Functional and comparative assessements of the octopus learning and memory system

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1. ABSTRACT

The octopus and its close relatives the cuttlefish and squid are the most advanced of the invertebrates, possessing the largest brains both in weight and cell numbers. Here I review recent studies of the neurophysiological properties of the vertical lobe system (VL) in the cephalopod brain, a system already thought to be dedicated to learning and memory. Summarizing from the point of view of comparative evolution, I relate these results to other systems where anatomical and electrophysiological data are available, mainly the insect mushroom bodies and the mammalian hippocampus. The emerging results suggest that a convergent evolutionary process has resulted in similar neural organization and activity-dependent long-term synaptic plasticity in all these learning and memory systems, even though the invertebrate systems conserve their typical anatomical and electrophysiological features. And finally, functional inferences based on the comparison with the insect mushroom bodies are discussed.

2. WHY STUDY COMPLEX NERVOUS SYSTEMS IN INVERTEBRATES?

The most challenging question in neuroscience is how nervous systems control complex behaviors. Although it is not easy to quantitatively define levels of behavioral complexity, there is a general consensus on some positive correlation between the level of behavioral complexity and the complexity of the nervous system (or, as a first estimate, the number of nerve cells). Such a relationship is most clearly demonstrated in invertebrates. These show the greatest diversity in behavioral complexity and this diversity correlates roughly with the huge diversity in complexity (or size) of their nervous systems. To examine the control of simple forms of behavior, we can study the nervous system of a nematode worm, such as *C. elegans* with only 302 neurons, while *Octopus vulgaris* with its c. 500 million neurons is most suitable for investigating complex behaviors. Such a comparative approach is intriguing because it allows us to ask whether the neuronal mechanisms in simple nematodes are conserved in the more
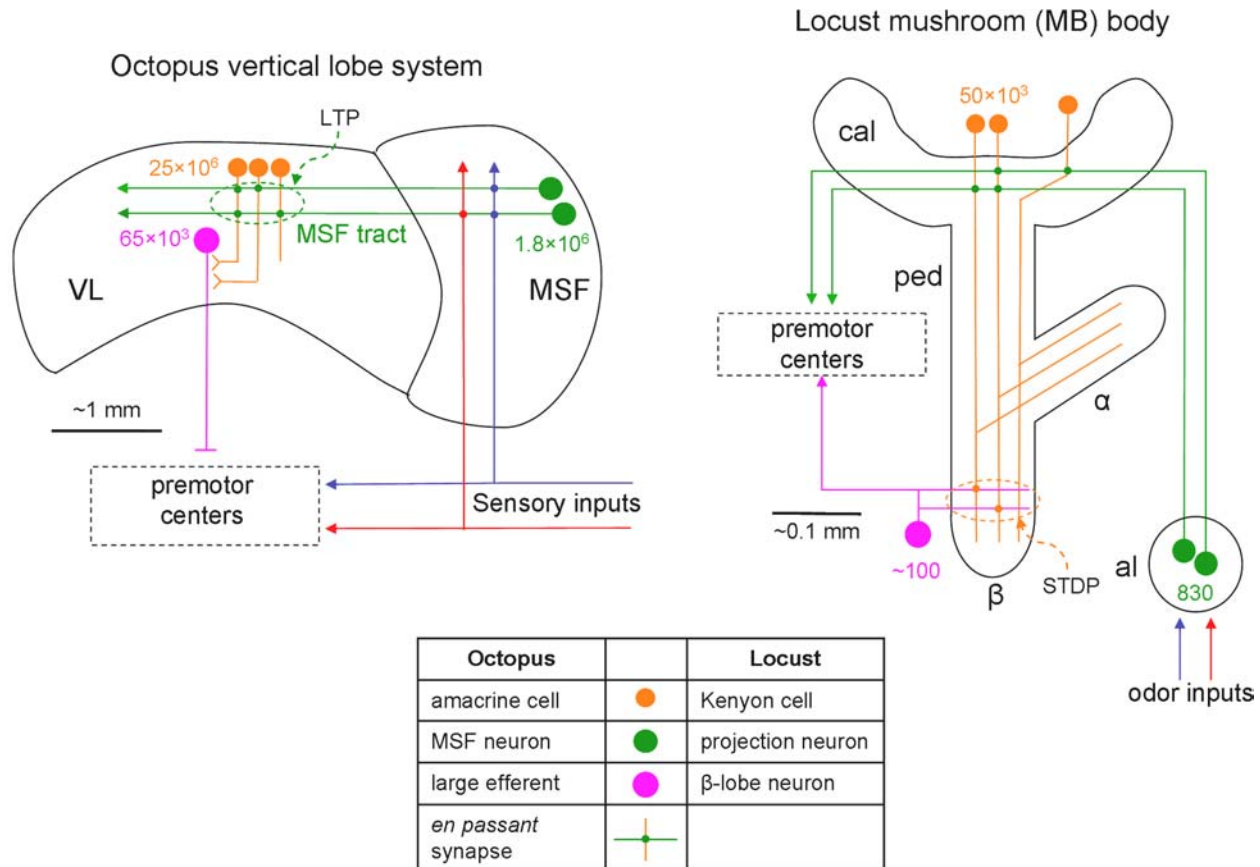
Figure 1. Principle neuronal elements and their connections in the octopus vertical lobe (VL) and locust mushroom body (MB) systems. The connectivity schemes are superimposed on schematic drawings to scale of the respective neural structures. The VL contains many more cells and is much larger than the MB (see approx. calibration bars). The various cell types are shown in different colors (see table) and their numbers are indicated in the schema. The parallels between the three cell types in the two systems are organized with respect to the amacrine and Kenyon cells; both are numerous, small, and intrinsic to their respective structures. The input lobe cells (green) innervate the amacrine and Kenyon cells *en passant*. In both cases the output cells appear to innervate premotor areas. The sites where synaptic plasticity has been found are encircled with broken line ellipses, i.e. LTP in octopus and spike-timing dependent plasticity (STDP) in the locust (56). (al) antenna lobe; (α) α-lobe; (β) β-lobe; (cal) calyx; (MSF) median superior frontal lobe; (ped) peduncle.

