Bridging Behavior and Physiology: Ion-Channel Perspective on Mushroom Body-Dependent Olfactory Learning and Memory in *Drosophila*

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An important body of evidence documents the differential expression of ion channels in brains, suggesting they are essential to endow particular brain structures with specific physiological properties. Because of their role in correlating inputs and outputs in neurons, modulation of voltage-dependent ion channels (VDICs) can profoundly change neuronal network dynamics and performance, and may represent a fundamental mechanism for behavioral plasticity, one that has received less attention in learning and memory studies. Revisiting three paradigmatic mutations altering olfactory learning and memory in *Drosophila* (*dunce*, *leonardo*, *amnesiac*) a link was established between each mutation and the operation of VDICs in Kenyon cells, the intrinsic neurons of the mushroom bodies (MBs). In *Drosophila*, MBs are essential to the emergence of olfactory associative learning and retention. Abnormal ion channel operation might underlie failures in neuronal physiology, and be crucial to understand the abnormal associative learning and retention phenotypes the mutants display. We also discuss the only case in which a mutation in an ion channel gene (*shaker*) has been directly linked to olfactory learning deficits. We analyze such evidence in light of recent discoveries indicating an unusual ion current profile in *shaker* mutant MB intrinsic neurons. We anticipate that further studies of acquisition and retention mutants will further confirm a link between such mutations and malfunction of specific ion channel mechanisms in brain structures implicated in learning and memory.

The ability to correlate behavior with experience, observed from unicellular organisms to mammals, implies the presence of a coupling surface connecting the external world with the net of processes operating in living beings. In all cases, the functional coupling surface relies heavily on components of the plasma membrane, such as receptors, transporters, and ion channels. Typically, in unicellular organisms, external perturbations give rise to conformational changes in specific elements of the coupling surface leading to changes in the levels of relevant internal parameters (H^+, Ca^{2+}, metabolites). Fluctuations in Ca^{2+} or metabolites can modify unicellular behavioral dynamics by, for example, transiently changing flagellar or ciliary beating patterns (Preston, 1990; Beck and Uhl, 1994; Sineshchekov and Govorunova, 1999; Hill et al., 2000). This repertoire of molecular mechanisms exploited by unicellular organisms to couple the external environment to behavior is conceptually identical to that present in animals endowed with a nervous system: a coupling surface (sensory surface) made of receptors or bare nerve endings forward environmental information to nets of neuronal components that are synaptically connected to motor centers that govern muscle function. This configuration accounts for stereotyped behaviors. However, animals endowed with a brain display, in addition, the ability to acquire and retain new behaviors in correlation with experience. The emergence of non-stereotyped behaviors is currently thought to rely heavily on neuronal plasticity in the brain (Martin et al., 2000; Kandel, 2001).

Synaptic transmission and action potential (AP) firing are the mechanisms by which physical or chemical environmental changes at the periphery induce changes in neuronal dynamics at the brain. At the synapse, an excitatory neurotransmitter activates chemically gated cation-selective channels at targeted post-synaptic elements, generating an excitatory post-synaptic potential (EPSP). Neuronal excitability can be defined as a propensity of neurons to generate, beyond a certain threshold, an output signal (the AP) given a certain input signal (the EPSP). This process depends on voltage-dependent ion channels (VDICs) located in the neuronal membrane. EPSPs and APs are essential to correlate perturbations at sensory surfaces triggered by external changes with the dynamics of neuronal nets and motor activity.

In mammals there is convincing evidence for differential VDIC expression in various brain structures, providing them with specific physiological properties (Mandel, 1992; Latorre and Labarca, 1996; Serodio and Rudy, 1998; Talley et al., 1999). VDIC expression can change during development (Iwasaki et al., 2000; Tansey et al., 2002) and accumulating findings indicate that VDIC properties can be modified by activity and neuromodulation (Wang et al., 2003; Yasuda et al., 2003; Frick et al., 2004; Misonou et al., 2004).

There is evidence that VDICs are important to plasticity in the *Drosophila* brain. Mutations that alter

