and show a good memory recall for the avoidance task, would indeed be related to non-associative and ascribable to a motivational drop. Having animals associated a punishment (the injection) with the stimulus presentation, their response would depend on this conditioning and not on an amnestic drug effect or on state-dependent learning.

However, considering that animals in the SW-SW condition attacked the stimulus associated with the negative reinforcement after the second injection; this show that the experimental procedure does not reveal a motivational drop. Previous experiments had unequivocally demonstrated that octopus' response to avoidance training tests is strictly stimulus-dependent (animals that remember to avoid to attack the red ball, were prone to attack a differently colored ball) and their behavior is not influenced by masking effects related to response depression or motivational drop (Wells and Young, 1970; De Simone, 1996).

These experiments lack of a possible control where the task is not associated to the inhibition of the attack as learned response by octopus (i.e., consecutive attacks to a positively reinforced stimulus) that should be utilized to further test protein synthesis dependence of learning and memory in octopus.

Appendix 3. Animals, care and experimental procedures

In the experiments included in this PhD Thesis, *Octopus vulgaris* (Mollusca, Cephalopoda) of both sexes (from 200 - 650 g, body weight) were utilized. Animals were caught in the Bay of Naples (Italy; for time span see main text). In order to standardize fishing procedures, the lab of Dr G. Fiorito utilized octopuses caught by our local fisherman (Mr. Di Liello). Di Liello's family mainly utilizes the "nassa" or a jig as fishing method although a series of "mummarella" may also be adopted occasionally (Lane, 1960; Lo Bianco, 1909). On arrival in the laboratory, each animal is numbered, sexed, weighed, and housed in an experimental tank with running seawater. The procedure utilized has been standardized during the past years and have been thoroughly summarized in Dr. L. Borrelli PhD thesis (2007). Here I will only briefly summarize the main issues related with animal maintenance, acclimatization, testing, morphometry, treatment, sacrifice and dissection.

A series of parameters are monitored with live animals in G. Fiorito lab, with the aim of controlling for possible environmental or physiological changes occurring during experiments. This with the aim of control for a certain homogeneity of such a distributed sample among different experiments. Environmental parameters: temperature (°C), oxygen levels (mg/l) and seawater inflow (measured as the time spent to fill a cylinder of one litre) were measured for each tank on a daily basis and using procedures that minimize the octopuses' disturbance. For maintenance of *O. vulgaris* the laboratory of Dr. G. Fiorito at the Stazione Zoologica Anton Dohrn utilizes tanks (usually 30 x 100 x 50 cm, in some cases larger) with running seawater. Octopuses are presented with a live crab attached to a cotton thread that is pulled up before the octopus could seize the prey (readiness to attack). The crab is provided as food day or every other day, depending on the experimental schedule (Amodio et al., 2014; Fiorito et al., 1990). In order to monitor for any potential difference in the overall food supply to each animal of the battery, each crab given to the octopuses on feeding days has been measured (carapax width, mm).

Dissection procedures for collection of samples were standardized following Young (1971). In particular, procedures of removal of brain parts were designed in order obtain the samples required in different experiments as fast as possible. Five minutes elapsed from the time that the anesthetized octopus was placed on the dissecting table to when the last sample was preserved. All dissecting tools were sterile; the experimenter wore gloves and the samples were collected in sterile plastic tubes. Unless otherwise stated, as samples taken the brain in its parts (the two optic lobes, left and right: OL; the supraesophageal mass: SEM; the subesophageal mass: SUB), and a small piece

of mantle. Preservation and/or fixation of samples depended from the procedure. For molecular biology experiments we utilized RNA Later and/or Trizol.

Appendix 4. Cellular analogue of learning and memory in octopus and underlying gene expression

During the course of this PhD project, we started to explore the possibility of benefit of the consolidated experience in the lab to test whether LTP in octopus induces changes gene expression including markers of epigenetic modifications. This has been not the case due to COVID-19 pandemics.

Long-term potentiation (LTP) is a form of synaptic plasticity that drives the strengthening of synaptic efficacy in an activity-dependent manner. It is considered to be *the in vitro* analogous for associative memory. Two phases of LTP can be distinguished: an early phase lasting few hours depending on protein phosphorylation and a long-term phase that, in analogy to long-term memory, depends on mRNA and protein synthesis (e.g., Impey et al., 1996; Abel et al., 1997; Jones et al., 2001; Abraham and Williams, 2008).

A robust hippocampal-like activity-dependent LTP has been observed at the level of the medium-superior-frontal to vertical lobe (MSF-VL) tract in *O. vulgaris* (Hochner et al., 2003). This form of synaptic plasticity has been considered to be involved in behavioral learning and memory (Shomrat et al., 2008; Shomrat et al., 2010; Shomrat et al., 2011). Vertical lobe (VL) appeared to be involved in memory consolidation from short to long-term.

