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CHANGES OF MEMBRANE CURRENTS DURING LEARNING

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SUMMARY

The integrated response of a population of neurones during conditioning results in long-term (days) changes of specific membrane currents within identified neurones. Prolonged elevation of intracellular calcium during conditioning causes a persistent increase of excitability by reducing K^+ currents (I_A and probably $I_{Ca^{2+}-K^+}$) in the membranes of identified somata. This Ca^{2+}-mediated reduction of K^+ currents, which encodes a learned stimulus association is thought to involve changes of Ca^{2+}-calmodulin-dependent phosphorylation of distinct membrane proteins. These changes are contrasted with the short-term regulation of currents by neurohormones during altered behavioural states such as arousal.

In searching for cellular mechanisms by which nervous systems store learned information, it is helpful to differentiate properties which are genetically programmed from those which are conferred by the interaction of an organism with its environment. With mechanisms of learning, as with those underlying developmental transformations, a chronology must be reconstructed – a sequence of processes which arise as a consequence of preceding conditions. To construct such a chronology, the cellular site at which learned information is stored must be accessible to analysis so that we may watch it during sequential transformations, and so that we can measure with appropriate morphological, biophysical and biochemical techniques the nature and magnitude of the critical changes.

Accessibility to sites of associative learning has been elusive for vertebrates, in which extracellular potentials have been recorded as correlates of acquisition and retention of conditioned behaviour (Woody, Vassilevsky & Engel, 1970; Woody & Black-Cleworth, 1973; Woody *et al.* 1974; Berger, Alger & Thompson, 1976; Cohen & MacDonald, 1976; Berger & Thompson, 1977, 1978; Brons & Woody, 1980; Thompson *et al.* 1982; Woody, 1982). It has also been largely true for invertebrates such as the bee, locust and the gastropod molluscs *Pleurobranchaea, Limax* and *Aplysia*. Hoyle and his colleagues recorded changes of impulses in certain locust motor neurones during learning (Hoyle, 1982). However, these changes have only been measured unequivocally for minutes, and their site of origin, and thus their

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accessibility, have not been determined. Furthermore, the synaptic organization of the neural elements which mediate the training response remains obscure.

Workers using *Aplysia* and *Pleurobranchaea* have had more success in identifying neurones within relevant sensory pathways (Castellucci, Pinsker, Kupferman & Kandel, 1970; Gillette & Davis, 1977; Gillette, Gillette & Davis, 1980). However, a close and specific correlation of neuronal changes with learned avoidance of food in *Pleurobranchaea* (as opposed to its being in a food-satiated state), has not been made, as it has for vertebrate conditioning. Nor has it been possible to measure correlated changes in identified neurones or neuronal aggregates of *Limax* or *Aplysia* during or after associative training. For dissected and reduced preparations, short-term correlations (Carew, Hawkins & Kandel, 1983) have been made, but their relevance to the neurophysiology of living animals must be established (see for example, Kanz, Eberley, Cobbs & Pinsker, 1979).

Furthermore, the type of learning involved with some of these gastropods has not been entirely resolved. Some assert that non-associative behavioural changes such as habituation and sensitization can underlie associative learning, such as classical conditioning (Kandel & Schwartz, 1982), although any relevance of non-associative to associative mechanisms remains to be demonstrated. Even those behavioural changes called 'associative' require further study before their 'associative' nature can be considered certain.

Mpitsos & Collins (1975) trained *Pleurobranchaea* to avoid food substances by pairing the food with shocks to the head. Unpaired presentations did not produce an effect. These experiments could be simplified by controlling the different states of satiation which might result from the training. Long-term sensitization of a neophobic reaction to the food substances by shock should also be examined. The recent experiments of Gelperin and his colleagues with *Limax* (Gelperin, Wieland & Barry, 1984; Sahley, Rudy & Gelperin, 1984) require these same controls, since all measures of avoidance behaviour following pairing of a food substance with quinine were made by comparison with responses to a 'safe' food. A neophobic reaction to a 'safe' food (on which the animal had been raised) might be entirely habituated, while quinine could sensitize the neophobic reaction to a food substance with which it was paired during training. An *apparently* associative behavioural change (i.e. one dependent on the pairing of a food substance with quinine or an electric shock) might be *simulated* by particular combinations of two non-associative behavioural effects such as habituation and sensitization.

In recent experiments on *Aplysia*, tactile stimulation of the gill withdrawal response, which habituates, has been paired with electrical stimulation of the tail, which causes sensitization (Carew, Walters & Kandel, 1981). This causes the animal to withdraw its gill more rapidly and reliably. However, the possibility exists that the shock-induced sensitization prevents the touch-induced habituation. This is suggested by the observation that repeated presentation of the shock alone produces, to a significant degree, the same effect as repeated, paired, presentations. Other characteristics of associative learning, such as the stimulus specificity shown for *Aplysia* (Carew *et al.* 1981), and the blocking shown for *Limax* (Sahley *et al.* 1984), have yet to be clearly distinguished from combinations of effects induced by single stimuli.

With these difficulties in mind, we have worked for some years with the nudibranch

mollusc *Hermissenda crassicornis* to localize sites of neuronal change which could play a causal role in associative learning, and which are accessible to quantitative analysis. We first found sites of convergence between three sensory pathways: the visual, vestibular and chemosensory (Alkon & Fuortes, 1972; Alkon, 1973, 1974*a*, 1980*a*, 1983; Alkon, Akaike & Harrigan, 1978). With simultaneous intracellular recordings from identified pre- and postsynaptic elements in thousands of adult nervous systems we constructed a working blueprint of the circumoesophageal nervous system (Fig. 1). Within this blueprint, the flow of sensory information could be traced from the input stages through the integrative centres to the output motor cells of relevant muscle groups. The effects of sensory stimuli such as light (transduced by five photoreceptors in each eye) and rotation (a gravitational stimulus transduced by thirteen hair cells in each statocyst, a primitive vestibular organ) could be studied at every level of integration: sensory cells, interneurones and motor neurones (Fig. 2A). Based on the known convergences between the visual and statocyst pathways, and the specific responses of the network to light and rotational stimuli with specific temporal relationships, we hypothesized that *Hermissenda* could be conditioned by repetition of paired light and rotational stimuli.

In fact, it has been possible to demonstrate most of those features which characterize vertebrate classical conditioning. Rotation, which serves as an unconditioned stimulus, elicits a consistent and reliable 'clinging' response (Alkon, 1974*b*) manifest by measurable contraction of the 'foot' (particularly the caudal half of the ventral surface). In addition, the animal moves towards a light source (Alkon, 1974*b*). This is accompanied by lengthening of its foot, again particularly the caudal half. After light and rotation are repeatedly paired (Alkon, 1974*b*) (i.e. maximal light precedes maximal rotation by 1·0 s), light elicits an entirely new response: contraction of the foot (Fig. 3), the response reflexly elicited by rotation. As a result of the conditioning procedure, light has taken on the meaning of rotation as measured by a new learned response (Lederhendler, Gart & Alkon, 1983). This learning behaviour is associative in that it is not produced by explicitly unpaired light and rotation, or light and rotation occurring with a randomly-varying interval during training (Crow & Alkon, 1978). It is stimulus specific, increases as a function of practice (i.e. it shows acquisition), can be retained for many weeks, extinguishes (Richards, Farley & Alkon, 1983) and shows savings. It also has a requirement for contingency (as well as contiguity) of the associated stimuli (Farley & Kern, 1983). This was shown by the degradation of the learning by interpolation of unpaired light or rotation stimuli. Finally, there is no long-term habituation or sensitization following training.

Intracellular recordings at each level of integration within the visual system of conditioned and control animals uncovered many learning-correlated changes of neuronal properties and response characteristics (Crow & Alkon, 1980; Farley & Alkon, 1982; Lederhendler, Goh & Alkon, 1982; West, Barnes & Alkon, 1982). Such recordings were made from progressively reduced preparations including living animals, isolated nervous systems and individual neurones isolated from all others, of conditioned and control animals. By this means it was possible to reconstruct a sequence of responses and changes during training.