complex nervous system of the octopus or whether the complex behaviors of the octopus involve the evolution of more advanced mechanisms and processes, perhaps convergently with those of advanced vertebrates.

3. THE VERTICAL LOBE OF CEPHALOPODS AND THE INSECT MUSHROOM BODY ARE UNIQUE BRAIN STRUCTURES INVOLVED IN THE ADVANCED BEHAVIOR OF THESE ANIMALS

The invertebrates whose highly advanced behaviors and learning and memory repertoires have been most studied are cephalopods mollusks, the cuttlefish *Sepia officinalis* and the octopus *Octopus vulgaris* (1-3), and insects, such as the fruit fly, locust, cockroach and honeybee (4, 5). Both groups have evolved special brain structures that participate in mediating these animals' advanced and complex behaviors including sophisticated learning and memory abilities - the vertical lobe (VL) in

modern cephalopods (coleoids) and the mushroom bodies (MB) in insects. These two special structures have a characteristic and unique anatomical organization. Firstly, they each contain a relatively large proportion of the neurons in the CNS (e.g. ~200,000 of ~1 million neurons in cockroach; ~25 million of ~140 million cells in the octopus CNS). These neurons are intrinsic to their structures and are the smallest in the nervous systems (Figure 1). Secondly, the octopus VL and the MB of several insect species are folded, like the vertebrate brain, possibly to accommodate the large number of cells (6, 7); the VL in the octopus has 5 gyri, although cuttlefish or squid do not.

