neurons during the REM periods of active sleep, act on other motor systems in a similar state-dependent way? The mechanisms responsible for the phasic contraction of the peripheral musculature during REM periods may reflect a general pattern that affects other somatomotor functions as well. For example, the striated muscles that move the orbits are active during REM periods. Despite the compelling heuristic value of the hypothesis that eye movements during active sleep are based on directed visualization of the dream experience, we believe there to be no convincing evidence that they are any more related to visual functions than the twitches and jerks of the limbs are to goal-directed movements. It is possible that the central neural areas that give rise to myoclonic activation of the limb muscles during REM periods also initiate a generalized pattern of twitches and jerks that affect all striated muscles. REM's are an example (*6*), as are irregular contractions of the middle ear musculature (*10*), and the erratic contractions of the respiratory muscles which result in irregular breathing patterns that are most prevalent during the REM periods (*11*).

We conclude that a strong motor facilitatory drive acts on spinal (and other) motoneurons during REM periods; when present, it encounters a somatomotor system subjected to enhanced inhibition. Consequently, during certain REM periods excitatory and inhibitory processes, both of which are postsynaptic, are simultaneously coactivated. We believe that, although paradoxical, these concurrently active but diametrically opposed processes could reflect proper adaptive responses to a widely activated nervous system; it seems logical for the organism to protect itself from the deleterious consequence of undirected and inappropriate movements when it is blind and unconscious.

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4. These patterns of discharge during REM periods do not seem to be simply a reflection of threshold stimuli occurring in conjunction with a tonically hyperpolarized membrane. For example, they were not observed when we first hyperpolarized the membrane during wakefulness or quiet sleep to the level present during REM periods and then induced spike activity by antidromic or orthodromic stimulation. These data indirectly indicate that lumbar motoneuron spike doublets, triplets, and quadruplets depend on processes operating exclusively during the REM episodes.
5. The cell soma is unlikely to be the source of partial spikes, because spikes originating in this region not only are greater in amplitude, but also are preceded by electrotonically conducted initial segment spikes (Fig. 2, C_1 and D_1) [L. G. Brock, J. S. Coombs, J. C. Eccles, *J. Physiol. (London)* **122**, 429 (1953)]. Partial spikes are also unlikely to represent activity generated in the dendrites, where motoneuron spikes occur only under conditions of abnormal hyperexcitability, for example, in the chromatolytic state after axotomy [J. C. Eccles, B. Libet, R. R. Young, *J. Physiol. (London)* **143**, 11 (1958); D. Purpura, in *The Neurosciences: A Study Program*, G. Quarton, T. Melnechuk, F. Schmitt, Eds. (Rockefeller Univ. Press, New York, 1967), p. 372]. We have also considered the possibility of damage to the neuron by microelectrode penetration which, theoretically, could result in partial spikes. This was not the case, because control spikes, initiated antidromically (Fig. 2, C_1 and D_1) or orthodromically (Fig. $2D_3$), were present during adjacent non-REM periods. Moreover, membrane potentials and the amplitude of the control spikes conformed to the most stringent standards for intracellular recording.
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An Opiate Binding Site in the Rat Brain Is Highly Selective for 4,5-Epoxymorphinans

Abstract. *In vitro binding studies have demonstrated the existence of multiple opiate receptor types. An additional site in the rat brain (termed the λ site) is distinct from the established types by its selectivity for 4,5-epoxymorphinans (such as naloxone and morphine). While the λ site displays a high affinity for naloxone in vivo and in vitro in fresh brain membrane homogenates, these sites rapidly convert in vitro to a state of low affinity. The regional distribution of the λ site in the brain is strikingly different from that of the classic opiate receptor types.*