Paired light and rotation (but not unpaired stimuli) enhance and prolong the depolarization of Type B photoreceptors during and following a light step. Repeated

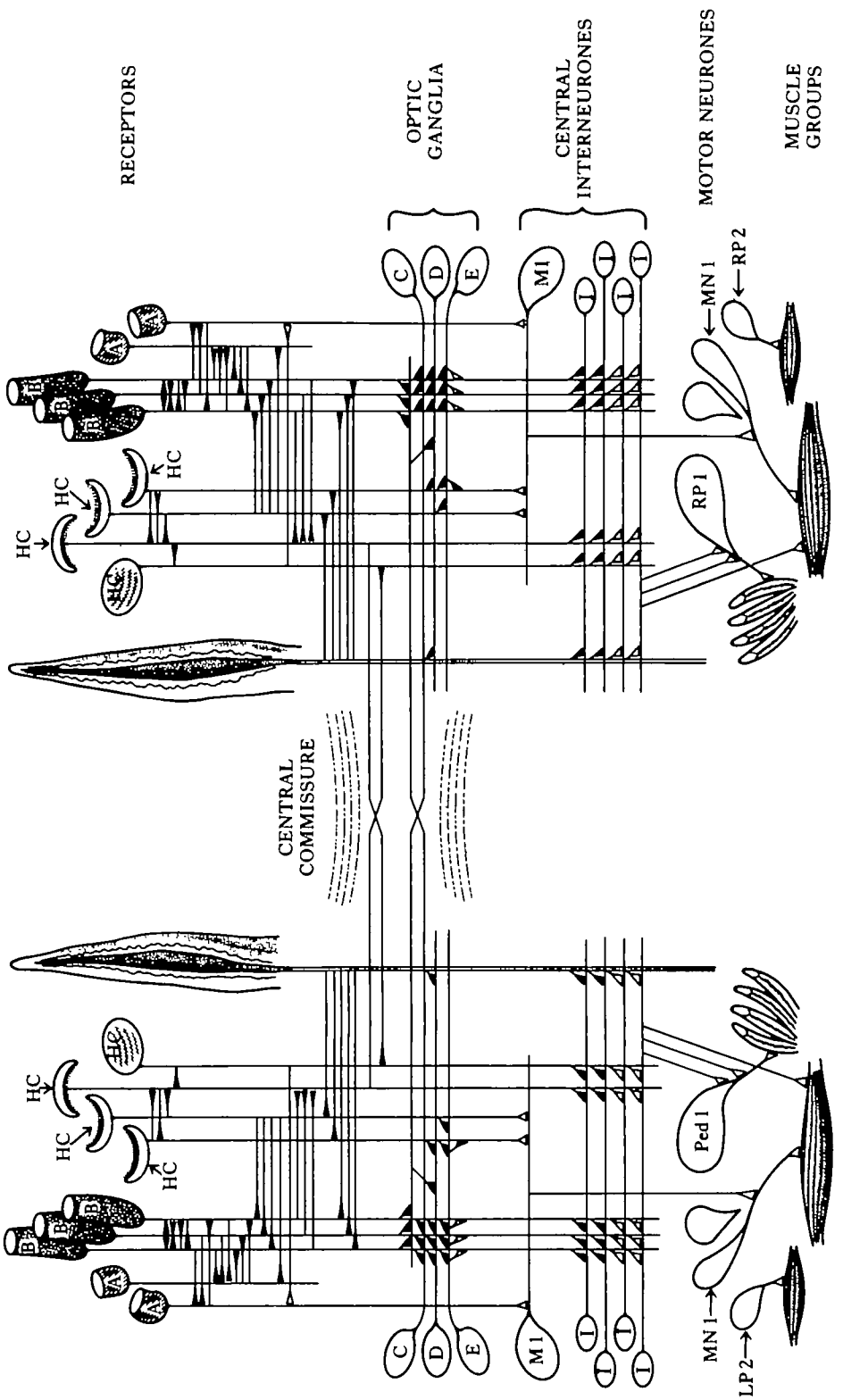


Fig. 1. Schematic summary of synaptic interactions in the neural systems of *Hermissenda crassicornis*. Interactions within and between three sensory pathways are included. The visual pathway begins with the five photoreceptors (two Type A and three Type B) of each eye. The afferent cells of the vestibular pathway, the thirteen hair cells in each statocyst, are labelled HC. The tentacle, represented as an intact structure in the diagram, has chemosensory receptors distributed on its surface. Inhibitory synaptic interactions are indicated by filled endings, excitatory interactions are indicated by open endings. Muscle groups, innervated by the motor neurones are pictured at the bottom of the figure. Each interaction represented was established to be reliably present in the adult nervous system by simultaneous pre- and postsynaptic intracellular recording. Not all known interactions are included (Alkon, 1982, 1983).