The connectivity patterns in the VL and MB are unique among invertebrate ganglia. The pioneering morphological work of Gray and Young (8, 9) showed that the projections of the median superior frontal lobe (MSF) cells to the VL are divergent, innervating many of the small intrinsic amacrine interneurons *en passant* (see schema in

Figure 1). In turn, these amacrine neurons converge onto a relatively small number of large efferent neurons, which form the sole output of the VL. J.Z. Young was the first to note this 'simple' network organization in the VL, describing it as matrix-like and pointing out the similarity to the mammalian hippocampus (10).

The insect MB appears more complex than the cephalopod VL (see schema in Fig 1; ((11)). It is a neuropilar structure, composed of a calyx (cal), where projection neurons (PNs) emerging from the antennal lobe (al) form synapses with Kenyon cells *en route* and terminate in the lateral horn. The Kenyon cell axons project down the peduncle (ped) and then bifurcate, sending branches to the α and β MB lobes. The calyx, or the input area, where the many Kenyon cells are innervated by a relatively small number of input neurons, is thus similarly organized to the VL.

Interference with the function of these unique matrix-like structures in insects and octopuses and cuttlefish affects the performance of behavioral tasks involving sensory processing and learning and memory (5, 11-18). This strongly suggests that deciphering the functioning of this form of neuronal organization may reveal the basic and possibly generic principles of networks subserving complex behaviors.

I now review in detail the octopus VL system; detailed information on insect MB are available elsewhere (e.g. reviews; (5, 11, 15-18). Finally, I will compare the functionality of the octopus VL with insect MB.

4. THE MORPHOLOGICAL ORGANIZATION OF THE OCTOPUS NERVOUS SYSTEM

Cephalopods have many more nerve cells than all other classes of Mollusca, e.g., ~20,000 neurons in *Aplysia californica* vs. about 500 million cells in the octopus (19). The octopus nervous system contains more neurons than the popular mammalian models, mouse and rat (20). Due to this enormously increased number of nerve cells and the encephalization of the ganglionic masses, the octopus central nervous system resembles the vertebrate brain more than the ganglionic chain of its close molluscan relatives, such as the gastropods and bivalves (9, 21, 22). Indeed, the size of the modern cephalopod central nervous system normalized to body weight lies within the same range as vertebrate nervous systems; smaller than birds and mammals but larger than fish and reptiles (23).

The octopus nervous system is divided into three main anatomical parts, which are arranged in a hierarchy of functional levels. Two of these major parts, the optic lobes and the nervous system of the arms, lie outside the brain capsule. The two large optic lobes contain ~120-180 million neurons. The arm nervous system, containing 2/3 of the ~500 million neurons in the octopus nervous system, may function rather autonomously, as it can generate coordinated stereotypical movements (24-27). The central brain, surrounding the esophagus and encased in a cartilaginous brain capsule,

contains ~40 to 45 million cells organized into 38 morphologically distinct lobes. Approximately 25 million of these neurons are concentrated in the VL. The lobes maintain a basically molluscan design and invertebrate ganglionic organization, with outer cell body layers and inner neuropil. As in other invertebrates, the neurons are monopolar; a single neurite emerges from the cell body and gives rise to a dendritic tree and axon(s) in the neuropil. Stimulation and lesion experiments have helped assign possible functions to several of these lobes (3, 10, 28, 29). In particular, recent single unit recordings in freely-behaving animals are beginning to reveal the organizational principles of the higher motor centers (30, 31) and field potentials recorded by electrodes placed above the VL, have shown neuronal activity during behavioral activity and, as in vertebrates, also during resting periods (32).