The opiate receptor system consists of multiple types of binding sites, such as the well-characterized μ, δ, and κ sites. This receptor multiplicity may underlie the diversity of pharmacological actions of the opiates (*1*). Few opiates display specificity for any of the receptor types, which makes it difficult to evaluate the pharmacological significance of each individual type. For example, naloxone is considered to be a general opiate antagonist with affinity for most of the established receptor types. Therefore, naloxone antagonism often serves to distinguish opiate- from nonopiate-mediated actions. However, recent reports demonstrate pharmacological actions of naloxone that may be independent of opiate receptor blockade (*2*). These include attenuation of barbiturate anesthesia (*3*), alleviation of shock symptoms (*4*), analeptic actions (*5*), reversal of neurological deficit after stroke (*6*), and effects on the adrenal cortex (*7*). Moreover, some reports suggest an agonistic action of naloxone in several experimental systems (*8*). The possibility that naloxone may bind to unique sites in the rat brain was first suggested by Squires and Braestrup (*9*), who identified two binding site populations, type 1, with high affinity for naloxone, and type 2, with low affinity. It was subsequently shown that naloxone indeed has high affinity to the μ sites (type 1) and lower affinity to the δ and κ sites (*10*). However, the δ and κ sites apparently are not identical to the type 2 binding sites, since Squires and Braestrup (*9*) and Hewlett *et al.* (*11*) demonstrated that diprenorphine, which binds with equal affinity [binding affinity (K_d), $2 \times 10^{-10} M$)] to the μ, δ, and κ sites (*10*), was incapable of displacing [^{3}H]naloxone from its low-affinity binding sites.

During recent in vivo opiate receptor binding studies we reported that buprenorphine, an opiate with binding properties similar to those of diprenorphine, also failed to fully prevent [^{3}H]naloxone binding in intact rat brain (*12*). This suggested that the type 2 (*9*) sites do not represent an artifact of tissue homogenization. Furthermore, in vivo titration of the additional naloxone binding sites in the presence of a blocking dose of diprenorphine (*13*) revealed a K_d of 36 μg of naloxone per kilogram (that is, the dose that occupies 50 percent of these sites in vivo), which is only five times larger than the K_d of naloxone against μ sites in vivo (*13*). Thus, the relatively high affinity of naloxone for these diprenorphine-insensitive sites in vivo stands in contrast to the rather low in vitro affinity of type 2 sites reported in (*9*) and (*11*). The present report explains this discrepancy by showing that the additional naloxone binding site, which we now label the λ site (*14*), rapidly loses

high affinity after homogenization of the brain. Moreover, these λ sites exhibit a unique selectivity for 4,5-epoxymorphinans.

The high- and low-affinity state of the λ site were studied in fresh and washed membrane homogenates (*15*), respectively. All incubations (*16*) were carried out for 20 minutes at 0°C in the presence of [^{3}H]naloxone, various concentrations of displacer, and a blocking concentration of $3 \times 10^{-7}M$ diprenorphine, which reduced [^{3}H]naloxone binding to an intermediate, plateau level (Fig. 1A). Diprenorphine at $3 \times 10^{-7}M$ was shown to fully block the classic opiate binding sites (μ, δ, and κ) by reducing the binding of [^{3}H]diprenorphine ($10^{-9}M$) and of tritiated D-Ala-D-Leu-enkephalin ($10^{-9}M$) to the level of nonspecific binding (measured in the presence of $10^{-5}M$ unlabeled diprenorphine and D-Ala-D-Leu-enkephalin, respectively). The diprenorphine-displaceable portion of [^{3}H]naloxone binding was assumed to largely represent binding to the μ site because of naloxone's pronounced preference for that site over the δ and κ sites (*10*). Separation of bound and unbound tracer was achieved by centrifugation (*17*) to exclude possible artifacts arising from binding of [^{3}H]naloxone to glass fiber filters. A modified filtration procedure (*17*) was also used to validate the results obtained by centrifugation and to measure on- and off-rates.

Binding of [^{3}H]naloxone (*16*) in fresh and washed membrane homogenates decreased proportionally with membrane homogenate dilution. Digestion of washed membranes with trypsin (2300 units of N_α-benzoyl-L-arginine ethyl ether per milliliter) (*18*) with a protease from *Streptomyces griseus* (6 U/ml) and from *Tritirachium album* (10 U/ml) largely reduced specific [^{3}H]naloxone binding to the λ site as well as to the μ sites (*19*), suggesting a proteinaceous character of the λ sites. Distinction of μ and λ sites in washed membranes was obtained with the protein-modifying reagent *N*-ethylmaleimide (*20*). This compound failed to affect the low-affinity state of the λ sites at a concentration ($5 \times 10^{-4}M$, 30 minutes, 37°C) that reduced μ binding to 28 percent of the control value.