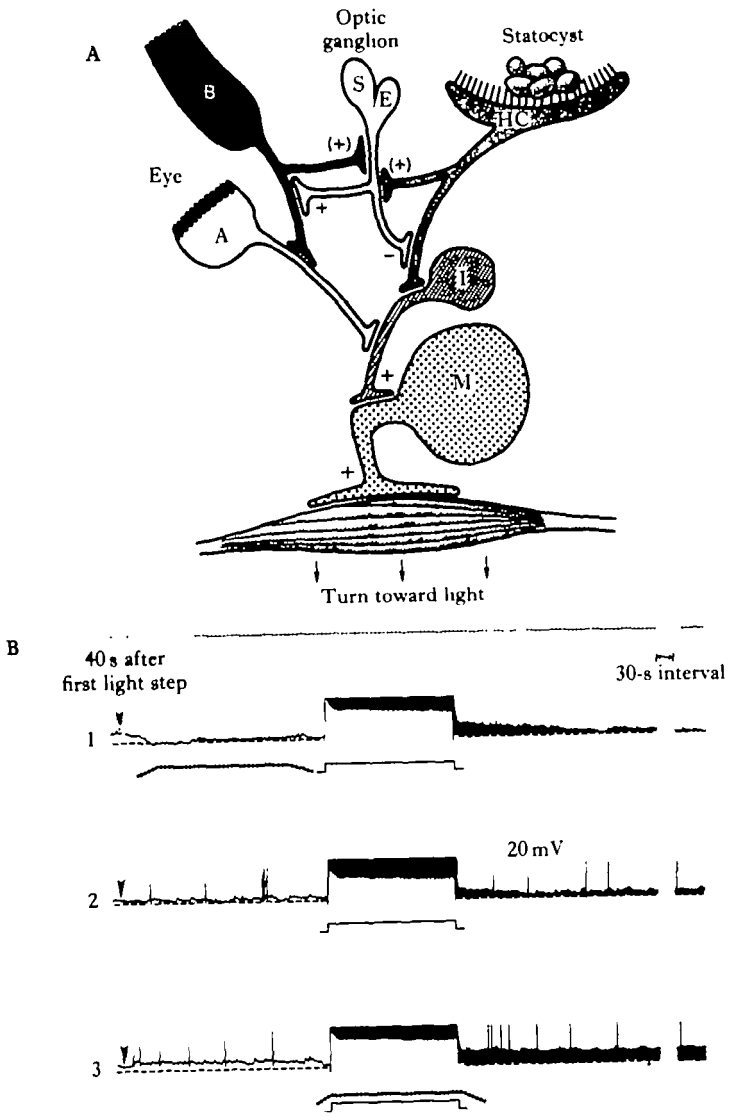


Fig. 2. (A) Schematic diagram of visual pathway and its convergence with the statocyst pathway. The Type B photoreceptor (B) causes monosynaptic inhibition of the medial Type A photoreceptor (A). The medial Type A photoreceptor causes monosynaptic excitation of ipsilateral interneurones (I), which are also excited by ipsilateral hair cells (HC). Ipsilateral hair cell impulses and Type B impulses cause a transient inhibition (not shown here) and are followed by long-lasting effective excitation (+) of the S/E optic ganglion cell and thereby the Type B cell (Goh & Alkon, 1984). (B) Intracellular voltage recordings of *Hermissenda* neurones during and after light and rotation stimuli. Responses of a type B photoreceptor to the second of two succeeding 30-s light steps (with a 90-s interval intervening). The cell's initial resting potential, preceding the first of the two light steps in (1), (2), and (3), is indicated by the dashed lines. Depolarization above the resting level after the second of the two light steps is indicated by shaded areas. (1) Light steps ($\sim 10^4$ erg cm^{-2} s^{-1}) alternating with rotation (caudal orientation) generating $\sim 1{\cdot}0$ g. The end of the rotation stimulus preceded each light step by 10 s. (2) Light steps alone. (3) Light steps paired with rotation. By 60 s after the first and second light steps, paired stimuli cause the greatest depolarization and unpaired stimuli the least. The minimal depolarization was in part attributable to the hyperpolarizing effect of rotation. Depolarization after the second presentation of paired stimuli was greater than that after the first. (Alkon, 1980*b*).

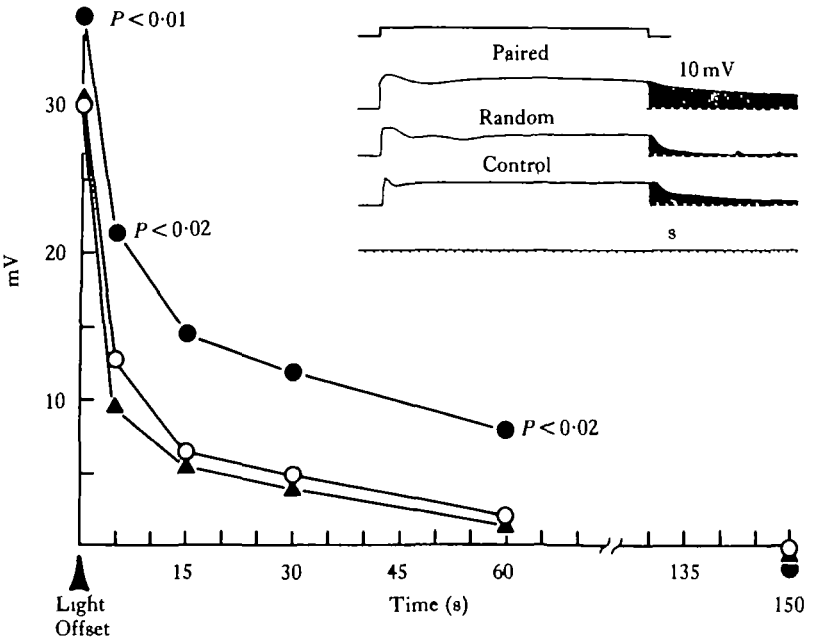
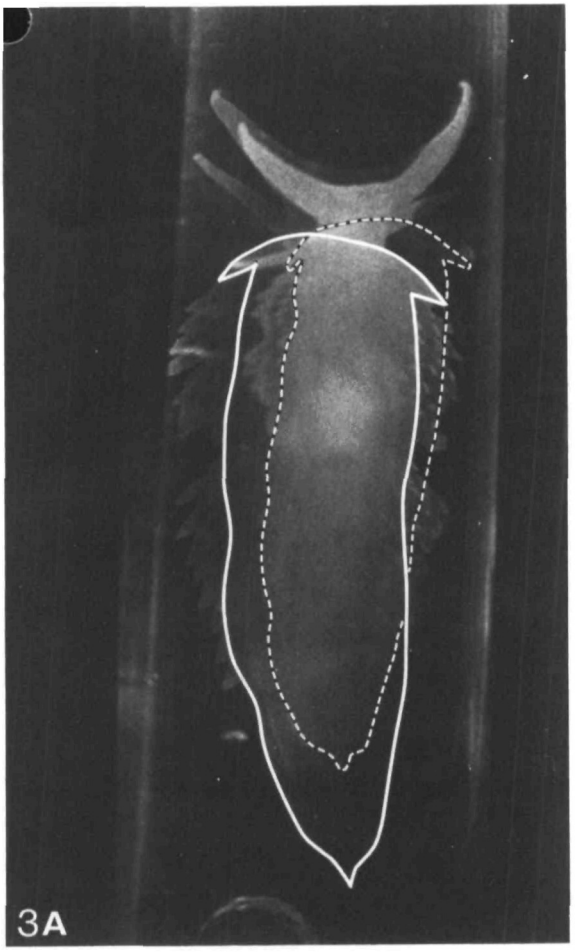


Fig. 4. LLD (long-lasting depolarization) responses of Type B photoreceptors. Values taken from actual voltage recordings at pre-chosen time points (0, 5, 15, 30, 60 s) following the first light step. Note that the paired LLD values are significantly greater than random and control, using a two-tail Mann-Whitney U test. The significance levels indicated refer to comparison of paired with respect to random values (West, Barnes & Alkon, 1982). Inset: responses to first light step of Type B photoreceptors from paired, random and control groups. Shaded areas indicate LLD following the light step (monitored by top trace). Note that the paired LLD is clearly larger than random and control (West *et al.* 1982).

pairings cause progressively greater light-elicited depolarizations, an accumulation of membrane depolarization (Alkon, 1980*b*) and an increased input resistance (Fig. 2B). After a training session (e.g. of 50 trials) the Type B cell remains depolarized for many minutes (Alkon, 1980*b*; Crow & Alkon, 1980). On days following training (the retention period) the Type B cell from conditioned animals is no longer depolarized but remains more excitable. In response to injection of positive current or to light stimuli (Fig. 4), the conditioned Type B cell shows a greater depolarization (West *et al.* 1982). The increased impulse activity accompanying light-evoked depolarization in turn causes more inhibition of the medial Type A photoreceptor and, thereby,

