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LEARNING CHANNELS. CELLULAR PHYSIOLOGY OF ODOR PROCESSING NEURONS WITHIN THE HONEYBEE BRAIN*

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To understand the cellular mechanisms of olfactory learning in the honeybee brain we study the physiology of identified neurons within the olfactory pathway. Here, we review data on the voltage-sensitive and ligand-gated ionic currents of mushroom body Kenyon cells and antennal lobe neurons *in vitro* and *in situ.* Both cell types generate action potentials *in vitro*, but have different voltage-sensitive K^+ currents. They express nicotinic acetylcholine receptors and ionotropic GABA receptors, representing the major transmitter systems in the insect olfactory system. Our data are interpreted with respect to learning-dependent plasticity in the honeybee brain.

Keywords: Voltage-sensitive currents – Kenyon cells – acetylcholine receptor – GABA receptor – insects

INTRODUCTION

Behavioural plasticity during memory formation correlates with neuronal plasticity within the central nervous system, which means that some neurons change their physiology while learning or memory formation occurs. Many invertebrates are powerful model systems for the study of cellular mechanisms of learning, including the marine snail, *Aplysia californica* [16], the pond snail, *Lymnaea stagnalis* [1], the fruitfly, *Drosophila melanogaster* [13] and the honeybee, *Apis mellifera* [21]. The mushroom bodies are the key neuropils within the insect brain for the search of the memory trace in Drosophila (e.g. [14, 20, 34]); in the honeybee both the mushroom bodies and the antennal lobes are involved in memory formation (e. g. [12, 19, 23]). Our goal is to understand the cellular mechanisms of olfactory learning in the honeybee brain. For this, we analyse the cellular physiology of mushroom body Kenyon cells and antennal lobe projection neurons using patch clamp techniques *in vitro* and

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in situ. This review provides an overview on the voltage-sensitive ionic currents and ionotropic transmitter receptors that are expressed by identified honeybee central neurons.

Whole-cell patch clamp recordings

Kenyon cells or antennal lobe neurons were dissected from honeybee *(Apis mellifera)* brain at various developmental stages (pupae and adults). Cells were taken into primary cell culture as described earlier [25]. When neurons were labelled with rhodamine-dextrane prior to recording a technique developed by Grünewald [9] was used. Patch clamp recordings under the whole-cell configuration were performed on cultured neurons after 3–6 days *in vitro* (for details of solutions, devices and data analyses see: [9]). To investigate ligand-gated membrane currents transmitters or agonists were pressure applied [4].

Voltage-sensitive ionic currents

Ionic currents of Kenyon cells

Cultured Kenyon cells generate TTX-sensitive overshooting action potentials upon injections of depolarizing currents [31]. The underlying voltage-sensitive ionic currents have been characterized in detail.

Voltage-gated Na^+ currents (I_{Na}) were isolated by adding $CdCl_2$ to the bath solution to inhibit voltage-sensitive Ca^{2+} currents and substituting Cs^+ and TEA for K^+ ions in the pipette solution to block K^+ currents [27, 31]. Thus, a classical TTX-sensitive, fast transient I_{Na} remains (Fig. 1A), that activates at voltages more positive than –40 mV and peaks at around –10 mV. Kenyon cells also express a small sustained I_{Na} (<1% of the total I_{Na}), which is voltage-sensitive, but shows little or no inactivation.

Three different voltage-sensitive K^+ currents are present in Kenyon cells [25, 27] (Fig. 1B, C). When inward I_{Na} are blocked with 100 nM TTX and Ca^{2+} currents with 50 μM $CdCl_2$, the most prominent K^+ current is a *shaker*-like K^+ current ($I_{K,A}$). This fast activating, transient current is sensitive to 4-aminopyridine. The kinetic and steady-state parameters were determined by Pelz et al. [25], who also provided a mathematical model of the $I_{K,A}$. Kenyon cells express a delayed rectifier type K^+ current ($I_{K,V}$), which is partially blocked by 100 μM quinidine and is less sensitive to TEA. The activation time constant is slightly voltage-dependent and rather fast, ranging between ~3.5 ms at 0 mV and ~1.9 ms at >60 mV. Using modelling approaches a slow transient K^+ current ($I_{K,ST}$) was postulated and subsequently identified experimentally [31]. This $I_{K,ST}$ is 4-aminopyridine-resistant and has a slower activation and inactivation time constant than the $I_{K,A}$. A Ca^{2+}-dependent K^+ current ($I_{K,Ca}$) reported by Schäfer et al. [27] could not be reproduced by recent studies [9] (Fig. 1B).

