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Depolarization-induced effects of Ca^{2+}-calmodulin-dependent protein kinase injection, in vivo, in single neurons of cat motor cortex

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Intracellular injection of calcium-calmodulin-dependent protein kinase followed by depolarization and depolarization-elicited impulse activity increased input resistance of neurons of the motor cortex of cats. Protein kinase alone or depolarization in the absence of protein kinase did not produce this effect. An analogous increase of input resistance can be produced in the type B photoreceptor of *Hermissenda* by applying protein kinase and sufficient depolarization to increase calcium conductance and internal Ca^{2+} concentration. Given previous studies linking changes in both types of neurons to the development of conditioning, the results suggest the possibility of shared biochemical steps in mechanisms of neuronal adaptation by vertebrate and invertebrate species.

The roles of cyclic nucleotide-dependent protein kinases[19,20,25] and calcium-calmodulin-dependent protein kinase[13–15] in mediating protein phosphorylation have been extensively investigated in a number of physiologic contexts. The cyclic AMP-dependent phosphorylations are thought to be mediated by type I and type II isoenzymes which have a common catalytic subunit, differ in their regulatory subunit, and have identical substrate specificities[10,23]. Protein kinases of this type have been shown to control voltage-dependent potassium currents in molluscan neurons[1,5,9,22].

Recently, Acosta-Urquidi et al.[2] found that intracellular iontophoresis of Ca^{2+}-calmodulin-dependent protein kinase (Ca^{2+}-CAMdPK) into the type B photoreceptor of the nudibranch *Hermissenda crassicornis* specifically controlled an early, voltage-dependent K^+ current (I_A). Previous observations suggest that long-term reduction of the I_A current in the type B cells plays a causal role in the acquisition and retention (for many days) of associatively conditioned behavior[3,4,6,12,16,17,18,27]. By contrast, iontophoresis of cyclic AMP-dependent protein kinase (i.e. its catalytic subunit) more specifically affected a delayed, voltage-dependent K^+ current (I_B)[5]. A reduction of the I_B current lasting for minutes occurred after exposing the photoreceptor to light alone[6]. Since these and other findings[24] implicated Ca^{2+}-CAMdPK-mediated phosphorylation in the production of conditioning-induced changes of membrane currents of *Hermissenda*, we injected Ca^{2+}-CAMdPK intracellularly into neurons of the motor cortex known to be involved in eyeblink conditioning in cats[8,29] to determine if similar voltage and kinase-dependent conductance changes could be induced.

The animals were comfortably restrained in cloth sleeves with their heads held by means of 4 bolts previously implanted under sodium pentobarbital (Nembutal) anesthesia[29]. Recordings were made with microelectrodes pulled from 1.5–2 mm o.d. theta tubing, filled with either 3 M KCl or with 5–40 units/ml of protein kinase (phosphorylase kinase, Sigma Chemicals) in 1.5 M KCl and 0.025 M Tris buffer and 0.475 M K^+-acetate, where 1 unit of enzyme activity was obtained from solutions of 0.01 mg protein/ml. The higher (40 units) dose level was used only in a few instances to check for increased concentration effects; none were noted. Electrode resistances

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ranged from 13 to 60 MΩ. The electrodes were connected to an amplifier with an input resistance of 10^{12} Ω and negative capacitance compensation, and then to an FM tape recorder with DC coupling and an upper band pass of 2500 Hz. Further details may be found elsewhere[29].

Forty-eight neurons of the pericruciate cortex were studied in 7 cats. The resting potentials of these cells averaged 50 ± 7 (±S.D.) mV. All but 10 of the recordings were obtained from awake preparations. The remaining 10 cells were studied in preparations given a single sedative dose (10–15 mg/kg i.p.) of sodium pentobarbital. No differences were found when recordings obtained from animals under light Nembutal sedation were compared with recordings from unanesthetized animals.