Fig. 3. (A) Time lapse measurement of *Hermissenda* foot contraction. On the left are actual photographs (from below) of *Hermissenda* foot immediately before and 3 s after the onset of rotation. On the right are traces of the photographs on the left to highlight the contraction of the caudal half of the foot within 3 s of rotation onset. This contraction, part of a 'clinging response' is reliably elicited by the UCS (rotation) before and after training. Light, the CS, only elicits this response after associative training in which light and rotation are repetitively paired (I. Lederhendler & D. L. Alkon, in preparation). (B) Median response ratios for acquisition, retention and re-acquisition of a long-term behavioural change in response to a light stimulus in *Hermissenda* (random rotation, ●; random light, □; unpaired light and rotation, △; random light and rotation, ▲; nothing, ■; paired light and rotation, ○). The response ratio [in the form of $1-A/(A+B)$] compared the latency during the test (A) with the baseline response latency (B). Group data consist of two independent replications for all control groups and three independent replications for the experimental group (Crow & Alkon, 1978).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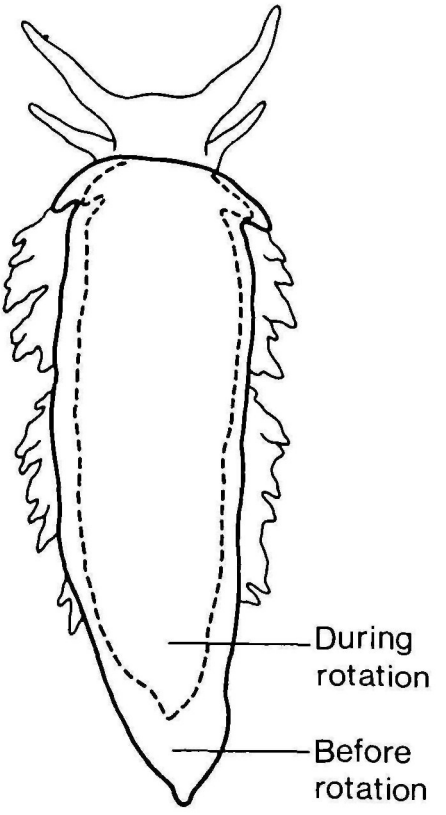
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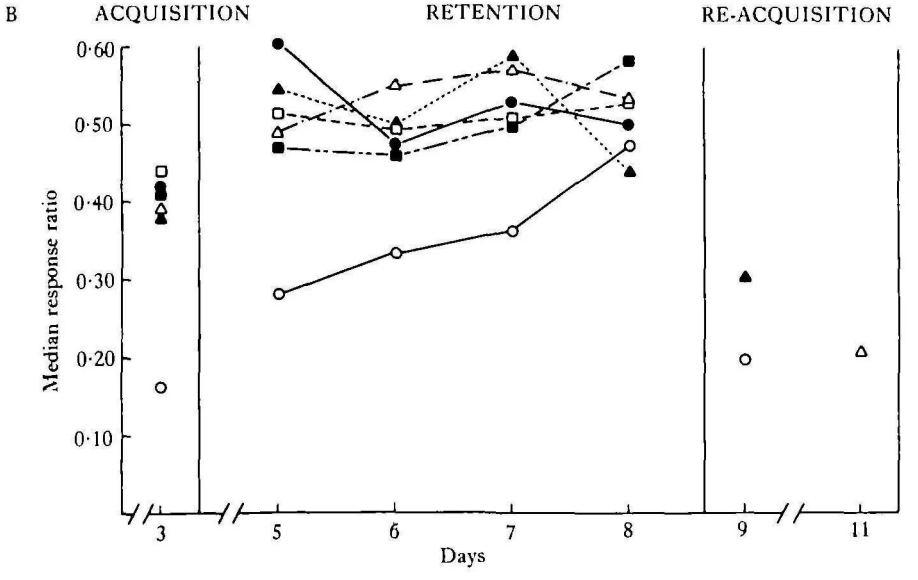
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Days

decreases excitation of interneurones which receive monosynaptic EPSPs from it (Farley & Alkon, 1982; Lederhendler *et al.* 1982; Goh & Alkon, 1984). Motor neurones which are monosynaptically excited by these interneurones are inhibited in this way by the Type B photoreceptor. In conditioned animals, increased light-evoked impulses in Type B cells decreases the excitation of motor neurones which in living animals cause turning toward a light source.

A number of other experiments taken together provide strong evidence that changes of Type B cells have a causal role in the production of associative learning. For example, the changes in Type B cells predict motor neurone changes (Lederhendler *et al.* 1982). The changes are present in Type B cells of conditioned but not control animals, physically isolated from all impulses and synaptic interactions with other neurones – i.e. these changes are intrinsic to the soma membrane (West *et al.* 1982). When these same changes are produced in Type B cells of living animals by pairing current injections (simulating the synaptic effects of stimulus pairing) with light, the learned behaviour measured on subsequent days is produced (Farley *et al.* 1983). This behavioural change does not follow unpaired light and current, or sham procedures.

Given that the Type B cell is a site for storage of learned information, is it accessible to biophysical and biochemical analysis? Fortunately, substantial changes induced by the conditioning were found to be intrinsic to the soma membrane of the Type B cell. These changes were sufficiently large and the spike initiating zone and synaptic endings were electrically close enough, to account for many of the observed changes of impulse activity and thus for the modified response of the visual pathway to light.

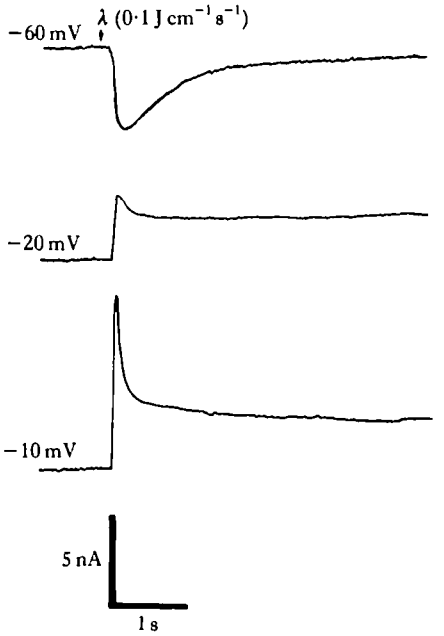

Fig. 5. Light-induced inward Na^+ current and voltage-dependent outward K^+ currents across the soma membrane of the Type B photoreceptor. Voltage clamp recordings from Type B photoreceptor. Top current recording shows inward Na^+ current during a light step whose onset is indicated by arrow. Middle and bottom current recordings are of outward K^+ currents (elicited in the dark by positive command pulses from −60 to −20 and from −60 to −10 mV), onsets also indicated by arrow (Alkon, 1982).

Since the roughly spherical soma can be isolated from the small diameter (~1–2 μm) and short axon, it is well suited for voltage-clamp studies (Alkon, 1979). Pharmacological blockers and substitution of ions permitted the separation of six distinct ionic currents across the soma membrane of a Type B cell (Fig. 5).