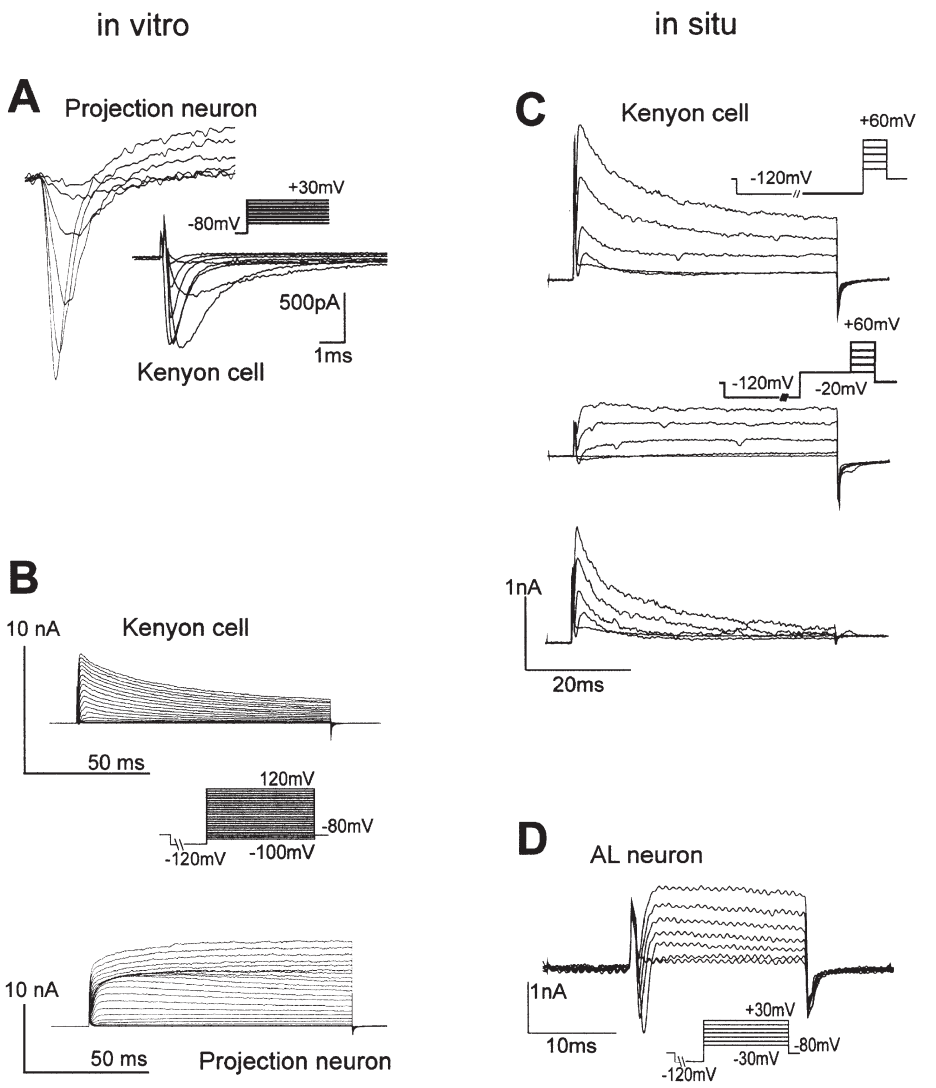

Fig. 1. Voltage-sensitive currents of cultured Kenyon cells *in vitro* (**A, B**) and *in situ* (**C, D**). **A.** Voltage-sensitive I_{Na} (under 20 mM TEA, 6 mM $BaCl_2$ instead of $CaCl_2$, 50 µM $CdCl_2$ in the external and 140 mM CsCl in the internal saline). Current amplitudes are larger in projection neurons than in Kenyon cells. Holding potential was –80 mV, throughout, command potentials are given in the insets. **B.** Different outward currents (external 100 nM TTX) in Kenyon cells and in projection neurons (note the different scale bars). Cells were clamped to –120 mV for 1s before the test pulses (ΔV = 10 mV) to completely remove K^+ current inactivation. **C.** Isolation of the transient $I_{K,A}$ in an *in situ* recording of a Kenyon cell (external 100 nM TTX, 50 µM $CdCl_2$). Currents were measured after a 1 s prepulse to –120 mV, which completely removes inactivation of $I_{K,A}$ (top trace) and after an inactivating prepulse to –20 mV (120 ms, middle trace). The subtraction (bottom trace) yields the inactivating K^+ currents, which closely resemble the *in vitro* currents [25]. **D.** *In situ* recording of an unidentified antennal lobe neuron. This cell shows pronounced inward currents (no blockers added), but lacks a transient outward current