The effects of Na^+ and guanyl nucleotides as regulators of the opiate receptor system (*21*) were tested on the binding of [^{3}H]naloxone ($0.5 \times 10^{-9}M$) to μ sites (diprenorphine-displaceable) and λ sites. While 100 m*M* NaCl increased μ binding (*19*) in fresh membrane homogenate at 0°C by 340 percent, it decreased λ binding by 20 percent. Guanylimidodiphosphate (50 μ*M*), a hydrolysis-resistant analog of guanosine 5′-triphosphate, did not affect μ binding but reduced λ binding by 25 percent. The combined effects of NaCl and guanylimidodiphosphate were identical to those of NaCl alone. At a higher temperature (20°C) the effects of Na^+ and guanylimidodiphosphate could only be measured upon [^{3}H]naloxone binding to the low-affinity state of the λ site, since the high-affinity state decays with a half-life of < 2 minutes. Under these conditions (20 minutes incubation at 20°C) Na^+ and guanylimidodiphosphate exhibited similar changes on μ and λ binding as those observed at 0°C. Therefore, μ- and λ-type binding are differently affected by sodium and guanyl nucleotides; however, the question of any regulatory functions of these agents with respect to the λ sites requires further study.

In experiments with a large number of opiate and nonopiate drugs the λ site exhibited a unique selectivity for 4,5-

Table 1. Binding parameters of the λ site. The binding affinity (K_d) estimates in fresh and washed membrane homogenate (*15*) correspond to the sets of fitted curves in Fig. 1, panels B and C, respectively. Each of the two parameter sets is the result of a simultaneous curve-fitting program (*22*) of five displacement experiments. All ten displacement experiments were repeated twice with similar results. The estimate of B_{max} in fresh membrane homogenate is considerably lower than that for washed membranes. The model (law of mass action, single binding site) does not account for the conversion of high-affinity λ sites to low-affinity λ sites, which should already have occurred to some extent during the 20 minutes of incubation at 0°C. A series of additional opioid and nonopioid compounds was tested under the same conditions for displacement of [^{3}H]naloxone from the λ site. The compounds tested displayed either very low or no affinity in both fresh and washed membrane homogenate. Median inhibitory concentrations were as follows ($\times 10^{-9}M$): noroxymorphone (400), *N*-normorphine (7,000), diprenorphine (10,000), buprenorphine (4,000), etorphine (1,000), and sufentanil (4,000). *N*-Allyl-*N*-normetazocine (SKF 10,047), levallorphan, dynorphin-(1–13), phencyclidine, and thebaine displaced 30 percent of the specific [^{3}H]naloxone binding at $10^{-5}M$. No measurable displacement at $10^{-5}M$ was found with codeine, norcodeine, fentanyl, alfentanil, pentazocine, ethylketocyclazocine, dextrallorphan, levorphanol, dextrorphan, adrenocorticotropin, β-endorphin, [Met^5]enkephalin, [Leu^5]enkephalin, γ-aminobutyric acid, bicuculline, glycine, strychnine, atropine, norepinephrine, propranolol, serotonin, dopamine, and haloperidol.

Drug	K_d ($\times 10^{-9}M$)	
	Fresh membrane homogenate*	Washed membrane homogenate†
Naloxone	6	44
Nalorphine	11	62
Naltrexone	29	107
Morphine	60	104
Dihydromorphine	104	144

*B_{max}, 22 pmole per gram of tissue. †B_{max}, 42 pmole.