5. THE LEARNING AND MEMORY SYSTEM OF *OCTOPUS VULGARIS*

5.1. Morphological organization of the octopus VL-MSF system

The octopus VL is composed of only two types of neuron (Figure 1). Approximately 25 million small amacrine interneurons (the smallest in the octopus brain) converge onto only 65,000 large efferent neurons. The axons of the large cells form the only output of the VL, the processes of the amacrine interneurons remain within the lobe. The lobe receives only two inputs of which numerically the largest is the 1.8 million axons from the median superior frontal (MSF) lobe (Figure 1; (9)). The other input comes from the direction of the subvertical lobe which lies below the VL. As in *Sepia officinalis* (32, 33), recent immunohistochemical results in the octopus suggest that these inputs include serotonergic afferents (34).

The MSF lobe is thought to integrate sensory information (9). The lobe appears to contain only one morphological type of neuron, whose axons project to the VL via a distinct tract running between the VL neuropil and its outer cell body layer (Figure 1). The VL amacrine cell neurites cross the MSF tract perpendicularly, enabling each of the 1.8 million MSF axons to make *en passant* synapses with many of the 25 million amacrine neurons in the VL (8, 9, 35). Gray and Young found special 'serial synapses' in the VL neuropile. These are bag-like structures in the amacrine cell dendrites, filled with densely packed clear synaptic vesicles. An MSF input neuron terminates presynaptically on an amacrine synaptic bag which, in turn, is presynaptic to the spines of the large efferent cells. Most probably these serial synapses relay signals from MSF axon terminals to the large cell spines.

A similar organization can be seen in the projections from the median inferior frontal lobe to the subfrontal lobe, which both play a role in tactile learning (3, 10, 29). As noted by Young, the similarity of this unique organization in these two systems supports the hypothesis that this anatomical organization is relevant for learning and memory.

5.2. Electrophysiological characterization of the MSF and VL neurons

Our preliminary results on the electrotonic and excitable properties of the MSF and VL neurons (36) suggest that the neurons in this system preserve both typical invertebrate morphological features and electrical properties. For example, whole-cell recordings from the cell bodies reveal that the amacrine interneurons are inexcitable. Decremental non-regenerative "A-spikes" can be recorded in the cell bodies of the large efferent neurons of the VL and in the MSF neurons, suggesting that the cell bodies and possibly the dendritic trees of these large cells are inexcitable, with action potentials being generated in the conducting axons. Cell bodies in other areas of the octopus brain are similarly electrically inexcitable (37-39).

Thus, while the neurons in these higher learning and memory areas of the octopus brain conserve typical invertebrate morphology and electrical function, their connectivity is similarly organized to that in areas in vertebrate brains with similar functions. As shown schematically in Figure 1 there is clearly some analogy in organization of the insect MB to the VL system. In order to understand the functional organization of this seemingly universal neural network, we have carried out a series of physiological and behavioral experiments in the octopus which I now describe.

5.3. Characterization of the MSF-to-VL synaptic connections

Due to the organization of the MSF-VL system, stimulating the tract from the MSF lobe generates field potentials in the VL close to the MSF tract. The potentials comprise a volley of action potentials in the axonal tract followed by a second, mainly negative-going field potential wave ~2-3 ms after the tract potential. This potential is a synaptic field potential (fPSP) most likely generated by the inflow of synaptic current into the inexcitable amacrine cell bodies (39). The short delay suggests that these synaptic connections are monosynaptic to the MSF axon terminals, as would be expected from the morphology (8, 9, 35). Similar field potentials can be recorded in isolated brain preparations, where the entire dorsal part of the supraoesophageal brain mass is dissected out and glued into a Petri dish (14). Similar field potentials from the highly organized octopus optic lobe were recently characterized in recordings from slice preparations (40).

Pharmacological analysis of the fPSPs suggests that, as in the equivalent synapses in the vertebrate central nervous system, the synapses between the MSF axons and the amacrine interneurons are glutamatergic. The synaptic response is AMPA-like, as it is blocked by CNQX, DNQX and kynurenate (39). APV or MK-801, NMDA receptor antagonists both in vertebrate and invertebrates did not affect the fPSPs (39).