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learning and retention in *Drosophila* associate with altered VDIC function (Cowan and Siegel, 1986; Griffith et al., 1994). Associative olfactory learning and retention is by far the most investigated experience-induced form of behavioral plasticity in which *Drosophila* is employed as an experimental model. Using a combination of genetic disruption and pharmacological ablation Heisenberg et al. (Heisenberg et al., 1985; de Belle and Heisenberg, 1994) showed that mushroom body (MB) integrity is required for the adequate performance of flies during olfactory conditioning. Early evidence for MB involvement in learning and memory in insects was derived from studies in the honeybee, which showed that selectively cooling the MBs after olfactory training causes retrograde amnesia (Erber et al., 1980). *In Drosophila*, MBs are bilateral structures formed by some 2,500 Kenyon cells, the intrinsic MB neurons (MBNs; Fig. 1). MBNs are third-order olfactory-neurons and preferentially express a set of genes whose mutation cause deficiencies in associative olfactory learning and retention (Dubnau and Tully, 1998; Roman and Davis, 2001; Waddell and Quinn, 2001). Odors are initially detected by the olfactory *receptor neurons* at the third segment of the antennae and at the maxillary palps (Stocker, 1994). Axons of receptor neurons project through the antennal nerve towards the antennal lobe, where they make contact with cholinergic *projection neurons* and GABAergic *interneurons*. Efferents from projection neurons send olfactory information through the *antennoglomerular tract* to the dendrites of MBs and to pre-motor regions in the lateral protocerebrum. The available evidence shows that while stereotyped behavioral responses to odorants arise from direct stimulation to the pre-motor elements, and persist even in absence of MBs, these brain structures are absolutely required for associative olfactory learning and retention (Hammer, 1997).

In *Drosophila* 144 genes coding for pore-forming channel subunits, known as α subunits, and auxiliary subunits, have been identified (Littleton and Ganetzky, 2000). Evidence indicates that ion channels are differentially expressed in the fly brain (Schwarz et al., 1990; Hong and Ganetzky, 1994). However, the type, kinetics and/or number of functional ion channels can be modified by neuronal activity and neuromodulatory substances (Siegelbaum et al., 1982; Benson and Levitan, 1983; Byrne and Kandel, 1996). These changes can affect the dynamics and performance of neuronal networks providing a regulatory mechanism of plasticity in the brain (Harris-Warrick and Marder, 1991; Byrne and Kandel, 1996). Here, we review the knowledge on the expression and modulation of ion channels in *Drosophila* MBNs, bridging MB physiology to MB-dependent behavioral traits.

ION CHANNELS IN MUSHROOM BODIES NEURONS

Voltage-dependent Na^+ channels (VDNCs) shape the raising phase of the AP, whereas voltage-dependent Ca^{2+}-channels (VDCCs) usually establish plateau-potentials. Because increases in intracellular Ca^{2+} concentration regulate a wide range of neuronal processes, from neurotransmitter release to changes in gene expression, operation of Ca^{2+}-selective channels is tightly regulated. Voltage-dependent K^+ channels (VDKCs) stabilize the membrane potential and regulate cellular excitability in diverse and subtle ways. In general, they lower the effectiveness of excitatory synaptic inputs into a neuron. They form the most abundant ion channel family, with around 30 different genes coding for VDKCs identified in *Drosophila* and more than 80 genes in mouse. Recently, K^+ channels have been shown to be crucial to neuronal plasticity in mammals (Frick et al., 2004; Murphy et al., 2004; Faber et al., 2005).

Since Wu et al. (1983) first reported that dissociated *Drosophila* larval neurons could be maintained in culture for more than a week, larval brain dispersions, from which MBNs can be identified by tissue-restricted expression of a reporter protein, have been used for the study of VDICs (Wright and Zhong, 1995; Delgado et al., 1998; Gasque et al., 2005). Olfactory learning is not an adult specific trait. *Drosophila* larvae are capable of olfactory learning (Aceves-Pina and Quinn, 1979), which also demands MB integrity (Heisenberg et al., 1985). A mutation in the gene *dunce* (*dnc*) expressed

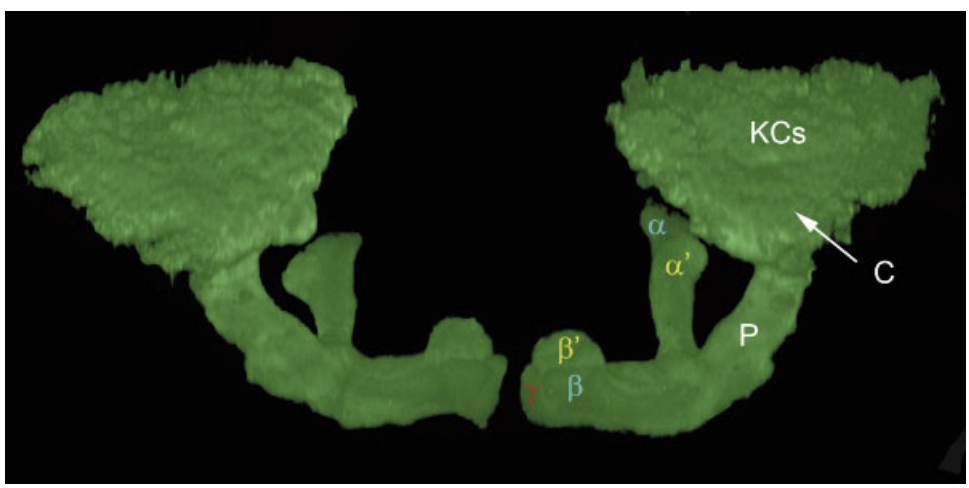

Fig. 1. Mushroom bodies. Somata of intrinsic mushroom body neurons, Kenyons cells (KCs), localize in the dorsoposterior cortex of each brain hemisphere. The Calyx (C) contains the dendritic fields of the KCs. Projection neurons in the antennal lobe (not shown) receive sensory information from the olfactory neurons located at the third segment of the antennae and maxillary palps and send it forward to the MB calyces. The Pedunculus (P) is formed by the axons of the KCs projecting anteriorly to the α, α′, β, β′, and γ lobes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