Ca^{2+}-CAMdPK was injected intracellularly by passing a steady negative current of 2.5 nA through the recording electrode for a period of 1 min following cell penetration. (One experiment utilized 6 nA, 10–12 ms, 10 Hz negative current pulses applied over a period of 1 min to inject Ca^{2+}-CAMdPK instead of steady current.) The effects on conductance were determined by passing weak, brief (0.5–1.0 μA, 10–12 ms, 10 Hz), positive and negative pulses across a Wheatstone bridge circuit. Examples are shown in Fig. 1. In the few instances in which the pulse duration was not long enough to reach a flat asymptote at a period beyond the time constant for charging the cell membrane capacitance, measurements of resistance were made at isochronal points just prior to the pulse offset measured before and after application of protein kinase and depolarization. As can be seen (Fig. 1A), neither low rates of spike discharge nor failure to reach asymptote prevented changes in input resistance comparable to those found in the ab-

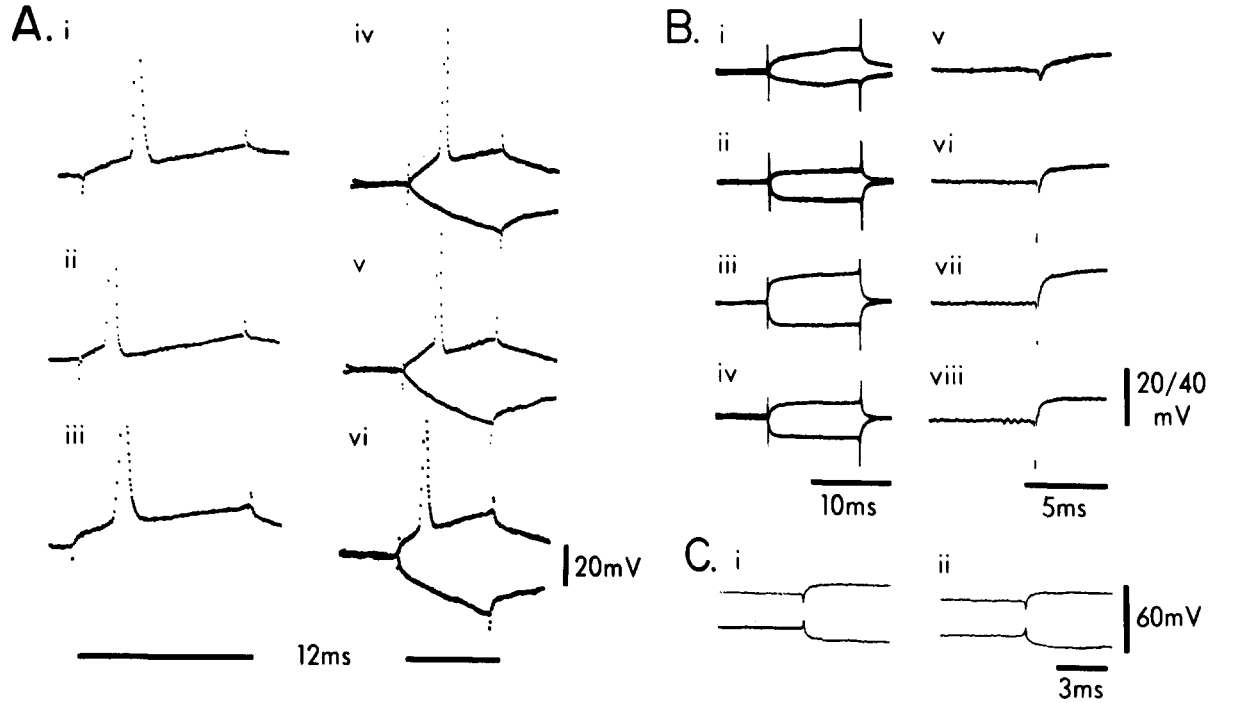

Fig. 1. Results of injection of Ca^{2+}-calmodulin-dependent protein kinase (Ca^{2+}-CAMdPK) and application of depolarizing current. A and B: two cells given Ca^{2+}-CAMdPK intracellularly prior to depolarization. A: input resistance: (i and iv) = before protein kinase injection; (ii and v) = after protein kinase injection; (iii and vi) = immediately after subsequent depolarization. (i, ii and iii) show the maintenance of bridge balance to null out changes in electrode resistance. B: input resistance (i) = before protein kinase injection; (ii) = after protein kinase injection; (iii) = immediately after depolarization subsequent to protein kinase injection; (iv) = 10 s after cessation of depolarization; (v–viii) = faster, amplified sweeps showing bridge balance at periods corresponding to those in the traces to the left. In A (ii) and B (v and vi) the bridge is slightly out of balance. C: effects on input resistance of depolarization alone, without administration of protein kinase; (i) = before depolarization; (ii) = after depolarization. Calibrations are as indicated below and to the right of each portion of the figure. In (A) 12 ms applies to both time calibrations and the 20 mV calibration applies to all records. (The tops of the spikes are cut off.) In (B), 20 mV applies to parts v–viii and 40 mV to parts i–iv.