5.4. Long term potentiation (LTP) of the MSF-VL synaptic input

We discovered this "hippocampal-like" electrical behavior only several years after the death of J.Z. Young (41), who had enthusiastically advocated the universality of basic principles of brain organization according to function. We were happy (but not surprised) to find that tetanic stimulation of the MSF tract with 3-4 pulse trains (20 pulses at 50 Hz) led to a large and enduring increase in the synaptic field potential, suggesting an activity-dependent long-term potentiation (LTP) of the synaptic connections (39). These results indicate the existence of robust synaptic plasticity at the main input synapse in the vertical lobe. This synaptic plasticity has long been considered the basis of neural learning and memory. Hebbian properties thought to be important for associative learning, such as co-operativity, specificity, gradation, and saturation, have also been demonstrated in the MSF-VL synaptic connection (39). Preliminary experiments in the inferior frontal lobe, the brain area related to tactile learning, showed similar activity-dependent LTP (Shomrat and Hochner, unpub. results).

The induction mechanisms of the LTP involve both postsynaptic-dependent and -independent mechanisms, as complete blocking of the postsynaptic response only blocked LTP induction in about half the cases (39). None of these induction mechanisms appear to involve NMDA-like receptors, because the induction was not affected by APV or MK-801 (Hochner et al., 2003) (39). This issue remains to be clarified in a more detailed study. However, if these findings hold, they are most significant, because NMDA-like currents have been discovered in the chromatophore muscle cells of squid (42) and NMDA-like immunoreactivity has also been reported in the brains of cuttlefish and octopus, although not associated with amacrine cells (43). Naturally, as in mammals, not all activity-dependent LTP need be NMDA-dependent. On the other hand, other synaptic sites in the octopus VL or elsewhere in the brain may possess NMDA-dependent plasticity. It is now well accepted that mollusks have NMDA-like receptors (44, 45) and several simple forms of associative learning in gastropods are mediated by mechanisms that can be blocked by APV (46, 47).

6. HOW THE VL AND ITS LTP ARE INVOLVED IN BEHAVIORAL LEARNING AND MEMORY?

LTP has long been recognized as a cellular mechanism involved in storing memories in the nervous system. This activity-dependent LTP is thought to strengthen the synaptic connections between neurons in the brain's memories stores that are active during specific learning. To test this hypothesis in the octopus brain, (14) used an LTP "saturation" procedure to block the ability of the VL to use LTP during learning. The rationale is that tetanization should artificially saturate LTP, leaving no capacity for activity-dependent plasticity for memorization of the behavioral task. This procedure was proposed as one of the rigorous tests for the importance of LTP in behavioral learning and memory (48).

To learn an avoidance task, octopuses received a mild electric shock each time they touched a red ball, until they reached a criterion of 4 trials without touching the ball (39). LTP was induced through global electrical tetanization of the MSF and VL area in anesthetized

octopuses (39). Octopuses, whose LTP was saturated about 75 minute before training did not remember the task well when tested for long-term memory the following day. Preventing the input from reaching the VL by transecting the axonal tract between the MSF and the VL (Figure 1) resulted in similar but more robust forgetting. These findings support the importance of LTP for creating long-term memories. As this is true for both an invertebrate and vertebrates, it would seem that LTP is a highly efficient mechanism for mediating learning and memory processes and thus was selected to mediate this behavioral function in animals which are phylogenetically very remote. However, we are still far from understanding how this cellular process is utilized for storing and recollecting memories in the brains of octopuses or other animals. Studies in the brain of the octopus, which though complex is far simpler than mammalian brains, may advance our understanding. Below I present the first steps in this direction.

The memory process can be divided into short-term memory (minutes to a few hours) and long-term memory that can store important events and facts for days or even our entire lifetime (49-51). In mammals, including humans (52), short and long-term memory are subserved by two separate systems in different locations in the brain. It is not understood how these two systems are interconnected, if at all. Shomrat *et al*. (39) showed that, in the octopus, short-term memory is stored outside the brain area that uses LTP to acquire long-term memory (the VL), since short-term learning during training for the avoidance task was not abolished by lesioning the MSF-VL tract nor by the tetanization procedure that blocked LTP.