both in larval and adult MB neuropil (Nighorn et al., 1991), which encloses the MB intrinsic neurites and their afferents, perturbs learning acquisition at both stages (Aceves-Pina and Quinn, 1979). Moreover, under proper training, adult flies can recall what they were taught as larvae (Tully et al., 1994), even though significant axonal rearrangements occur during metamorphosis (Armstrong et al., 1998; Lee et al., 1999). The expression pattern of several molecular MB markers is conserved in larval and adult brains (Nighorn et al., 1991; Rogero et al., 1997; Crittenden et al., 1998). A number of these proteins, including the VDKC Shaker, have been implicated in olfactory learning and memory (Cowan and Siegel, 1986; Dubnau and Tully, 1998; Roman and Davis, 2001). Thus, there is a strong suggestion that MBs play similar roles in larvae and in adults, very likely employing the same molecular machinery. Consequently, larval brain dispersions seem an adequate model to study VDICs and their relationship to neuronal plasticity underlying learning acquisition and memory formation. However, acutely dissociated or cultured MBNs derived from larvae seem to lack VDNCs and therefore are unable to fire APs (Wright and Zhong, 1995; Gasque et al., 2005). This precluded investigating whether and how mutations that alter acquisition and retention affect regulation of electrical activity and synaptic transmission in MBNs. Paradoxically, Wu et al. (1983) documented that larval neurons in primary culture are sensitive to veratridine, a VDNC activator. In these neurons veratridine-induced lethality is blocked by tetrodotoxin (TTX), a VDNC blocker, indicating the presence of VDNCs. We found that in cultured larval MBNs VDNCs are inactivated at holding potentials commonly used to investigate Na^+ currents (around −80 mV). By recording from more negative holding voltages (−140 mV), we succeeded in recording voltage-dependent, TTX-sensitive inward currents (Fig. 2).

Two recently developed methodologies promise a great advance in the field of MB neuronal physiology. Su and O'Dowd (2003) utilized a culture system that supports formation of excitatory and inhibitory synaptic connections between neurons harvested from central brain region of late-stage *Drosophila* pupae. Because in vivo wiring synaptic connectivity depends on electrical activity (Goodman and Shatz, 1993), these pupal MBNs in primary culture would be expected to express functional VDNCs and VDCCs. In fact, *Plectreurys* toxin (PLTx)-sensitive VDCCs, of major physiological relevance (see below), were recorded from these neurons (Jiang et al., 2005). Cultured pupal MBNs offer a unique opportunity to study in a controlled system how VDIC modulation and synaptic connectivity and strength are interconnected. Such studies might be complemented with recordings in situ (Gu and O'Dowd, 2006).

Wilson et al. (2004) developed a protocol that allows whole-cell patch-clamp recordings from projection neurons and local interneurons of the antennal lobe in living animals. Adult flies were wax-fixed within a small hole in an aluminium foil, and bathed by a saline solution. A window was cut in the dorsal head cuticle and the perineural sheath was picked away from the antennal lobe. This technique has been extended to directly recording synaptic inputs into MBNs in living flies (Murthy and Laurent, abstract at the Neurobiology of *Drosophila* Meeting 2005, Cold Spring Harbor Laboratory; Turner et al., abstract at the Neurobiology of *Drosophila* Meeting 2005, Cold Spring Harbor Laboratory).