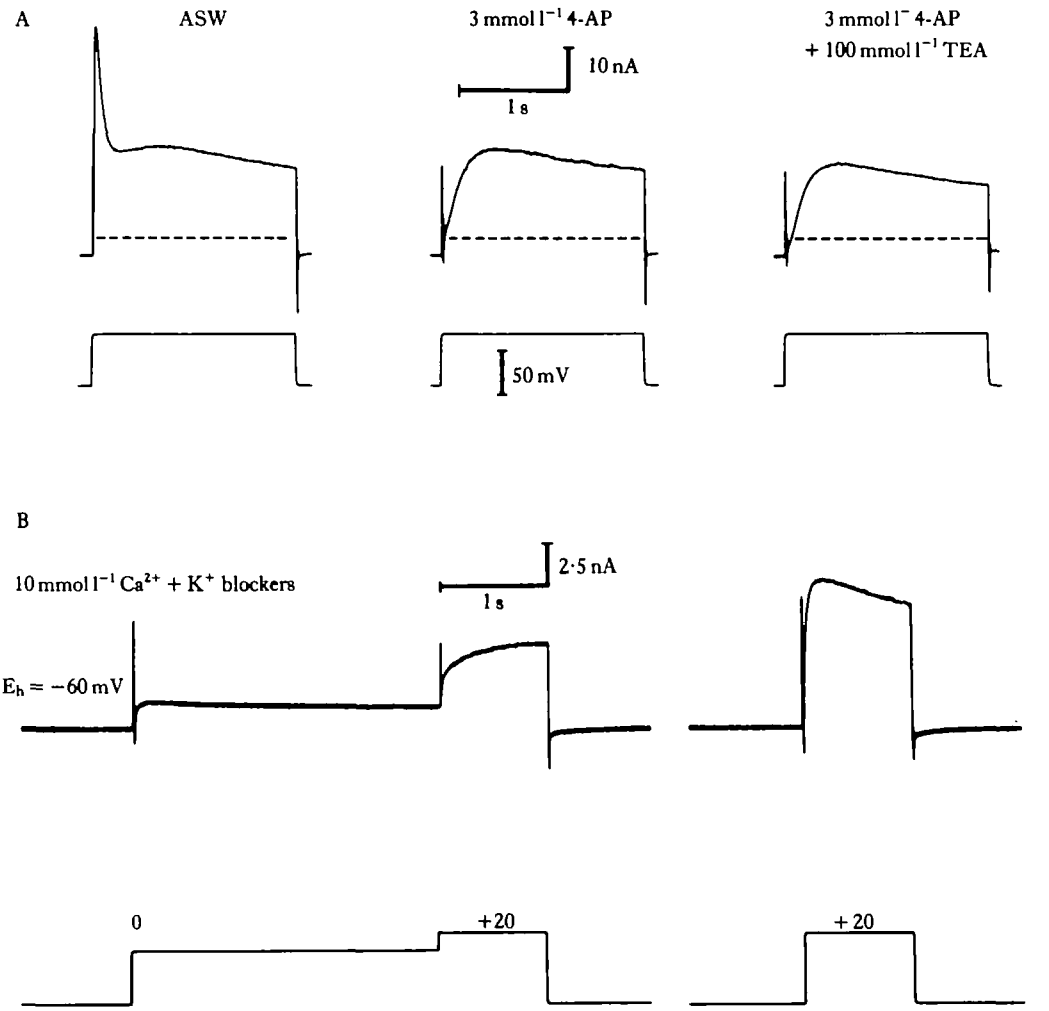

Fig. 6. (A) Voltage-dependent outward currents across the membrane of the isolated Type B cell soma. From left to right, ASW, 3 mmol l^{-1} 4-aminopyridine (4-AP) added to ASW, 4-AP and 100 mmol l^{-1} tetraethylammonium (TEA) ion added to ASW. Note that addition of 4-AP and TEA remove only a small portion of the late outward current elicited by command to 0 mV from a holding potential of −60 mV. The dashed lines indicate the level of the non-voltage-dependent or 'leak' current. (B) Voltage-dependent outward current in presence of 4-AP and TEA. Voltage-dependent activation of outward calcium-dependent K^+ current (I_C) in Type B photoreceptor, and reduction by pre-pulse depolarization. Current (top) and voltage (bottom) records from voltage-clamp experiment illustrate that a 3 s depolarization to 0 mV elicits a small net outward current (top left), followed by a larger outward current when the membrane is stepped to +20 mV (top middle). The pre-pulse depolarization reduced by ~40 % the outward current normally evoked by an 80 mV step from −60 to +20 mV (top right) and slowed the rise time as well. Bathing solutions included 10 mmol l^{-1} 4-AP and 100 mmol l^{-1} TEA to block the fast (I_A) and delayed (I_K) K^+ currents (Alkon, Farley & Hay, 1984).

(1) An early rapidly-activating and inactivating outward K^+ current (Shoukimas & Alkon, 1980; Alkon, Lederhendler & Shoukimas, 1982*a*), blocked by 1–3 mmol l^{-1} 4-aminopyridine. It resembles I_A currents previously identified (Connor & Stevens, 1971).

(2) A slowly-activating outward K^+ current with much less inactivation, blocked by 100 mmol l^{-1} tetraethylammonium ion. It is similar to delayed rectifying currents described for many neurones (Shoukimas & Alkon, 1980).

(3) A slowly-activating outward K^+ current with substantial and prolonged inactivation (Fig. 6), which depends on elevation of intracellular Ca^{2+} and is therefore blocked by injection of Ca^{2+}-chelators such as ethylene glycocol tetracetic acid. It can thus be termed a Ca^{2+}-dependent K^+ current or I_C (Alkon, Farley, Hay & Shoukimas, 1983*b*; Alkon, Farley, Sakakibara & Hay, 1984).

(4) A rapidly-activating voltage-dependent inward Ca^{2+} current which shows no inactivation and is blocked by 2–4 mmol l^{-1} Cd^{2+} (Fig. 7; Alkon *et al.* 1983*b*, 1984).

(5) A light-induced inward Na^+ current which rapidly inactivates (Fig. 5; Alkon, 1979).

(6) A light-induced outward K^+ current which substantially inactivates and results from a light-induced release of intracellular Ca^{2+} (Fig. 8). This current differs from the third one in that it arises from a light-induced rather than a voltage-dependent rise of intracellular Ca^{2+}.

'Blind' experiments on Type B somata indicate that increase of excitability induced

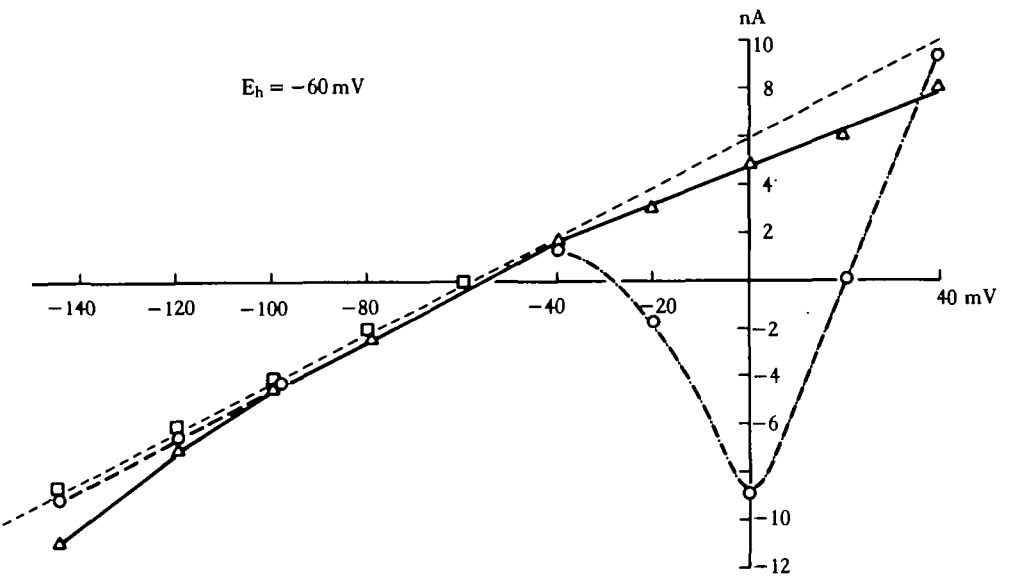

Fig. 7. Steady-state current-voltage plot of voltage-dependent inward current present in Type B photoreceptor. Under conditions of high external Ba^{2+} (100 mmol l^{-1}) and blockade of the voltage-dependent K^+ currents (O–·–·–O), the current is inward over the range of −40 to +40 mV (absolute), reaching its peak at ~0 mV. Removal of Ba^{2+} and Ca^{2+} from the bath, and addition of the calcium-channel blocker Cd (10 mmol l^{-1}) substantially reduced after 5 min the inward current at all levels of membrane potential (Δ——Δ). The residual deviations from the extrapolated leak current values (□- -□) may be due to incomplete block of the inward current (Alkon, Farley & Hay, 1984).

by conditioning arises from a persistent decrease of voltage-dependent K^+ current (Fig. 9; Alkon *et al.* 1982*a*; Forman *et al.* 1984). These include the I_A and the $I_{Ca^{2+}-K^+}$ outward K^+ currents. A number of other studies were undertaken to determine some of the biophysical steps leading to this long-term reduction of K^+ currents. It was already known that progressively enhanced light-evoked depolarization and cumulative membrane depolarization of the Type B cell occurs during acquisition of associative learning (Alkon, 1980*b*). During prolonged depolarization, prolonged elevation of intracellular Ca^{2+} was demonstrated by differential absorption spectrophotometry (Connor & Alkon, 1982, 1984). Elevation of intracellular Ca^{2+} by injection of Ca^{2+} causes prolonged reduction of both I_A (Fig. 10; Alkon, Shoukimas & Heldman, 1982*b*) and $I_{Ca^{2+}-K^+}$ currents (Alkon *et al.* 1984; Alkon & Sakakibara, 1984).