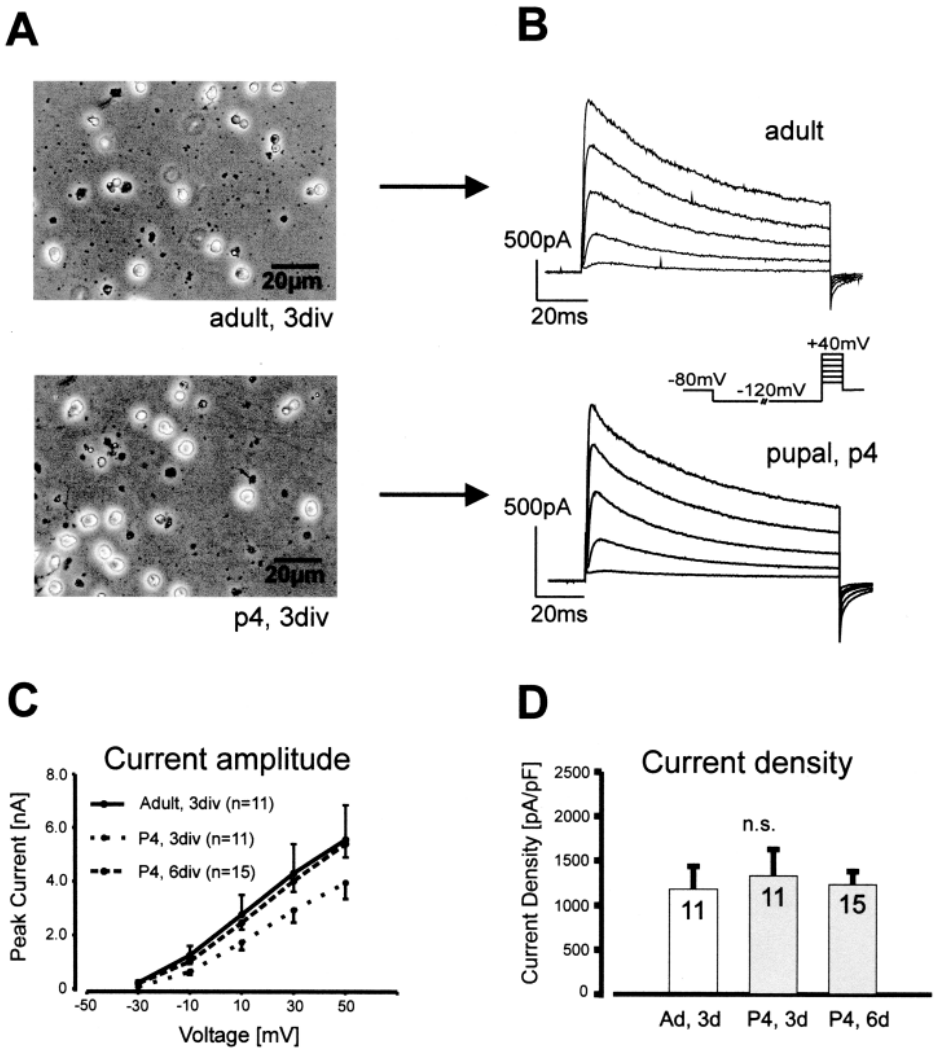

Fig. 2. Comparison of pupal and adult currents. **A.** Cultured Kenyon cells from a freshly emerged (top) or a pupal (P4, below) honeybee after 3 days *in vitro*. **B.** Whole-cell K^+ currents of adult and pupal neurons (scaled to the same amplitude) at various depolarizing command potentials (–20 to +60 mV, 20 mV steps, preceded by a 1 s prepulse to –120 mV, inset). Inward currents were blocked by external 100 nM TTX and 50 µM $CdCl_2$. **C.** Current-voltage relationship of the peak K^+ current is similar in pupal and adult Kenyon cells after 3–6 days *in vitro* (div). **D.** Current densities (pA/pF) of adult and pupal neurons do not differ ($p = 0.90$; $df = 2$; ANOVA, Statistica for Windows, version 5.5, StatSoft, Tulsa, OK). Peak current amplitudes were measured at a command potential of +50 mV, cell capacitances were determined for each cell by the compensation procedure of the patch amplifier

The voltage-sensitive Ca^{2+} currents (I_{Ca}) of Kenyon cells show rapid activation and slow inactivation [9, 27] (no figure). The voltage threshold for activation of the I_{Ca} was about –35 mV, it peaked at command potentials between 0 and 10 mV and had a reversal potential at around 35 to 45 mV. Their peak current amplitude is small (mean <–200 pA) and current rundown is substantial, hindering a more detailed biophysical description of the I_{Ca}.

Overall, the voltage-sensitive whole-cell currents of honeybee Kenyon cells are similar to those described in other insects [30]. We incorporated our spike and ionic current data into a physiology-based Hodgkin-Huxley type model, which simulates most of our current- and voltage-clamp data correctly [31].

Adult and pupal currents

Most of the patch clamp experiments were performed on neurons from pupal stages (P4–P6), because the development of the mushroom bodies and antennal lobes is almost mature at these stages [28], but the cells can be dissociated easily without enzymatic treatments. But how mature is the current phenotype of the pupal cells? We compared the whole-cell K^+ currents of Kenyon cells from a midpupal stage (P4) with those from an adult bee just after emergence. Adult Kenyon cells can be taken into culture (Fig. 2A), but that requires collagenase/dispase treatment before trituration (1 mg/ml, 30 min at RT). Contrary to pupal neurons they hardly grow any processes *in vitro*. The whole-cell currents are indistinguishable and the current amplitudes and current densities do not differ (Fig. 2B–D). This indicates that the current phenotype is already mature at midpupal stages P4–P6.

Ionic currents of antennal lobe projection neurons

We compared the ionic currents of cultured Kenyon cells and their presynaptic neurons, projection neurons from the antennal lobes. To identify the projection neurons *in vitro* we retrogradely labeled their somata prior to recording by dextrane rhodamine [9]. The somata diameters of cultured Kenyon cells (7–10 μm) are smaller than those of projection neurons (10–25 μm), resembling their sizes in the intact brain. Both neuron types generate action potentials upon depolarization and express very similar I_{Na} and I_{Ca}. By contrast, the voltage-sensitive K^+ currents of the two neuron classes differ. Whereas Kenyon cells express pronounced *shaker*-like $I_{K,A}$, this current is almost absent in projection neurons (Fig. 1B). This resembles the situation in the Drosophila brain, where *shaker* channel proteins are highly expressed in the mushroom body, but not the in antennal lobes [26]. Ca^{2+}-dependent K^+ currents ($I_{K,Ca}$) are expressed by projection neurons but not by Kenyon cells (Fig. 1B). The differential current expression of antennal lobe and mushroom body neurons indicates that the current phenotypes are conserved through culturing procedures. Figure 4 schematically summarizes the different voltage-sensitive conductances of Kenyon

cells and projection neurons. Since the main currents during an action potential are I_{Na} and $I_{K,V}$ the modulation of the spike shape may be achieved by $I_{K,A}$ in Kenyon cells or transient $I_{K,Ca}$ in projection neurons, respectively. Additional work is required to reveal whether these currents are differentially modulated during learning.