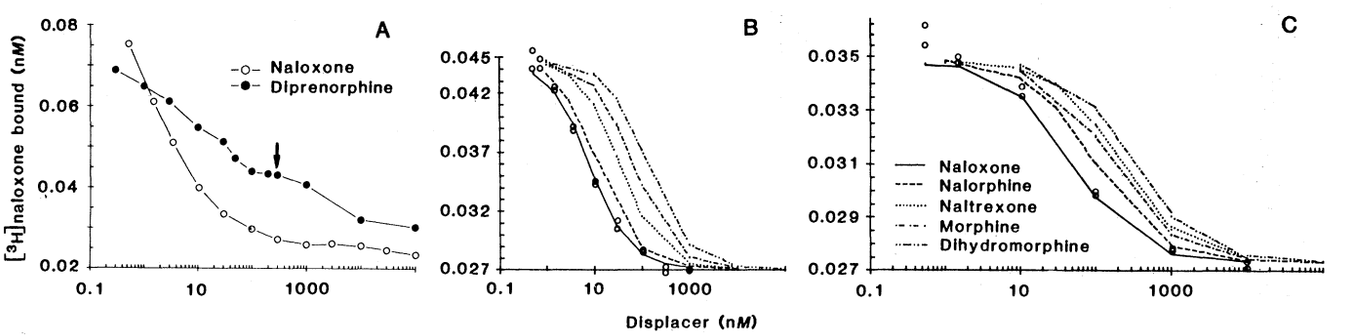

Fig. 1. (A to C) Displacement curves for [^{3}H]naloxone. (A) Fresh membrane homogenate was incubated for 20 minutes at 0°C with $0.5 \times 10^{-9}M$ [^{3}H]naloxone and increasing concentrations of unlabeled naloxone and diprenorphine (each point represents the mean of a triplicate incubation). The arrow indicates the concentration of diprenorphine ($3 \times 10^{-7}M$) chosen as a block for all binding studies at the λ site. (B and C) Fitted displacement curves at the λ site in fresh (B) and washed (C) membrane homogenates. Incubation (*16*) was followed by centrifugation (*17*). Each group of five displacement curves was fitted simultaneously (*22*). The estimated binding parameters are given in Table 1. The circles represent unmodified experimental data for unlabeled naloxone displacing [^{3}H]naloxone. For clarity all other data points have been omitted, but a similarly good fit was achieved in the case of nalorphine, naltrexone, morphine, and dihydromorphine displacing [^{3}H]naloxone.

Table 2. Regional tissue distribution of μ and λ sites in rat brain. The brain was dissected in accordance with the procedure of Glowinski and Iversen (*25*). Each region was homogenized in 80 volumes of ice-cold tris-HCl buffer and immediately incubated for 20 minutes at 0°C with $0.5 \times 10^{-9}M$ of [^{3}H]naloxone, either alone or in the presence of $3 \times 10^{-7}M$ diprenorphine or $3 \times 10^{-7}M$ diprenorphine plus $10^{-5}M$ naloxone. Bound and unbound tracer were separated by centrifugation (*17*). The high-affinity state of the λ site was stable at room temperature during the dissection procedure (~ 10 minutes). Values of bound [^{3}H]naloxone are means ± standard deviations for three experiments.

Region	Concentration of μ binding (pmole/g, wet weight)	Concentration of λ binding (pmole/g, wet weight)
Cerebellum	0.39 ± 0.05	1.42 ± 0.22
Medulla oblongata and pons	1.21 ± 0.10	0.32 ± 0.02
Hypothalamus	1.59 ± 0.27	0.79 ± 0.18
Striatum	1.71 ± 0.04	1.04 ± 0.08
Hippocampus	3.86 ± 0.11	1.27 ± 0.10
Cortex	3.23 ± 0.21	1.83 ± 0.37
Midbrain and thalamus	3.71 ± 0.28	0.61 ± 0.14

epoxymorphinans such as naloxone, nalorphine, naltrexone, morphine, and dihydromorphine. Simultaneous fitting of the displacement curves was performed on the basis of a law of mass action model by means of the NIH computer system PROPHET (*22*). Figure 1 shows the fitted curves for the high (fresh membranes) and low (washed membranes) affinity state of λ sites. The computer estimates of the maximum number of binding sites (B_{max}) and K_d are shown in Table 1. A gradual decline of K_d during the preparation of washed membranes (*15*) was observed by obtaining several naloxone displacement curves throughout the membrane preparation procedure. These results strongly suggest that the same binding sites are detected in different affinity states in fresh and washed membrane homogenates.