sence of spiking (Fig. 1B) from being detected. The electrode capacitances were such that it was possible to separate electrode and cell resistances components reliably by adjusting the balance of the Wheatstone bridge.

Sixteen cells were given Ca^{2+}-CAMdPK followed by a 30-s period of steady depolarization (1.0 nA). This group of cells showed an increase in input resistance in comparison with a control group of 15 cells given depolarization only, without injection of Ca^{2+}-CAMdPK. Eight of the cells in the control group were studied with electrodes containing Ca^{2+}-CAMdPK and 7 cells were studied with electrodes containing KCl alone. Most of the control cells (13 of 15) showed a small decrease in resistance, as has been previously observed in prolonged intracellular recordings from neurons of this region[32]. The average change of input resistance after Ca^{2+}-CAMdPK plus depolarization was $+2.6 \pm 2.1$ (S.E.M.) MΩ, while that after depolarization alone was -3.2 ± 1.3 MΩ. The difference between groups was significant ($P < 0.05$, Student's t-test, two-tailed). There were no significant differences between experimental and control groups in sizes of action potentials, resting potentials or in initial input resistances.

The increase of input resistance did not occur after injection of (Ca^{2+}-CAMdPK) alone (Fig. 2C). Postiontophoretic measurements of input resistance in 26 cells given Ca^{2+}-CAMdPK were no different from those in 7 cells given equivalent negative currents through electrodes containing only KCl, nor were levels of resistance increased after injection of Ca^{2+}-CAMdPK in the cells that subsequently showed increases of input resistance after Ca^{2+}-CAMdPK plus depolarization (Figs. 1A, B and 2C).

Not all cells given Ca^{2+}-CAMdPK plus depolarization responded with an increase of input resistance. Twelve of the 16 cells given Ca^{2+}-CAMdPK showed an increase of input resistance immediately after the cessation of depolarizing current application (aver-