In situ *patch clamp recordings*

Ionic currents recorded in the culture dish may differ from those of neurons within their brain environment. We therefore studied, whether whole-cell currents recorded *in situ* are similar to those *in vitro*. The ionic currents recorded at the somata of antennal lobe neurons or Kenyon cells *in situ* closely resemble those recorded from cultured cells (Fig. 1C, D). Although the somata of insect neurons do not participate in spike generation the somata express voltage-sensitive currents *in situ*. Outward currents of Kenyon cells show the typical $I_{K,A}$ and a partially TEA-sensitive $I_{K,V}$. Also antennal lobe neurons show pronounced inward currents and transient as well as sustained outward currents as observed *in vitro*. Similarly, recordings of currents *in situ* and *in vitro* of honeybee antennal motoneurons revealed no measurable differences [17]. Several technical problems, however, prevent the routine application of *in situ* patch recordings from honeybee neurons. First, the success rate of patch clamp recordings from neurons within the living brain is low and the gigaseal is seldom stable. Second, the space-clamping conditions of *in situ* recordings are compromised, because of seal problems and because the neurite is still attached to the soma. This hinders a direct comparison of the biophysics of *in situ* and *in vitro* currents. Third, we failed to stain the neurons for a morphological identification. Nevertheless, given that these difficulties will be solved, *in situ* recordings are inevitable for analysing the cellular physiology of neurons within a biological environment and for assaying different physiological properties of morphologically different Kenyon cells.

Ligand-gated ionic currents

The range of transmitter receptors in insects is highly diverse and the physiological and pharmacological properties of the insect receptors are very different from their vertebrate counterparts (reviews: e.g. [3, 10, 15]). Behavioural pharmacological experiments are necessary to unravel the contribution of a given transmitter system to learning or memory formation. However, before such studies can be correctly interpreted, we have to study the pharmacology and physiology of the underlying transmitter receptor. Which transmitters do Kenyon cells express?

Acetylcholine is the major excitatory transmitter within the insect brain and most neurons of the olfactory pathway are probably cholinergic [18, 33]. Accordingly, pressure applications of acetylcholine induced inward currents (at negative command potentials) in cultured Kenyon cells (Fig. 3A, B). This acetylcholine-induced current