Other 4,5-epoxymorphinans lacking the *N*-alkyl substituent, such as noroxymorphone and *N*-normorphine, had considerably lower affinity than dihydromorphine, whereas thebaine, codeine, and norcodeine had no access to λ sites. Compounds such as *N*-allyl-*N*-normetazocine (σ ligand), ethylketocyclazocine (κ ligand), sufentanil (μ ligand), [Leu^5]enkephalin (δ ligand), and the oripavines (diprenorphine, buprenorphine, and etorphine, considered to be "universal" ligands), did not bind to the λ site at concentrations two to four orders of magnitude higher than their K_d at their respective high-affinity site. The endogenous opioid ligand β-endorphin and adrenocorticotropin displayed no access to the λ site at $10^{-5}M$. The putative endogenous κ ligand dynorphin-(1–13) displaced approximately 38 percent of [^{3}H]naloxone binding to λ sites at $10^{-5}M$. These findings (Table 1) clearly demonstrate that the λ site is distinguished from any of the known opiate receptor types. Moreover, a series of nonopioid neurotransmitters and their analogs (Table 1) did not bind to the λ site, thus separating it from established binding sites in neural tissue.

Table 2 summarizes the regional distributions of μ sites (*19*) and λ sites (high-affinity state) in the rat brain. The μ site distribution is similar to that found previously (*23*). The distinct distribution pattern of the λ site strongly indicates that μ and λ sites represent distinct types of binding sites rather than different conformations of a general "opioid receptor." This is supported by the high concentration of λ sites in the cerebellum, which contains negligible amounts of classical opioid receptors (*24*).

The unique selectivity of the λ site contrasts with the more promiscuous binding behavior of the other known opiate receptor types, a feature that may facilitate elucidation of the function of the λ site. Moreover, the present study demonstrates the importance of considering changes in receptor binding behavior during routine in vitro membrane preparation. The high affinity of naloxone to the λ site in vivo (*13*) suggests that these sites are occupied after normal pharmacological doses and that their potential functional significance must be considered.

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14. The λ stands for laudanum, an obsolete term for opium extract, which contains the 4,5-epoxymorphinans, such as morphine.
15. For detection of the high-affinity state of λ sites, male Sprague-Dawley rats (120 to 140 g) were decapitated and their brains were rapidly removed. The cerebellum was discarded and the remainder was homogenized in 80 volumes of ice-cold 50 m*M* tris-HCl buffer (*p*H 7.4) with a Brinkman blender. The resulting preparation (fresh membrane homogenate) was used immediately for incubation (total time after death, including incubation, < 30 minutes). For detection of the low-affinity state of λ sites the brain minus cerebellum was homogenized, centrifuged at 40,000*g* for 15 minutes, exposed to 20°C for 30 minutes, centrifuged again, and diluted in 60 volumes of ice-cold tris-HCl buffer (washed membrane homogenate).
16. The incubation was performed at 0°C in 1.0 ml of fresh or washed membrane homogenate (*15*) in the presence of $0.5 \times 10^{-9}M$ [^{3}H]naloxone (specific activity, 55 Ci/mmole) and $3 \times 10^{-7}M$ diprenorphine. Naloxone has a half-life of dissociation from the high- and low-affinity states of λ sites of 2.5 and 0.8 minutes, respectively. The association of [^{3}H]naloxone ($0.5 \times 10^{-9}M$ in the presence of $3 \times 10^{-7}M$ diprenorphine) and of [^{3}H]diprenorphine ($3 \times 10^{-9}M$) reached maximum values within 3 minutes; therefore an incubation period of 20 minutes was sufficient to establish equilibrium.
17. In the case of centrifugation the incubation was carried out in centrifuge tubes (1.5 ml). At the end of incubation the tubes were centrifuged for 5 minutes at 12,800*g* in a cooled centrifuge (Eppendorf-Brinkman 5412). The supernatant was removed by aspiration and the inner surface of the tube was washed three times with 1.0 ml of ice-cold tris-HCl buffer. In the case of filtration Whatman GF/B filters (2.5 cm) were used. To prevent binding of [^{3}H]naloxone to these filters, 2 ml of $10^{-4}M$ unlabeled naloxone was pipetted onto the filter, followed immediately by the incubation mixture. The valve was opened rapidly and the filter was washed three times with 2 ml of ice-cold buffer. Binding of [^{3}H]naloxone to filters accounted for less than 0.3 percent of the total radioactivity present and was included in nonspecific binding (0.8 percent, in the presence of $10^{-5}M$ naloxone). The washing step took no more than 10 seconds, or considerably less than the dissociation half-life times (*16*). Both procedures led to identical results.
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19. Specific binding of [^{3}H]naloxone to μ sites in fresh and washed membrane homogenate (*15*) was obtained by subtracting [^{3}H]naloxone binding in the presence of $3 \times 10^{-7}M$ diprenorphine from binding in the absence of diprenorphine. Specific binding of [^{3}H]naloxone to λ sites was calculated as the difference of [^{3}H]naloxone binding in the presence of $3 \times 10^{-7}M$ diprenorphine with and without $10^{-5}M$ naloxone added.
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Membrane Changes in a Single Photoreceptor Cause Associative Learning in *Hermissenda*