Among the about 40 distinct interconnected lobes of the central nervous system of the octopus, VL is considered a pivotal centre for learning and memory processing. Until now two types of neurons, both monopolar cells, have been identified in the vertical lobe: about 25 million amacrine interneurons (AM, ~ 5 µm diameter) and around 65.000 large efferent neurons whose axon project outside the vertical lobe (LN), about 15 µm diameter on average (Gray, 1970; Young, 1971). The high number of amacrine cells make the vertical lobe, one of the of the mostly cell-dense lobes of the central nervous system. Furthermore, the presence of 5 cylindrical gyri in *O. vulgaris* increase volume and complexity to this structure (Young, 1971). The vertical lobe receives inputs from the around 1.8 million neurons of the median superior frontal lobe (MSF) that is consider to integrate sensory information (Young, 1971). The MSF-VL neural architecture is a fan-out fan-in association matrix (Tal Shomrat et al., 2011). The MSF neurons diverge (fan-out) to innervate the VL amacrine

cell neurites perpendicularly, therefore the MSF axons are able to make *en passant* synapses with the amacrine neurons in the VL (Shomrat et al., 2011). The amacrine cells then converge (fan-in) to innervate the large efferent neurons via “special serial synapses”, named this way because the amacrine cells neurites are postsynaptic site for the MSF axon terminals and are presynaptic to the long efferent neurons dendrites.
Similar neural architecture is observed for inferior frontal-subfrontal lobes connections, involved in chemotactile learning and memory (Wells, 1978 for review).

Since neurons of MSF and VL axons are organized as a tract, a significant extracellular local field potential is generated by the summation of action field potentials that propagate following MSF stimulation. The MSF-VL tract was studied through intracellular and extracellular recording configuration. Intracellular configuration is allowed by recording from LN cell bodies; extracellular recording is achieved by recording their spiking activity in the axonal bundles that project from the VL. The observation of physiological tissue responses from both recording assets along with pharmacological intervention, allowed the identification of the MSF-AM as a glutamatergic synapse, and the AM-LN as a cholinergic synapse (Shomrat et al., 2011). Tetanization of MSF evokes, with a distance-dependent delay, a local field potential (LFP) that is a triphasic tract potential (positive–negative–positive), followed by a negative, glutamatergic fPSP. Four high frequency trains to MSF tract (20 pulses at 50 Hz with 10 intertrain intervals) enabled Hochner and coworkers (2003) to observe a long-term potentiation (LTP). LTP involves the glutamatergic MSF-AM synapse, but the activity-depend plasticity extends to LN, since the facilitation at the first fan-out layer increased AM cells neurotransmitter release, increasing LN spiking activity (Shomrat et al., 2011).
Tetanization of the MSF drove a LTP of nearly fourfold fPSP amplitude increase with no effect on tract potential (TP). Saturation occurs since further stimulation drove no longer enhancement. This activity-dependent synaptic plasticity only occurs at the fan-out glutamatergic site (while in cuttlefish the site for synaptic plasticity is the cholinergic fan-in connection (Shomrat et al. 2011, reviewed in Turchetti-Maia, 2019).
Octopus LTP presents the essential associative induction properties of specificity, cooperativity and associativity (Hochner et al. 2003). The LTP of the octopus VL has a bimodal nature, being for a half NMDA-independent and for half NMDA-dependent. These two forms of plasticity are probably spatially separated (Hochner et al., 2003b; Turchetti-Maia et al., 2019).

An important, unsolved aspect is whether the synaptic plasticity depends on presynaptic, postsynaptic or both events.

NO is a well-known retrograde messenger in mammalian hippocampus contributing to presynaptic neurotransmitter release. In octopus NO was reported to be involved more likely in LTP expression than in its induction. NO-synthase inhibition abolished LTP expression or erased the induction and maintenance of a second LTP.

Furthermore, LTP dependence on protein synthesis has been investigated. Protein synthesis did not affect LTP induction or its maintenance, since anisomycin had no effect on LTP during a prolonged testing period (Turchetti-Maia et al., 2018).

In vertebrates (zif-268, NTRK2, Stmn) and invertebrates (Htt) gene transcription has been observed to be induced following LTP induction, probably in order to subserve LTP duration. Some of these genes, among which is the immediate-early gene zif-268 than activate late effectors that also contribute to LTP maintenance (Abraham et al., 1993; Abraham et al., 1992; Nguyen et al., 1994). Accordingly, transcription, as well as epigenetic machinery, inhibition proved not only a transcriptional, but also an above epigenetic control in LTP expression (Levenson et al., 2004b; Vecsey et al., 2007).

Although *de novo* protein synthesis is apparently unrequired for LTP induction and maintenance, gene transcription and epigenetic mechanisms have never been investigated in *Octopus vulgaris* for a potential contribution to neurotransmitter release or postsynaptic response enhancement.

Due to COVID-pandemics I have been unable to report here preliminary data including relative gene expression profile after induction of LTP in *O. vulgaris*. Future research may benefit of the samples collected (experiments not reported here). It will be interesting to evaluate whether zif-268, NTRK2, Stmn and Htt, to cite some, appear differentially expressed in the VL in areas connected such as MSF when compared with other areas of the supra- and sub-esophageal mass of *O. vulgaris*.

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