The biophysical sequence (Fig. 11) underlying the transformation of the Type B membrane during learning is most probably as follows: repeated pairings of light and rotation elicit cumulative membrane depolarization. This arises from (1) the genetically specified synaptic organization and (2) the response of this network during the

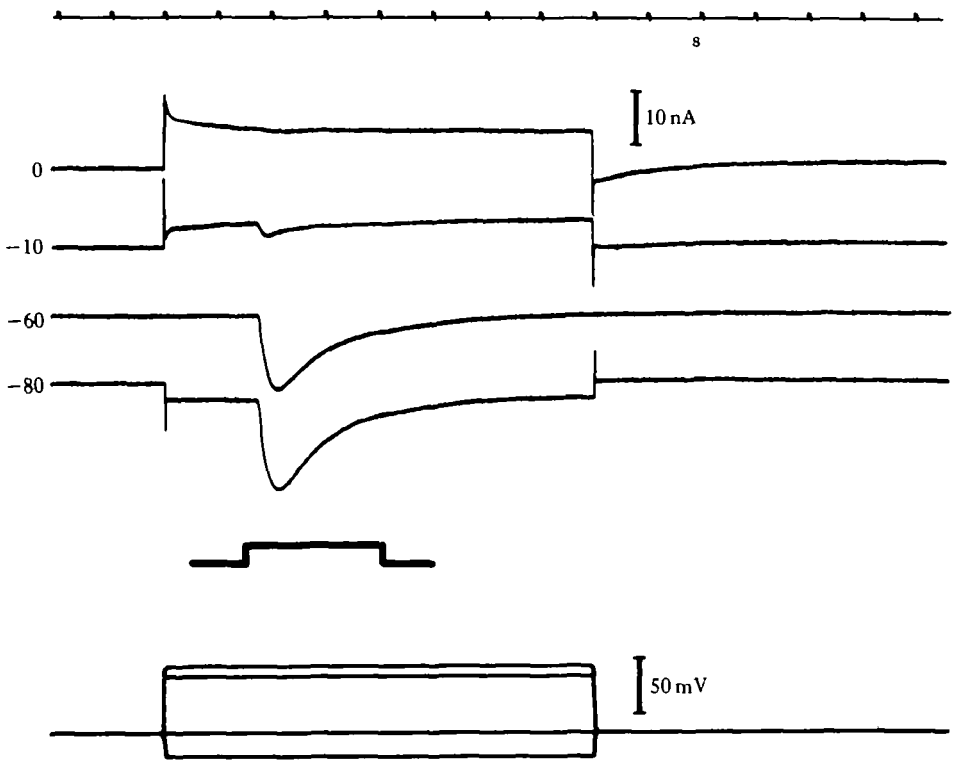

Fig. 8. Light-induced Ca^{2+}-dependent K^+ current. Current records (in response to command depolarizations indicated on the left) obtained during voltage clamp of an isolated Type B photoreceptor soma in 0 Na^+, 300 mmol l^{-1} K^+-artificial sea water. The light-induced current is inward below the equilibrium potential for potassium flux, approximately 0 mV. At 0 mV there is no appreciable light-induced current. This current was reduced by intracellular injection of EGTA but not affected by 5 mmol l^{-1} Cd^{2+} in the external bathing medium, lowering of external Ca^{2+} or Na^+, or substitution of Ba^{2+} for Ca^{2+}. The trace beneath the current records indicates the duration of the light stimulus ($\sim 10^{-3}$ J cm^{-2} s^{-1} at 510 nm).

interaction of the animal with its environment during the training stimuli. Cumulative depolarization is accompanied by prolonged elevation of the intracellular calcium within the soma, and perhaps in the axon and terminal branches. Elevated calcium causes inactivation of voltage-dependent K^+ currents (I_A and most probably $I_{Ca^{2+}-K^+}$) which outlasts by many days the cumulative depolarization and elevation of intracellular

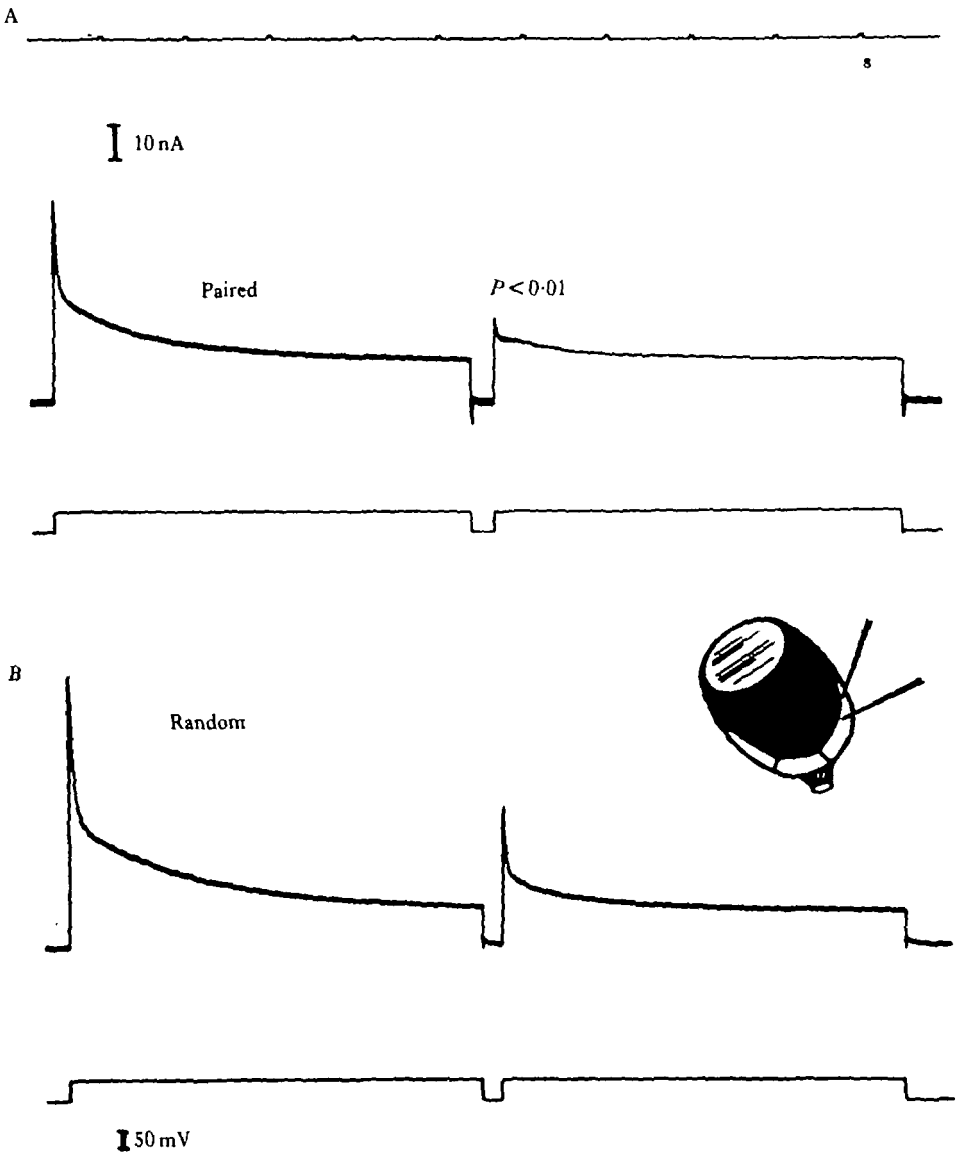

Fig. 9. Outward K^+ currents of Type B cell (A and B). Outward currents elicited by command pulses to 0 mV. Initial peaks are the early rapidly inactivating K^+ currents (I_A). Late outward K^+ currents, largely $I_{Ca^{2+}-K^+}$, attain a maximum value approximately 300 ms after the onset of the command pulse. Paired I_A is smaller than random I_A for both first and second command pulses. The ratio of paired I_A for second pulse to I_A for the first pulse is smaller for paired than for random. Cartoon depicts impalement of medial Type B cell with two microelectrodes (Alkon, Lederhendler & Shoukimas, 1982*a*).