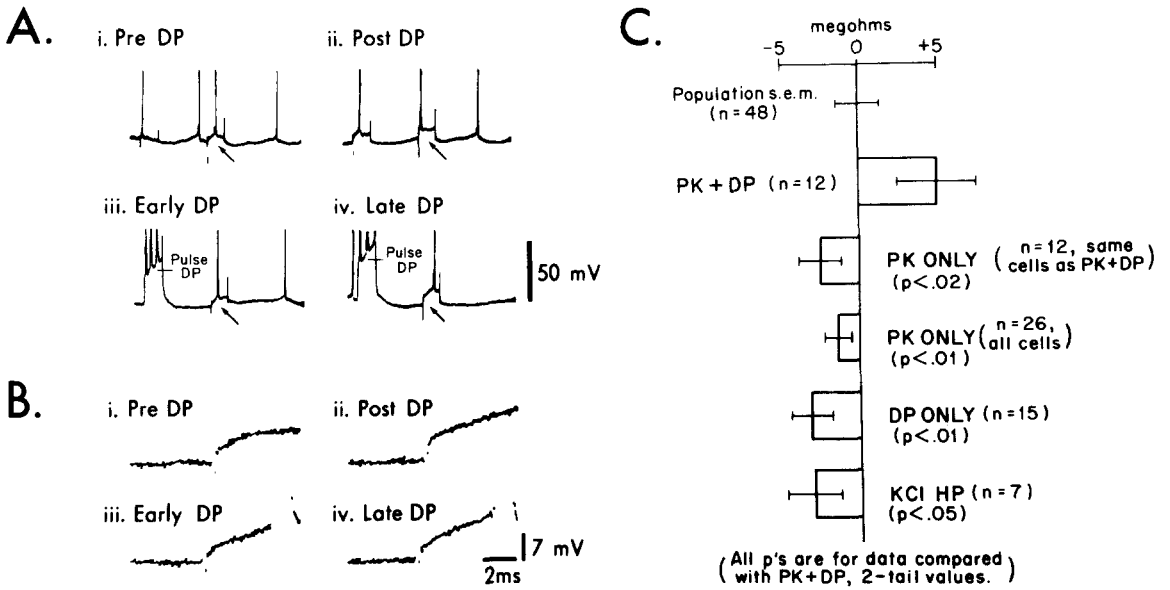

Fig. 2. Response of a cell given protein kinase (Ca^{2+}-CAMdPK) and pulse depolarization. A: input resistance: (i) = after protein kinase administration but before depolarization; (ii) = after protein kinase and depolarization; (iii) = early during depolarization after protein kinase; (iv) = later during depolarization after protein kinase. The bridge currents are 0.5 and 1.0 nA in (i), 0.75 and 1.0 nA in (ii), and 4.0 and 1.0 nA in (iii) and (iv). B: faster, amplified sweeps during the initial periods of pulse application at similar times as above to show that the electrode resistance component, which is so fast that it fails to activate the phosphors on the storage oscilloscope at this sweep speed (compare with traces in A), has been balanced sufficiently well that changes in electrode resistance do not contribute significantly to the measured change in cell input resistance shown by arrows in A. Voltage calibrations are as shown; the time calibrations in A are given by the 10 ms bridge pulses. C: bar graphs of average change in input resistance before and after protein kinase plus depolarization in responsive cells (PK+DP), after protein kinase only (PK only), after depolarization alone (DP only), and after passage of negative current, i.e. hyperpolarization, without iontophoresis of protein kinase (KCl HP). Standard errors of the means are shown for each different group of cells.

age = +5.0 ± 2.5 MΩ). The remaining 4 did not. The increase of input resistance after Ca^{2+}-CAMdPK plus depolarization (relative to the resistance prior to depolarization) was significant at $P < 0.02$ (Student's *t*-test, two-tailed) for comparisons made within the same cell before and after depolarization (Fig. 2C). The magnitude of the increased resistance in the twelve responsive cells was sizeable and differed greatly ($P < 0.01$, Student's *t*-test, two tailed) from the level of input resistance in the 15 cells given depolarization alone. The increase in resistance was transient, slowly returning to pre-treatment values over a 10–60-s period. The shorter duration of resistance change in these cells than in the type B photoreceptors of *Hermissenda*[2] may reflect differences in the efficacy of the exogenous enzyme used on the available substrates in these two different types of cells or in their sizes and resulting dilutional effects on injectates. As discussed later, the failure of some cortical cells to respond to Ca^{2+}-CAMdPK plus depolarization might be related to the previous finding that only two-thirds of the cells of the motor cortex respond with increases of input resistance to application of acetylcholine or cyclic GMP[26,32].