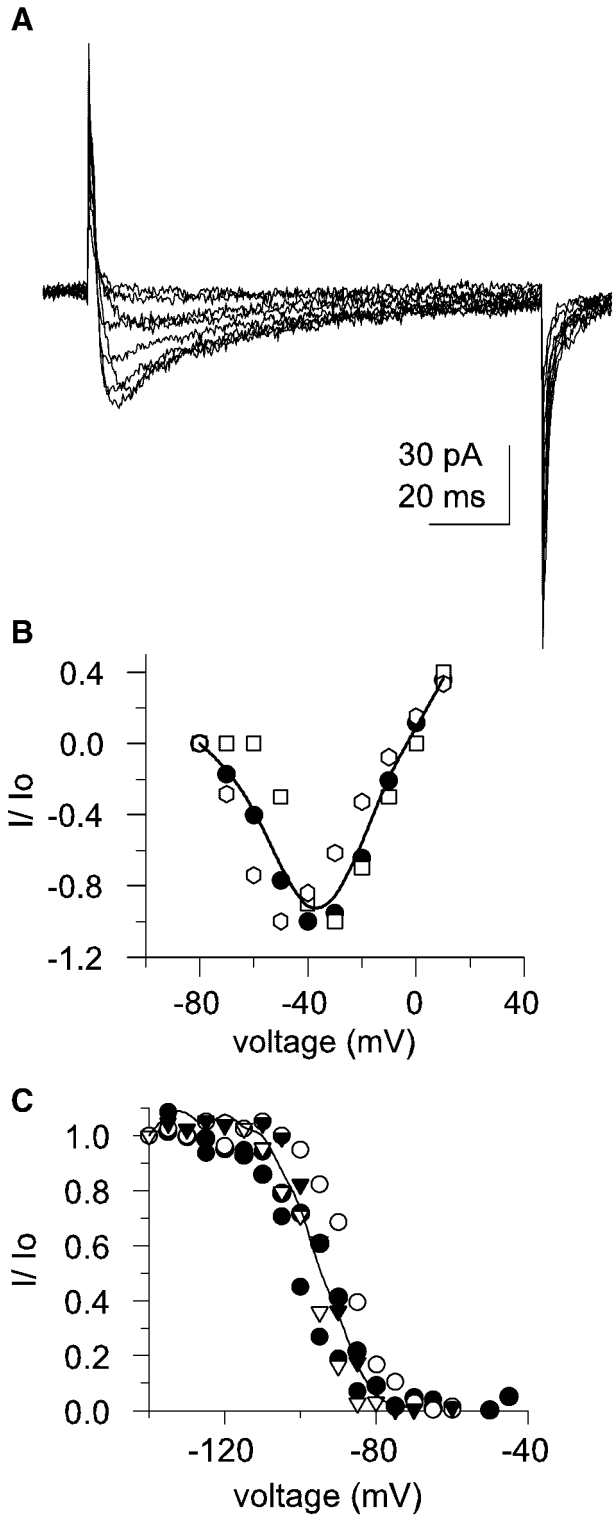

Fig. 2. Functional expression of *Drosophila* larval Kenyon cell voltage-dependent Na^+ currents requires a very hyperpolarized holding potential. **A**: Traces of voltage-dependent Na^+ currents recorded at increasing membrane depolarizations from a holding voltage of −140 mV. **B**: I–V plot of voltage-dependent Na^+ currents. **C**: Steady-state inactivation plot, obtained from voltage-dependent Na^+ currents.

Some recordings have also been performed in dissociated MBNs from other insects, including the honeybee (Table 1). However, the lack of molecular genetics in these models has precluded deeper insights

TABLE 1. Ion channels related to olfactory learning and memory in *Drosophila* and their honeybee counterpart

	Drosophila			Honeybee	
	Immunological evidence	Physiological evidence	Relation to MB-dependent learning	Immunological evidence	Physiological evidence
CDKC	Yes; Slo protein detected in the MBs[a]	No	Slo is regulated, in vitro, by the learning gene *leo*[b]	No	Yes; kinetic and pharmacological properties similar to Slo[c]
VDCC	No	Yes; voltage-dependent Ca^{2+} current mainly encoded by a PLTx-sensitive channel[d]	Required for cell-autonomous spontaneous Ca^{2+} oscillation that, in vivo, are regulated by the middle-term memory gene *amn*[e]	No	Yes; partially sensitive to a very high verapamil concentration[c]
VDKC (delayed-rectifier)	No	Yes[f,g,h]	Dowd-regulated by cAMP, which is fundamental for olfactory learning[g,i]	No	Yes[c]
VDKC (Shaker)	Yes[j]	Yes; Shaker encodes a 4-AP-sensitive, PaTx-sensitive A-type current in a subset of MBNs[f]	Shaker mutants display short-term olfactory learning deficits[k]	No	Yes; Shaker encodes the major 4-AP-sensitive A-type current[l]

4-AP, 4-aminopyridine; *amn*, *amnesiac*; CDKC, Ca^{2+} dependent K^+ channel; *leo*, *leonardo*; MBNs, mushroom body neurons; PaTx-2, phrixotoxin-2; PLTx, *Plectreurys* toxin; Slo, Slowpoke; VDCC, voltage-dependent Ca^{2+} channel; VDKC, voltage-dependent K^+ channel.
[a]Becker et al. (1995).
[b]Zhou et al. (1999, 2003).
[c]Schafer et al. (1994).
[d]Jiang et al. (2005).
[e]Davis (2001); Rosay et al. (2001); Jiang et al. (2005).
[f]Gasque et al. (2005).
[g]Delgado et al. (1994).
[h]Wright and Zhong (1995).
[i]Roman and Davis (2001).
[j]Schwarz et al. (1990); Rogero et al. (1997).
[k]Cowan and Siegel (1986).
[l]Pelz et al. (1999).

into the molecular components encoding these currents, as well as the role they have in coding olfactory learning and memory.