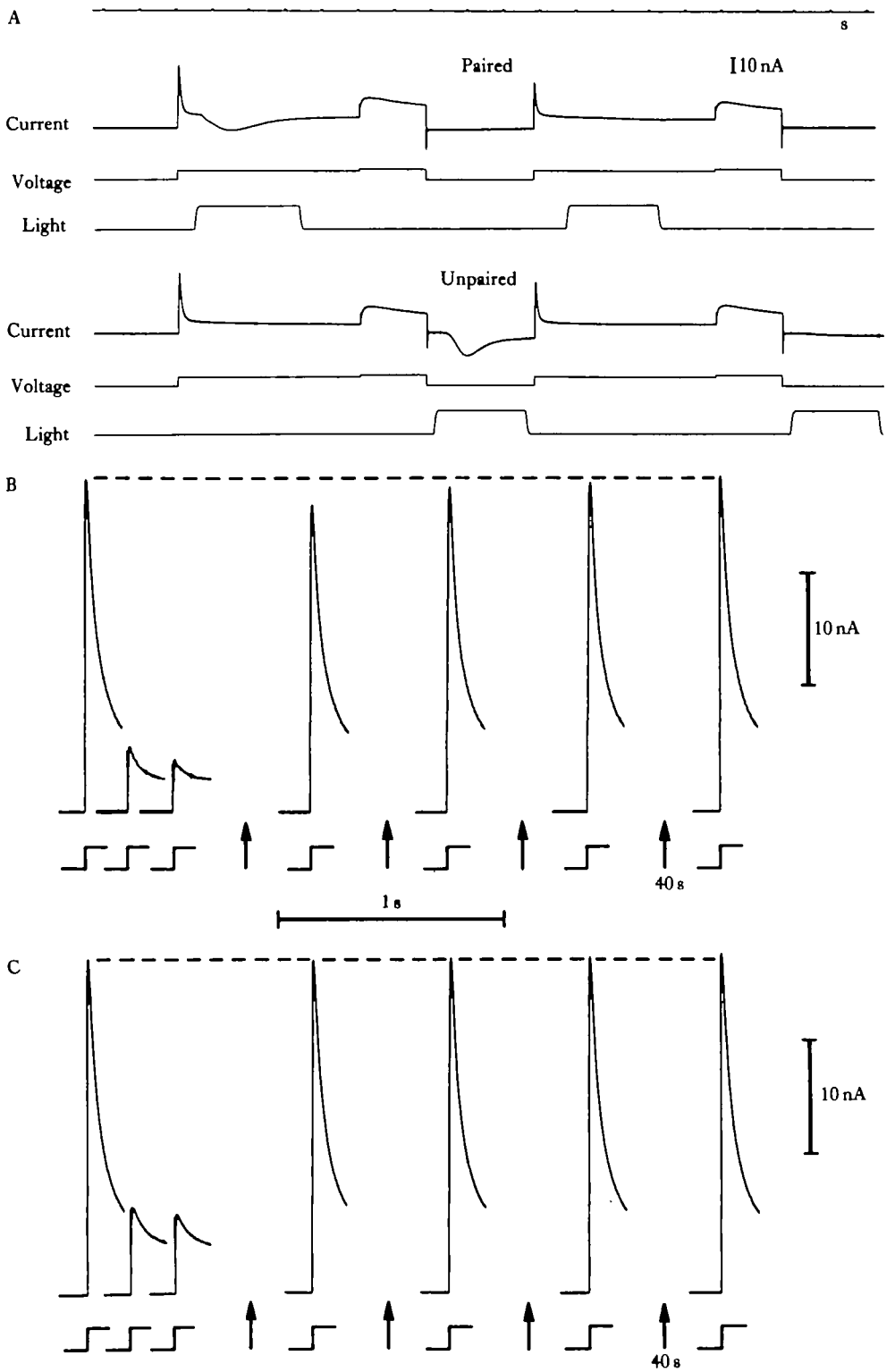
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Fig. 10A–C

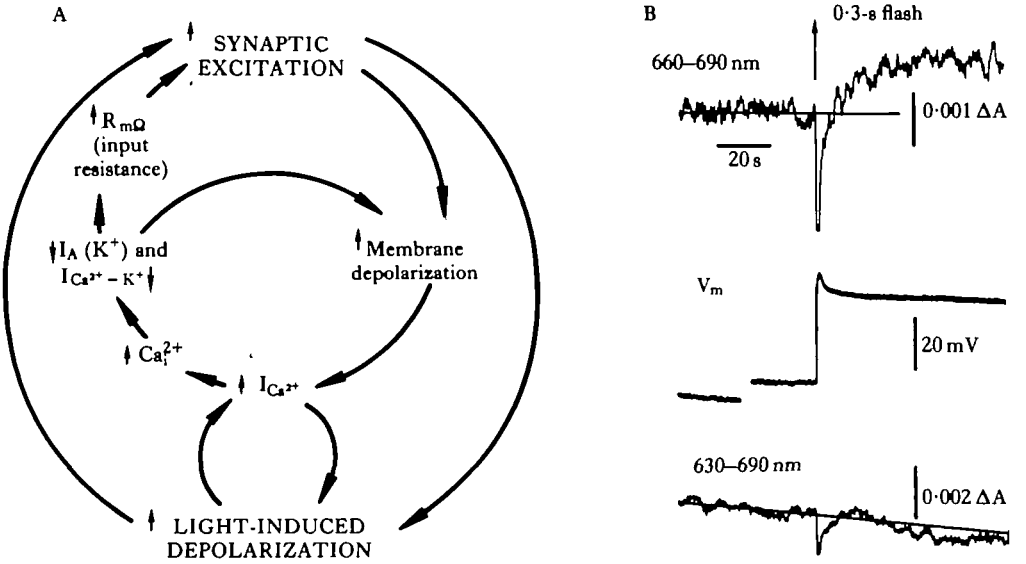

Fig. 11. (A) Regenerative synaptic and light induced excitation of the Type B photoreceptor. Light-induced depolarization facilitates synaptic excitation and *vice-versa* in response to temporally associated light and rotation. Analysed in biophysical terms, synaptic depolarization causes inactivation of I_A and $I_{Ca^{2+}-K^+}$ and enhancement of a voltage-dependent Ca^{2+} current. Increased intracellular Ca^{2+} causes further inactivation of I_A and $I_{Ca^{2+}-K^+}$ and thus a further increase of effective input resistance. These in turn cause more membrane depolarization. (B) Absorbance changes at 660–690 (monitoring intracellular Ca^{2+}) and 630–690 nm wavelength pairs (top and bottom records) and membrane voltage response (middle) following a 0·3 s light flash (Connor & Alkon, 1984).

Ca^{2+}. Reduction of voltage-dependent K^+ currents increases the excitability thus permitting an enhanced depolarization to a light stimulus. The greater depolarization and the greater number of impulses that will be generated cause (1) greater inhibition of the medial Type A photoreceptor, (2) increased inhibition of ipsilateral interneurones, (3) decreased depolarization of ipsilateral motor neurones and finally (4) decreased turning toward a light source.

The change from transient (lasting minutes to hours) cumulative depolarization

Fig. 10. (A) Positive command pulses paired (upper records) and unpaired (lower records) with light steps. Each command step to 0 mV is followed after 30 μs by a brief step to +10 mV. The interstimulus interval illustrated here was used for paired *vs* unpaired comparisons (Alkon, Shoukimas & Heldman, 1982*b*). (B) Rates of I_A decrease and recovery during and following repetitive command depolarizations. Current responses to only three of the first five depolarizing steps used are shown. The steps following arrows were given at 40-s intervals after the five depolarizing steps used for quantitation of differences. These five command depolarizations (2·2 s) to 0 mV occurred with a cycle time of 4·0 s. Each 2·2 s step was followed by a second command (800 ms) to −10 mV. (B) Command depolarizations paired with light. A light step (2·0 s) was presented 150 ms after the onset of each command depolarization. Light intensity: $10^{-3.5}$ J cm^{-2} s. (C) Command depolarizations alone. Arrows indicate 40-s intervals. Lower rectangular traces under B and C indicate onset of 60 mV command steps. Note that I_A (peak-inward currents) decreases to lower values in B than in C, and peak I_A takes minutes to return to original values in B but not in C. The first three currents included in B and C are the first, second and fifth currents elicited by the five successive command steps (Alkon, Lederhendler & Shoukimas, 1982*a*).