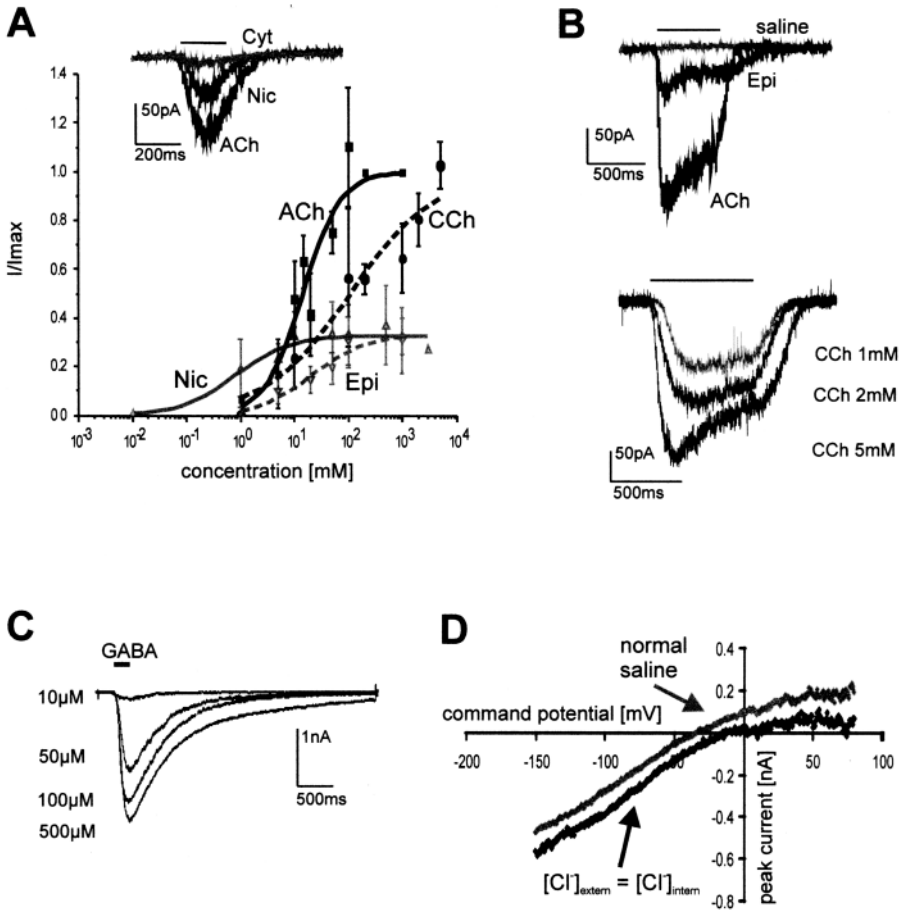

Fig. 3. Acetylcholine- and GABA-induced currents in honeybee Kenyon cells. **A.** Inset: Pressure applications (bar, 200 ms) of acetylcholine (ACh), nicotine (Nic) or cytisine (Cyt) induced inward currents of different amplitudes (concentration 20 µM; pulse potential –110 mV). The dose-response curves indicate different potencies for the various agonists. The peak current amplitude normalized to the maximum amplitude induced by 200 µM ACh (I/Imax) is plotted against the different concentrations; several concentrations of a given agonist were tested on a given cell. **B.** Epibatidine (Epi, top) is only a partial agonist of the nicotinic receptor (concentrations of EPi, ACh was 20 µM). Pressure applications of saline never induced any currents. Concentration-dependency of currents induced by carbamylcholine (CCh, bottom). At 5 mM CCh no further increase of the current amplitude can be observed; at this concentration ACh (200 µM) elicited the same peak current amplitude (not shown). **C.** Concentration dependency of GABA-induced currents. Pressure applications of GABA (pulse duration 200 ms, bar; pulse potential –110 mV) at the concentrations indicated induced rapidly activating inward currents of different amplitudes. Data were obtained from an individual cell. **D.** Ion substitution experiments using voltage ramps (–150 mV to +80 mV). Two ramps were run with different Cl^- concentrations in the pipette and the external solution (26.4 mM and 154 mM, respectively: normal saline; data from one cell) and with equimolar Cl^- concentrations (26.4 mM; N = 4). The reversal potential shifted from approximately –41 mV to 0 mV

is a cation-selective current through a ionotropic nicotinic acetylcholine receptor (nAChR). The receptor is equally permeable for K^+ and Na^+ ions and has a high Ca^{2+} permeability [4]. Acetylcholine and carbamylcholine are full agonists, nicotine and epibatidine are partial agonists [32]. The vertebrate neuronal nAChR antagonists, dihydro-β-erythroidine and methyllycaconitine are highly potent blockers. The honeybee receptor is a typical insect neuronal receptor, which resembles the vertebrate neuronal receptors formed by α7, α9 or α10 subunits. Its physiology is consistent with a role of the nAChR for fast excitatory synaptic transmission between projection neurons and Kenyon cells. The peak amplitude of the acetylcholine-induced current is reversibly reduced by bath applications of 100 μM octopamine or serotonin (Wersing and Grünewald, unpubl., no figure). This modulation may be one mechanism of experience-dependent cellular plasticity within Kenyon cells, because these biogenic amines are important neuromodulators of olfactory learning [22].

The mushroom body contains prominent GABA-immunoreactive feedback neurons [2, 6, 7]. GABA applications induce Cl^- currents through ionotropic, picrotoxin-sensitive GABA receptors in cultured Kenyon cells [29] (Fig. 3C, D), indicating an inhibitory role of GABAergic transmission within the honeybee mushroom bodies. The peak current amplitude of the GABA-induced current was increased by bath applications of the Ca^{2+} ionophore A 23187 (10 μM, [29]). This indicates that the level of inhibition may be altered through a postsynaptic mechanism.