LEARNING MUTANTS AND VOLTAGE-DEPENDENT K^+ CHANNELS

Early evidence that ion channels are relevant to associative olfactory learning in the fruit fly was provided by Cowan and Siegel (1986), who found that this process is deficient in a *shaker* mutant. The *shaker* gene codes for a family of VDKCs that, through alternative splicing, give rise to either rapidly inactivating A-type currents or non-inactivating currents (Iverson et al., 1988; Timpe et al., 1988; Iverson and Rudy, 1990; Stocker et al., 1990). Immunohistochemical studies revealed that Shaker channels are preferentially expressed in the *Drosophila* MB neuropil (Schwarz et al., 1990; Rogero et al., 1997), suggesting that they play an important role in MB physiology. Additionally, the kinetic properties of the inactivating K^+ current recorded from honeybee MBNs indicated that in these neurons, the A-type current is dominated by a Shaker-like component (Pelz et al., 1999). Shaker channels are important to the regulation of excitability (Tanouye et al., 1981) and loss of the Shaker K^+ conductance in *Drosophila* photoreceptors significantly limits the amplification of the graded-voltage signals (Niven et al., 2003). Peripheral larval synapses in *shaker* mutants are hyperactive, with grossly enlarged, asynchronous nerve-evoked responses (Jan et al., 1977). Importantly, they lack post-tetanic potentiation (PTP) (Delgado et al., 1994), a form of synaptic plasticity present in peripheral and central synapses thought to be associated with short-term memory (Silva et al., 1996). The fact that *shaker* is deficient in olfactory learning suggests that, by regulating transmitter release and synaptic plasticity, VDKCs can play key roles in MB physiology. However, until recently, a link between Shaker channels and *Drosophila* MBN physiology was missing. Recent work by Gasque et al. (2005) provided evidence that lack of Shaker channels alters MBN physiology. Using whole-cell patch-clamp recordings from the soma of acutely dissociated MBNs, we found that a fraction of MBNs lacks A-type currents contributed by Shaker channels. As a result, outward currents are reduced in amplitude and the remaining outward currents show significantly slower inactivation. Further work using patch clamp recording is necessary to investigate the role of Shaker in regulating AP firing and synaptic transmission in MBNs and their relation to learning and retention.

cAMP SIGNALING CASCADE AND ION CHANNEL MODULATION

A variety of genetic, behavioral, biochemical, immunohistochemical, electrophysiological, and imaging inquiries have been performed in *dnc* and *rutabaga* (*rut*) *Drosophila* mutants to study the role of the cAMP cascade in short-term memory in *Drosophila*. *Dnc* is a mutant deficient in a cAMP phosphodiesterase, and has abnormally elevated cAMP levels (Byers et al., 1981; Davis and Kiger, 1981). *Rut* has a diminished Ca^{2+}/calmodulin-sensitive adenylate cyclase activity, needed to promote cAMP production (Livingstone et al., 1984; Levin et al., 1992). Behavioral studies showed that both mutants are deficient in short-term olfactory memory (Dubnau and Tully, 1998). In addition, inmunohistochemistry indicates that components of the cAMP cascade are preferentially expressed in the MBs (Nighorn et al., 1991; Han et al., 1992). In electrophysiological experiments at larval peripheral synapses, *rut* and *dnc* mutants were found to lack PTP (Zhong and Wu, 1991).

Additionally, cAMP analogs effectively abolished PTP in *wt* synapse (Zhong and Wu, 1991). Importantly, a K^+-channel blocker, 3,4−diaminopyridine, was reported by Delgado et al. (1992) to be effective in rescuing PTP in *dnc* and in restoring PTP in *wt* synapses exposed to cAMP analogs. These data provide evidence that cAMP-regulated channels might be important to synaptic plasticity in *Drosophila*. More recently Cheung et al. (2006) documented the presence of hyperpolarization-activated, cAMP-regulated channels modulating synaptic plasticity in the *Drosophila* neuromuscular junction (NMJ). Their presence at synapse in the MBs remains to be investigated.

cAMP can regulate ion channel activity either directly or through phosphorylation by protein kinase A (PKA). Patch-clamp recording in somas of acutely dissociated MBNs by Wright and Zhong (1995) first documented the presence of cAMP-regulated K^+ channels. Further work by Delgado et al. (1998) in MBNs in primary culture showed that cAMP-regulated VDKCs are present in only a fraction of MBNs and that this fraction is significantly larger in MBNs compared to the general neuron population. These results agree with previous evidence that components of the cAMP cascade are preferentially expressed in MBs (Nighorn et al., 1991; Han et al., 1992).