Abstract. *Single type B photoreceptors in intact, restrained* Hermissenda *were impaled with a microelectrode and exposed to either paired or unpaired presentations of light and depolarizing current to simulate natural stimulus effects during conditioning with light and rotation. Paired, but not unpaired, stimulus presentations produced cumulative depolarization and increased input resistance in type B cells. These membrane changes are similar to those observed after pairings of light and rotation are administered to either intact animals or isolated nervous systems or when light is paired with electrical stimulation of the vestibular system in isolated nervous systems. One and two days after treatment, pairing- and light-specific suppression of phototactic behavior was observed in recovered animals. These findings indicate that the membrane changes of type B cells produced by pairing light with current injections cause acquisition of the learned behavior.*

Previous research with the nudibranch mollusk *Hermissenda crassicornis* has demonstrated striking correlations between the associative suppression of phototaxis and biophysical changes intrinsic to two of the three type B photoreceptors in the animal's eyes during both acquisition and retention of associative learning (*1–8*). We now report that membrane changes of type B cells are causally related to the associative modification of phototaxis that occurs with pairings of light and rotation.

During acquisition of associative learning, type B cells undergo a cumulative depolarization (*2, 3*) and probable increase in intracellular Ca^{2+} (*4, 6*) that arise from a pairing-specific synaptically mediated enhancement of the long-lasting depolarization response of type B cells to light (*2, 5*). This synaptic enhancement is a unique consequence of the temporal convergence of electrical activity in the visual and statocyst (vestibular) sensory pathways upon optic ganglion cells and statocyst hair cells and is responsible for cumulative depolarization of the type B cell (*3*).

During retention of learning, type B cells from conditioned animals exhibit enhanced light-induced generator potentials and impulse activity frequencies, as well as increased input resistances in the dark, both immediately (*7*) and for several days (*8, 9*) after training. Type B cells both encode (change during acquisition) and store (exhibit changes during retention) information concerning pairings of light and rotation (*10, 11*).