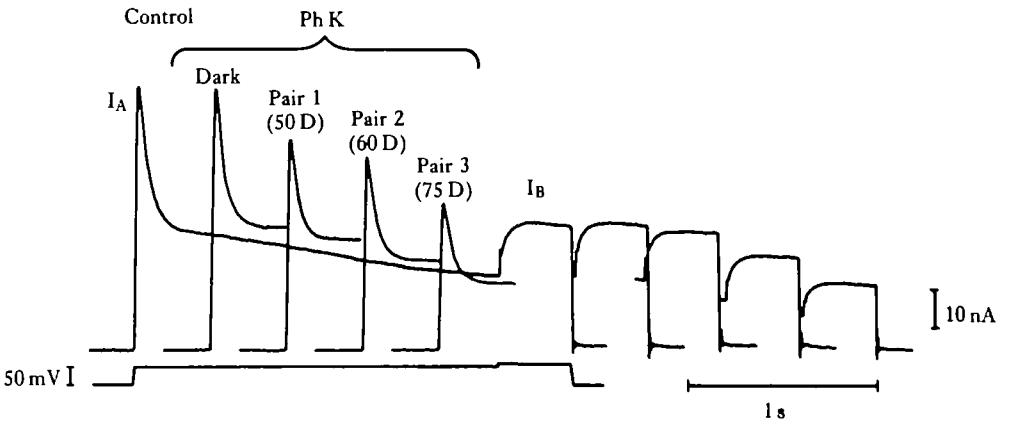

Fig. 12. Family of K^+ currents (I_A traces at left and matched I_B traces at right) illustrate the effect of a single PhK (phosphorylase kinase) injection (60 nC) under two conditions: in darkness (Dark) and after a 20-s pairing (Pair 1-3) of a light step with a variable command pulse to deliver a Ca^{2+} load. Control, control before injection. I_A was elicited from a V_H of −60 mV, and decays within ~1 s to the steady state. 1·9 s later, a second superimposed command pulse (+10 mV) elicits I_B (delayed K^+ current) uncontaminated by I_A. (Full record only shown for control condition.) Note no effect of PhK injection under dark conditions but a reduction of I_A and a small (but not statistically significant) reduction of I_B after the first pairing to a +50 mV command (Pair 1, 50 D). Further reduction of I_A and also I_B occurred after two additional pairings associated with increasing command steps (60 and 75 D, respectively) (Acosta-Urquidi, Alkon & Neary, 1984).

and elevated intracellular Ca^{2+} to a longer lasting reduction of open voltage-dependent K^+ channels can most reasonably be thought to be mediated by biochemical steps. Ca^{2+}-calmodulin phosphorylation of proteins which regulate or are a part of the K^+ channels themselves is implicated. Electrophoretic analysis revealed a difference in phosphorylation of low molecular weight proteins from the eyes of conditioned but not control *Hermissenda* (Neary, Crow & Alkon, 1981). Phosphorylation of these proteins was in addition shown to be Ca^{2+}-dependent (Alkon, 1983). Phosphorylation of one of these proteins (25 000 M_r) also changed under conditions which inactivated the same voltage-dependent K^+ current (I_A) that was reduced during days on which the associative learning was retained (Neary & Alkon, 1983). Finally, iontophoresis of a Ca^{2+}-calmodulin-dependent protein kinase (phosphorylase kinase) caused a long-lasting reduction of the I_A current (Fig. 12) which required some initial elevation of intracellular Ca^{2+} (Acosta-Urquidi, Alkon & Neary, 1982; Acosta-Urquidi, Neary & Alkon, 1984).

The essential link between the stimulus pattern (here paired light and rotation) and the change of ionic channels with learning is the training-induced *voltage* change of the Type B cell. This voltage change is an integrated effect of the entire visual-statocyst network as it responds to pattern repetition. This voltage change, and the calcium-mediated reduction of K^+ channels it causes, contrasts with other neurohumorally-mediated phenomena. Such generalized physiological states as arousal are accompanied in vertebrates, for example, by increased levels of circulating epinephrine that exerts diffuse effects *via* neural receptors. In recent years neurohumoral agents such as octopamine, dopamine and serotonin have been suggested to have a similar role in invertebrates. Serotonin and octopamine, for example, have profound effects on the development and the ability to sustain muscular tension

f crayfish (Jacobs & Atwood, 1981), and on neuromuscular junctions of the lobster (Kravitz *et al.* 1980). Serotonin also produces effects on *Aplysia* similar to those observed after delivering a noxious stimulus such as shock or a 'pinch' (Camarado, Siegelbaum & Kandel, 1984).

These effects of pharmacological agents differ in many respects from the learning of an association between two specific stimuli as analysed for *Hermissenda*. First, a learned stimulus association is quite specific, while 'state' changes elicited by neurohumoral agents are generalized to many targets throughout the body. Second, the associative learning in *Hermissenda* persists for many weeks, while the pharmacological effects do not outlast the presence of the neurohumoral agent. Thus, there is no evidence that the neurohumorally-induced cellular changes involve storage of information, which persists beyond the initial experience of, for example, an arousal state. Nor mechanistically would the persistence of arousal effects be useful for the animal unless the arousing stimuli had been associated with other temporally-related events. Moreover, the effects of serotonin and octopamine are mediated by cyclic nucleotide phosphorylation (Batelle & Kravitz, 1978) rather than Ca^{2+}-calmodulin-dependent phosphorylations involved in the learned association in *Hermissenda* (Acosta-Urquidi *et al.* 1982, 1984; Alkon *et al.* 1983*a*). The serotonin-induced effects on *Aplysia* neurones are also independent of calcium (Siegelbaum, Camardo & Kandel, 1982). It may, however, be premature to compare directly calcium-dependent modification of *Hermissenda* membrane channels during associative learning with mechanisms of arousal and sensitization. Thus, although serotonin has been thought to be responsible for long-term reduction of potassium channels during sensitization, their prolonged reduction has never been correlated with long-lasting sensitization of intact animals. Furthermore, it has been recently shown that serotonin is not released or synthesized from presynaptic interneurones which have long been thought to convey the sensitizing effects on the synaptic endings of the sensory cell (Hawkins *et al.* 1981; Kistler *et al.* 1983).

Ultimately some intelligent and testable extrapolation from relatively simple to more complex nervous systems may become possible. Most promising to date are the properties of ionic channels within neural membranes. The voltage-dependent calcium channels of the *Hermissenda* Type B photoreceptors have been found in *Paramecium* (Naitoh & Eckert, 1974), spinal cord motor neurones (Barrett & Barrett, 1976), dendrites of inferior olive neurones (Llinas & Yarom, 1981*a*,*b*) and pyramidal cells of the hippocampus (Clark & Wong, 1983). Voltage-dependent K^+ currents such as I_A and $I_{Ca^{2+}-K^+}$, are equally widespread. Prolonged membrane depolarization is known to occur in vertebrate nervous systems and could modify membrane excitability in exactly the same way as in *Hermissenda*. The necessary physiological prerequisites exist within vertebrate neural systems to use an invertebrate mechanism for associative learning. It will be extremely interesting to induce prolonged depolarization in cells with these prerequisites in brain slices to see if prolonged excitability changes, analogous to those of *Hermissenda*, would result. Encouragement has already been provided by the finding of prolonged elevation of intracellular calcium in somata of hippocampal pyramidal cells in response to a depolarizing stimulus (Krnjevic, Morris, Reifenstein & Ropert, 1982). It will be far more difficult, of course, to correlate such biophysical changes with associative learning of a cat or

dog and then to prove that these changes are intrinsic to particular neurones. It will be more difficult still to reach any conclusion as to what portion of a discrete association learned by a cat or dog resides within a given set of neurones and is critical for its recall.

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