Delgado et al. (1998) extended their studies to *dnc* mutants to find that the number of neurons displaying cAMP-regulated K^+ currents was dramatically reduced compared to *wt*. This result documented for the first time that deficiencies in the cAMP cascade alter MBN physiology and suggest ion channel regulation by cAMP as a feasible physiological mechanism underlying learning and memory regulation in *Drosophila*. In addition the above work also showed that MBs are not homogeneous regarding outward current profiles, in agreement with findings by Wright and Zhong (1995) and Gasque et al. (2005). MBs morphology defines at least three neuronal types (Crittenden et al., 1998; Lee et al., 1999). Two types of neurons branch their axons to give rise to a vertical and a median lobe (α/β and α'/β', respectively). The third type composes the γ median lobe. However, it remains to be established whether the variety of neurons found in culture reflects actual electrophysiological differences that also exist in MBs in vivo.

LEONARDO-DEPENDENT SHORT-TERM MEMORY AND Ca^{2+}-DEPENDENT K^+-CHANNELS

Leo is the ζ isoform of the *Drosophila* 14-3-3 protein and was originally identified because of its MB-enriched expression (Skoulakis and Davis, 1996). Mutations that compromise expression of *leo* in adult MBs and the ellipsoid body result in short-term memory deficit (3 min after training under olfactory classical conditioning) that is proportional to the reduction in protein expression (Skoulakis and Davis, 1996). Conditional expression of wild-type *leo* rescues olfactory learning defects in *leo* mutants, supporting an acute, and not a developmental requirement of Leo during olfactory conditioning (Philip et al., 2001).

Members of the 14-3-3 family are ~30 kDa acidic proteins found in all eukaryotes, from plants to mammals. They appear to act as molecular anvils that induce conformational changes in their binding partners that can alter their enzymatic activity, or mask or reveal functional motifs that regulate their localization, activity, phosphorylation state, and/or stability. Today, more than 100 binding partners have been identified and they include transcription factors, biosynthetic enzymes, cytoskeletal proteins, signaling molecules, apoptosis factors, and tumor suppressors (Dougherty and Morrison, 2004). Additionally, 14-3-3 proteins also have adaptor functions, mediating interactions between two different binding partners.

How Leo specifically participates in olfactory learning is presently unknown. However, in the NMJ of *Drosophila* embryos, Leo regulates pre-synaptic function. *leo* mutants show impaired synaptic plasticity, including lack of PTP, possibly due to the interaction of Leo with PKC or related kinases that regulate the size of the readily releasable vesicle pool at pre-synaptic active sites (Broadie et al., 1997).

We believe that Leo might additionally control synaptic function by its interaction with Ca^{2+}-dependent K^+-channels (CDKCs). CDKCs modulate cell excitability and AP waveform (Elkins et al., 1986; Latorre et al., 1989; Kaczorowski et al., 1996) and because they are regulated both by $[Ca^{2+}]_i$ and membrane potential, these channels are molecular *loci* for the integration of electrical and biochemical signals engaged in the regulation of neuronal plasticity. Recently, CDKCs have been implicated in long-term potentiation in mammals (Faber et al., 2005; Ngo-Anh et al., 2005).

In *Drosophila*, the gene *slowpoke* encodes a CDKC of large conductance, that when expressed in *Xenopus* oocytes induces a voltage dependent current activated by micromolar intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Adelman et al., 1992; DiChiara and Reinhart, 1995). Becker et al. (1995) documented Slo expression in the soma and axons of the MBNs in fly brains, overlapping with the expression of Leo (Skoulakis and Davis, 1996). Even though there are no electrophysiological studies of CDKCs in *Drosophila* MBNs, a Ca^{2+}-dependent outward current has been recorded from dissociated honeybee MBNs (Schafer et al., 1994; Table 1); and the kinetic and pharmacological profiles of this current strongly resemble the properties of the recombinant *Drosophila* Slo channel (dSlo).

Neuronal dSlo co-immunoprecipitates with an accessory soluble subunit called Slob (*Slo*wpoke *b*inding protein; Schopperle et al., 1998). Leo interacts with the Slob-Slo complex and diminishes the Slo current by shifting the activation curve of the Slo channel toward more positive values (Zhou et al., 1999). Slo-Slob-Leo complex formation requires phosphorylation of Slob by the Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII). Using conditional expression of CaMKII in transgenic *Drosophila* flies, Zhou et al. (1999) showed that Leonardo-dependent modulation of Slo varies dynamically, and depends on the phosphorylation state of Slob. Thus it is conceivable that the abnormal *leo* behavioral phenotype associates to deficient dSlo channel modulation in the MBs. In *leo* mutants Slo channels would be expected to activate at more hyperpolarized voltages to reduce excitability, disrupting transmitter release, and experience-dependent changes in synaptic strength.