The organization of the learning and memory system in the octopus demonstrates a sophistication that has not yet been described in other animals. In the octopus, the short-term and long-term systems work in parallel but not independently. We found that the long-term storage area, in addition to its capacity for acquiring (or controlling the acquisition outside the VL) of long-term memories, also regulates the rate at which the nervous system acquires short-term memories during training. We inferred this capacity from the opposite effects of tetanization and transection on the rate of learning during training. Transecting the tract significantly reduced the rate at which the octopuses learned the task but, surprisingly, the tetanized animals learned the task faster than the control animals. This regulatory mechanism, by which the neuronal output of the VL can modulate short-term learning, is probably a useful adaptive mechanism where faster learning (faster understanding of risk) is significant for the octopus' survival.

7. SUMMARY

7.1. Neurophysiological reflections arising from the comparison with the insect mushroom body

In this review I have presented our invertebrate model, the cephalopod mollusk *Octopus vulgaris.* Our interest in this unique invertebrate is based on its well documented complex behavioral repertoire comparable to those of vertebrates. This complex behavior is controlled by a brain that still maintains much of the simpler organization of the molluscan nervous system. Our research is exploiting this feature in our attempt to understand how cellular processes function in neuronal circuits to control behavior.

I have drawn functional parallels between the insect antennal lobe-mushroom body system and the median superior frontal lobe-vertical lobe (MSF-VL) system of cephalopods. As noted, these are two examples of special brain structures that most likely evolved to subserve the unique behaviors shown by these two classes of animals. While both systems still require much research, especially in the octopus, it seems that they have both converged to an organization enabling the neural system to represent sensory/neural information in sparse codes. Recent experimental and theoretical studies are pointing to the existence of a common underlying principle in sensory information processing, namely that information is carried by the simultaneous activity in a relatively small fraction of neurons within a large population. This is commonly referred to as 'sparse coding' (53). Sparse representations are important for building a system with a large representational capacity (54, 55). The huge octopus VL system (with roughly one order of magnitude more cells than in rodent hippocampus) may use this type of organization for detection, discrimination, memorization and recall of a vast number of representations.

One difference between the insects and the octopus is that the octopus shows a robust activity-dependent LTP at the first input layer in the VL (MSF-to-amacrine synapse; Figure 1). In locusts, there is no plasticity at this first synaptic station in the MB (54) but rather a spike timing-dependent plasticity (STDP) at the Kenyon cells' output (56). The authors suggested that the many Kenyon cells support multiple invariant sparse representations of odor information in a neural format useful for storage and association at later stages in the system. Thus, plasticity at this level is not required.

Alternatively, the difference in sites of plasticity in the insect and octopus learning systems may correlate with the difference in membrane properties; the Kenyon cells have excitable axons, whereas the amacrine cells are inexcitable. The formation of sparse activity in Kenyon cells is attributed to a unique mechanism, which transforms synaptic inputs into sparse spike codes (55), a mechanism which cannot be implemented in the inexcitable amacrine cells (36). In other words, while insects seem to use a 'digital' coding mechanism, the octopus VL system may use an 'analog' coding system.

This difference in the site of plasticity correlates with a difference in synaptic transmitters. The glutamatergic transmission in the octopus MSF-amacrine cell synapses is similar to the transmission from mechanosensory cells in the gastropods *Aplysia* that also show activity-dependent synaptic plasticity (46, 57). However, the PN- Kenyon cell synapses in the insect MB calyx, that, according to some studies, lack long-term plasticity (53), are cholinergic (58-60). Long-term synaptic

plasticity appears to be a property mainly of glutamatergic transmission, but not necessarily of NMDA.

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