Cellular physiology of central olfactory neurons in honeybees

How do the descriptions of the ionic currents of odor processing neurons fit into a working model of the ionic bases of olfactory learning? Figure 4 presents a diagram of the cellular physiology of the neurons that are involved in olfactory learning and memory formation. It focuses on the mushroom body calyces, because we assume that this is one site where learning-induced plasticity occurs in the honeybee brain and because the pathway of the conditioned stimulus (CS, odors) converges there with the reward pathway. Activation of the CS pathway (represented by antennal lobe projection neurons) activates nicotinic receptors, which induce a depolarization and a Ca^{2+} influx into Kenyon cells (I_{ACh}). Odor stimuli also lead to an excitation in mushroom body feedback neurons [8], which provide an inhibitory signal through ionotropic GABA receptors (I_{GABA}). When an unpredicted reward is presented to the honeybee, octopamine (OA) is released from modulatory neurons such as the VUMmx1 neuron [11]. The insect octopamine receptor (OAR) is coupled to an adenylyl cyclase (AC) via a G-protein, which ultimately leads to the activation of the cAMP-dependent protein kinase A (PKA, [24]). Activation of the octopamine receptor also induces an intracellular Ca^{2+} signal [5] (Wersing, pers. comm.). The internal Ca^{2+} level increases the I_{GABA} [29] and decreases the I_{ACh} (Wersing, pers. comm.). The coincident activation of the CS and reward pathway, i.e., the CS precedes the reward, should lead to a pairing specific effect in individual Kenyon cells. These effects are probably Ca^{2+}-dependent and may involve the activation of the protein

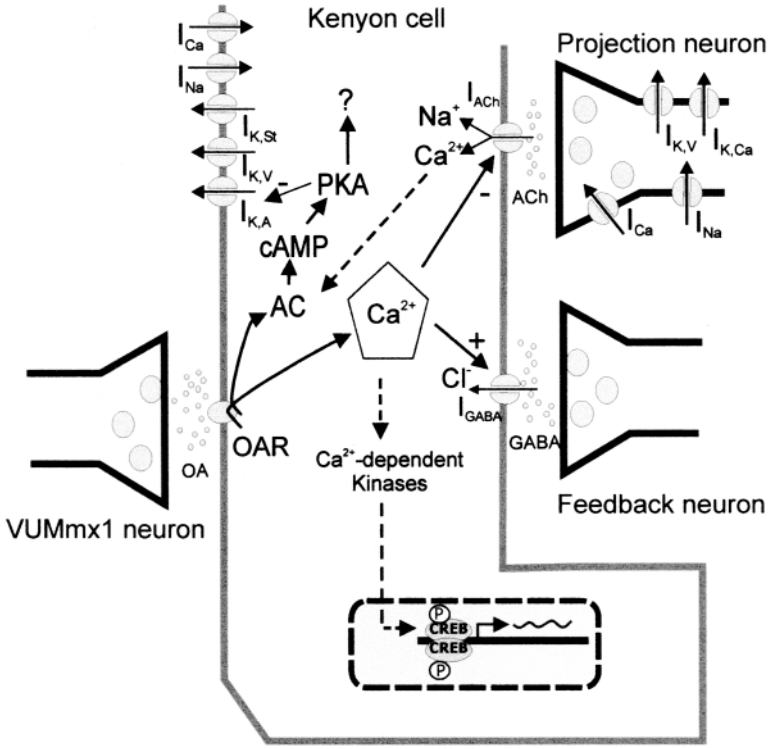

Fig. 4. Schematic diagram of the cellular physiology of honeybee central olfactory processing neurons (see text for details)

kinase A, modulations of the nicotinic receptor or the *shaker*-like K^+ current ($I_{K,A}$, Grünewald, in prep.). They induce long-term changes, which depend upon the activation of Ca^{2+}-dependent kinases such as CamKII and induce protein synthesis [24]. Experiments are currently underway to unravel the cell physiological consequences of *in vitro* coincident activation of the CS and the reward pathway.

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