Ca^{2+} OSCILLATIONS AND MIDDLE-TERM MEMORY CONSOLIDATION

Transient increases in $[Ca^{2+}]_i$ regulate a wide range of cellular processes in neurons, ranging in time scale from submillisecond neurotransmitter release at pre-synaptic terminals to the essentially permanent changes in gene expression that occur during various forms of brain plasticity (Ghosh et al., 1994; Ghosh and

Greenberg, 1995; Augustine et al., 2003; Koh and Bellen, 2003). In vivo studies in *Drosophila* using tissue-restricted expression of the Ca^{2+}-sensitive luminescent protein, apoaequorin, reveal spontaneous rhythmic Ca^{2+} oscillations in the MBs (Rosay et al., 2001). These oscillations reflect synchronized Ca^{2+} waves over a population of MBNs (Davis, 2001). The Ca^{2+} oscillations described by Rosay et al. (2001) also occur in isolated brains, indicating these events are independent of sensory input. Ca^{2+} oscillations disappear when extracellular Ca^{2+} is removed or when L-type Ca^{2+} channel blockers are added to the bath solution, but are unaffected by drugs that perturb intracellular Ca^{2+} stores (Rosay et al., 2001). These results suggest that the Ca^{2+} oscillations originate in the plasma membrane. Additionally, a collection of drugs affecting VDNCs and VDKCs were effective in modifying or eliminating Ca^{2+} oscillations.

Evaluation of the biophysical mechanisms underlying the Ca^{2+} oscillations requires the ability to resolve Ca^{2+} dynamics in single MBNs. Very recently, Jiang et al. (2005) used Fura-imaging to demonstrate that cultured pupal MBNs generate spontaneous Ca^{2+} transients in a cell autonomous manner, at a frequency similar to Ca^{2+} oscillations in vivo. Removal of external Ca^{2+}, addition of the general Ca^{2+} channel blocker Co^{2+}, or addition of PLTx, an insect-specific Ca^{2+} channel antagonist (Leung et al., 1989), abolished Ca^{2+} transients. Pupal MBNs display a PLTx-sensitive voltage-dependent Ca^{2+} current which might mediate spontaneous Ca^{2+} oscillations in MBs.

PLTx has been shown to inhibit synaptic transmission at the *Drosophila* larval NMJ (Branton et al., 1987). In this synapse Dmca1A Ca^{2+} channels are localized at the pre-synaptic terminal and control transmitter release (Kawasaki et al., 2000, 2004). Such PLTx-sensitive Ca^{2+} channels might mediate the cell autonomous Ca^{2+} oscillations in the MBNs.

A striking difference between in vivo rhythmic transient $[Ca^{2+}]_i$ increases and in vitro spontaneous Ca^{2+} oscillations is that rhythmic oscillations are inhibited by the VDCC blocker verapamil (5 μM; Rosay et al., 2001) whereas the spontaneous transients are refractory to this blocker (Jiang et al., 2005). Though verapamil sensitivity was not assayed in the VDCCs recorded in *Drosophila* MBNs, it has been tested in the Ca^{2+} currents recorded from honeybee MBNs. In these neurons, the Ca^{2+} current was only partially blocked by 100 μM verapamil (Schafer et al., 1994). Since rhythmic $[Ca^{2+}]_i$ transients in vivo require synchronized MBN activity (Davis, 2001), it has been proposed that verapamil-sensitive VDCCs participate in interneuronal communication leading to MBN synchronization, but not in the basic mechanism of Ca^{2+} wave generation in individual Kenyon cells (Jiang et al., 2005).

Synchronous Ca^{2+} oscillations in MBNs could be relevant for memory consolidation. A mutation in the gene *amnesiac* (*amn*), whose molecular product is required for middle-term memory (Feany and Quinn, 1995; Waddell et al., 2000), increases dramatically the amplitude of oscillations (Rosay et al., 2001). *Amn* codes for a putative neuropeptide that shares similarity with the pituitary adenylate cyclase activating peptide (PACAP) of mammals and is expressed in the dorsal paired neurons situated medially to the MBs (Feany and Quinn, 1995; Waddell et al., 2000). These neurons profusely innervate the ipsilateral MB lobules. Blocking vesicle-mediated secretion from the dorsal pair medial cells during associative olfactory training does not interfere with immediate recall but abolishes memory retrieval at 60 min (Waddell et al., 2000), mimicking the *amn* phenotype. It has been proposed that release of Amn neuropeptide upon MB axons produces relatively long-lasting physiological changes required for middle-term memory consolidation and retrieval, mediated by synchronous $[Ca^{2+}]_i$ oscillations (Davis, 2001). Expression of new genes by Amn stimulation can be discarded since middle-term memory does not seem to require protein synthesis (Dubnau and Tully, 1998).