Steady depolarization, per se, may not be required for increases in resistance to occur after administration of protein kinase. Fig. 2 illustrates a cell in which, after intracellular application of Ca^{2+}-CAMdPK, pulse depolarization sufficient to produce repeated spike discharge resulted in an increase in resistance comparable to that seen after administration of Ca^{2+}-CAMdPK and steady depolarization. I–V plots made from injections of depolarizing and hyperpolarizing currents confirmed an increase in slope resistance of 3.6 MΩ over the period of depolarization shown in Fig. 2A and B. We showed earlier that on delivering depolarizing pulse currents equivalent to those of this figure during application of acetylcholine or cyclic GMP to neurons of these same cortical regions, the response to these agents was changed from a transient to a persistent increase in resistance (cf. refs. 26,31,32).

A number of parallels were found between the action of protein kinase in neurons of the motor cortex of cats and that in the type B photoreceptor of *Hermissenda*. First, hyperpolarizing administration of protein kinase by itself produced no measurable change of input resistance. The small decrease in resistance observed in the present studies was previously found to be characteristic of long term, intracellular penetrations of such neurons, i.e. with or without injection of protein kinase[32]. Second, levels of depolarization sufficient to produce repeated spike discharge, when applied subsequent to intracellular administration of protein kinase, produced an increase in input resistance. In *Hermissenda* this effect has been ascribed to a voltage-dependent increase in calcium conductance across the plasma membrane[2,3,7]. Since a calmodulin-dependent protein kinase was injected, the entry of calcium would provide a means by which it could be activated.

It is not established, in vivo, whether calcium conductances exist in cells of the motor cortex of cats or if internal calcium concentration can be increased by depolarization in these cells in the same manner as has been directly demonstrated for the type B photoreceptors of *Hermissenda* with differential absorption spectrophotometry[11]. Nonetheless, there is extensive evidence that strong depolarization can act in this manner to increase internal calcium concentration in many different types of neurons. Moreover, recent studies in hippocampal neurons report a 4-aminopyridine-dependent outward current much like that found in the photoreceptors of *Hermissenda*[21]. Thus, it is not unreasonable that a depolarization-induced inward flux of Ca^{2+} might modulate an early outward K^+ current controlling a prolonged change in the input resistance of vertebrate neurons. A change in input resistance has been postulated to support the increases in excitability to intracellularly injected currents that occur in these cells after conditioning[29]. These cells appear to be necessary in order for this type of conditioning to occur[30,33].

The conductance changes underlying comparable alteration of neural excitability have been demonstrated in the type B photoreceptors of *Hermissenda* after conditioning[3,4,6,16,17,18,27]. Although the process of association in the cat is far more complex at the cellular level than might have been guessed from earlier psychophysiologic observations[28], applications of stimuli such as those used in associative conditioning would be expected to produce pulse-like depolarization with superimposed impulses. Stimuli of sufficient salience to produce depolarization well beyond the firing threshold of the cell could activate a calmodulin-dependent protein kinase by elevating intracel-

lular calcium and thereby alter conductances across the cell membrane and the transmission properties of the cell. The observations of this report suggest the possibility of shared biochemical steps controlling adaptation in neurons involved in the acquisition of conditioned behavior by both vertebrate and invertebrate species.

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1 Acosta-Urquidi, J., Alkon, D. L., Olds, J., Neary, J. T., Zebley, E. and Kuzma, G., Intracellular protein kinase injection simulates biophysical effects of associative learning on *Hermissenda* photoreceptors, *Soc. Neurosci. Abstr.*, 7 (1981) 944.

2 Acosta-Urquidi, J., Neary, J. T. and Alkon, D. L., Ca^{2+}-dependent protein kinase regulation of K^+(V)-currents: a possible biochemical step in associative learning of *Hermissenda*, *Soc. Neurosci. Abstr.*, 8 (1982) 825.

3 Alkon, D. L., Membrane depolarization accumulates during acquisition of an associative behavioral change, *Science,* 210 (1980) 1375–1376.