Despite these relatively invariant correlations between phototaxic behavioral changes produced by learning and electrophysiological changes of type B cells, it remains to be demonstrated that the membrane changes in type B cells produced by pairing light with rotation are causally related to the learned change in behavior. We sought to establish such a relation by (i) substituting depolarization of a B cell induced by an intracellular

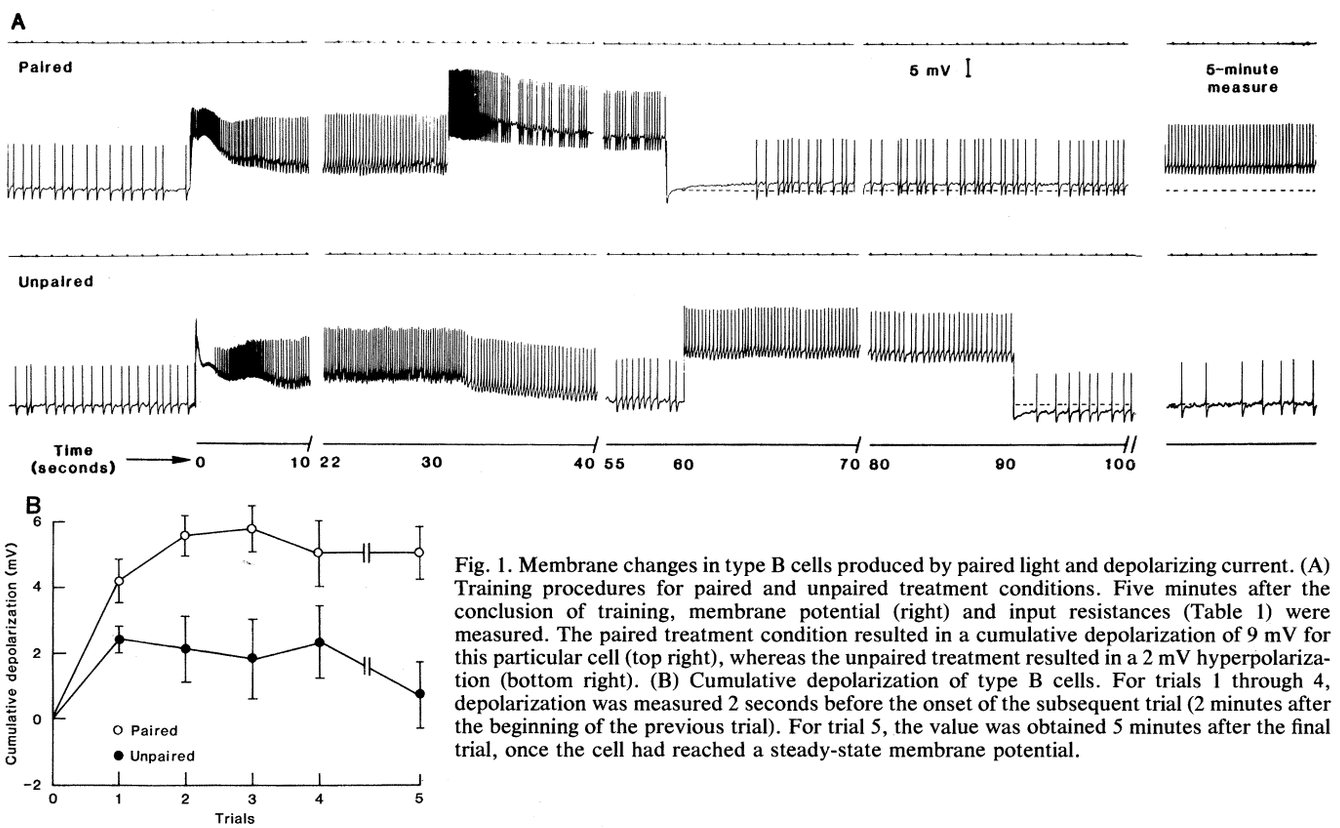

Fig. 1. Membrane changes in type B cells produced by paired light and depolarizing current. (A) Training procedures for paired and unpaired treatment conditions. Five minutes after the conclusion of training, membrane potential (right) and input resistances (Table 1) were measured. The paired treatment condition resulted in a cumulative depolarization of 9 mV for this particular cell (top right), whereas the unpaired treatment resulted in a 2 mV hyperpolarization (bottom right). (B) Cumulative depolarization of type B cells. For trials 1 through 4, depolarization was measured 2 seconds before the onset of the subsequent trial (2 minutes after the beginning of the previous trial). For trial 5, the value was obtained 5 minutes after the final trial, once the cell had reached a steady-state membrane potential.