Each set of ipsilateral MB lobes is innervated by a single dorsal medial neuron. Secretion of the neuropeptide by the dorsal medial neurons might act as a paracrinal signal that influences synchronization of the electrical activity of the target neurons. Evidently, a cAMP-dependent pathway is the favored mediator of the AMN regulation. However, Rut adenylate cyclase does not seem to be involved in the AMN response because preliminary results with *rut* mutants did not reveal major effects on oscillation amplitude, period, or waveform (Rosay et al., 2001). On the other hand, middle-term memory does seem to depend on PKA activity because a temperature sensitive allele of the catalytic subunit, *DCO*, decreases memory retention at 1 h, exactly as in *amn* (Li et al., 1996; Dubnau and Tully, 1998). Thus, a Rut-independent PKA-dependent regulation of electrical activity, leading to synchronous $[Ca^{2+}]_i$ oscillations associated to verapamil-sensitive Ca^{2+} channels could mediate the Amn-dependent middle-term memory formation and retrieval.

CONCLUDING REMARKS

Genetic analysis of *Drosophila* behavior has allowed unbiased identification of genes engaged in associative learning and retention. It has paved the way to dissect the neuroanatomy and biochemistry required for these behavioral traits (Dubnau and Tully, 1998; Roman and Davis, 2001; Waddell and Quinn, 2001). In spite of this progress, physiology still remains a gray area in understanding associative learning and memory in the fruit fly. A review of available evidence points at ion channels as key players in the physiology of MBs, structures in the *Drosophila* brain needed for the emergence of olfactory associative learning and retention. MBNs are the targets of mutations that alter acquisition and retention. In turn, mutations that affect olfactory learning impair ion channel function directly or indirectly, by altering regulatory mechanisms. Mutants displaying deficient olfactory associative learning and retention have also been found to display abnormal transmitter release and synaptic plasticity at larval NMJ and the evidence also implicates ion channels in some of these synaptic deficiencies. It is puzzling, however, why genetic loci that encode ion channels and related proteins in *Drosophila* have not been identified in screens for memory deficit mutants, even though reverse genetics has shown their requirement (Cowan and Siegel, 1986).

Understanding the mechanisms of associative learning and retention demands unveiling the physiology of Kenyon cells, the intrinsic neurons of the MBs. It is apparent that the ability to genetically manipulate *Drosophila* and to combine behavioral studies with physiological approaches makes this insect a most attractive model to investigate fundamental aspects of associative behavior. At this stage it is possible to investigate the way ion channels participate in shaping MB neuronal plasticity; and how mutations that target specific ion channels affect fly behavior. However, we

anticipate that not all channels should be involved in regulating olfactory learning and retention.

It is important to note here that the best-established mutants of learning and memory in *Drosophila* correspond to mutants exhibiting deficiencies in acquisition and/or short- and middle-term retention. Such mutants display altered transmitter release and plasticity at the NMJ, associated essentially to the pre-synaptic terminal. However, little is known on the effects of such mutations in MB synapses. Moreover, little is known about the mechanisms mediating long-lasting memory in *Drosophila* and whether or not such a phenomenon associates to long-term potentiation, as in mammals. MBNs in primary culture have provided significant insights on their properties. Pupal neurons derived from MBs, introduced by O'Dowd and collaborators (Jiang et al., 2005), offer an attractive experimental model to monitor electrophysiological aspects of MBN physiology as well as to investigate synaptic plasticity both in normal and mutant synapses. On the other hand, their small size had precluded patch-clamp recording directly from MBNs in the fly brain. Recent progress indicates that this limitation has been overcome at last (Gu and O'Dowd, 2006; Murthy and Laurent, abstract at the Neurobiology of *Drosophila* Meeting 2005, Cold Spring Harbor Laboratory; Turner et al., abstract at the Neurobiology of *Drosophila* Meeting 2005, Cold Spring Harbor Laboratory) providing researchers with a crucial tool to further inquire on the physiological aspects of associative learning in the fruit fly brain.

The basic rules of brain plasticity from which associative behavior emerges seem to be essentially similar in all animals endowed with a nervous system. Understanding how associative learning operates in a fly will provide precious information on how associative learning operates in all brains. Some may think this is not good enough and will rather wait until the mystery of the poet brain is unveiled. But to most it will not seem a small achievement, to know how the workings of the fly brain, in harmony with the rest of the body machinery, do of the light fly a self in the kingdom of animals.

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