4 Alkon, D. L., Voltage-dependent calcium and potassium ion conductances: a contingency mechanism for an associative learning model, *Science,* 205 (1979) 810–816.

5 Alkon, D. L., Acosta-Urquidi, J., Olds, J., Kuzma, G. and Neary, J. T., Protein kinase injection reduces voltage-dependent potassium currents, *Science,* 219 (1983) 303–306.

6 Alkon, D. L., Lederhendler, I. and Shoukimas, J., Primary changes of membrane currents during retention of associative learning, *Science,* 215 (1982) 693–695.

7 Alkon, D. L., Shoukimas, J. J. and Heldman, E., Calcium-mediated decrease of a voltage-dependent potassium current, *Biophys. J.*, 40 (1982) 245–250.

8 Brons, J. F. and Woody, C. D., Long-term changes in excitability of cortical neurons after Pavlovian conditioning and extinction, *J. Neurophysiol.*, 44 (1980) 605–615.

9 Castellucci, V. S., Kandel, E. R., Schwartz, J. H., Wilson, F. D., Nairn, A. C. and Greengard, P., Intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase simulates facilitation of transmitter release underlying behavioral sensitization in *Aplysia, Proc. nat. Acad. Sci. U.S.A.*, 77 (1980) 7492–7496.

10 Cheung, W. Y., Calmodulin: an overview, *Fed. Proc.*, 41 (1982) 2253–2257.

11 Connor, J. A. and Alkon, D. L., Light-induced changes of intracellular Ca^{2+} in *Hermissenda* photoreceptors measured with Arsenazo III, *Soc. Neurosci. Abstr.*, 8 (1982) 944.

12 Crow, T. J. and Alkon, D. L., Associative behavioral modification in *Hermissenda*: cellular correlates, *Science,* 209 (1980) 412–414.

13 DeLorenzo, R. J., Role of calmodulin in neurotransmitter release and synaptic function, *Ann. N.Y. Acad. Sci.,* 356 (1980) 92–109.

14 DeLorenzo, R. J., Postsynaptic densities: calmodulin and calcium-dependent phosphorylation, *Trans. Amer. Soc. Neurochem.*, 11 (1980) 81.

15 DeLorenzo, R. J., Calmodulin in neurotransmitter release, *Fed. Proc.*, 41 (1982) 2265–2272.

16 Farley, J. and Alkon, D. L., Neural organization predicts stimulus specificity for a retained associative behavioral change, *Science,* 210 (1980) 1373–1375.

17 Farley, J. and Alkon, D. L., Associative neural and behavioral change in *Hermissenda*: consequences of nervous system orientation for light and pairing specificity, *J. Neurophysiol.*, 48 (1982) 785–807.

18 Farley, J., Richards, W., Ling, L., Liman, E. and Alkon, D. L., Membrane changes in a single photoreceptor cause associative learning in *Hermissenda*, *Science,* 221 (1983) 1201–1203.

19 Greengard, P., Cyclic nucleotides, phosphorylated proteins, and the nervous system, *Fed. Proc.*, 38 (1979) 2208–2217.

20 Greengard, P. and Kebabian, J. W., Role of cyclic AMP in synaptic transmission in the mammalian peripheral nervous system, *Fed. Proc.*, 33 (1974) 1059–1067.

21 Gustaffson, B., Galvan, M., Grafe, P. and Wigstrom, H., A transient outward current in a mammalian central neurone blocked by 4-aminopyridine, *Nature (Lond.),* 299 (1982) 252–254.

22 Kaczmarek, L. K., Jennings, K. R., Strumwasser, F., Nairn, A. C., Walter, U., Wilson, F. D. and Greengard, P., Microinjection of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture, *Proc. nat. Acad. Sci. U.S.A.*, 77 (1980) 7487–7491.

23 Krebs, E. G. and Beavo, J. A., Phosphorylation-dephosphorylation of enzymes, *Ann. Rev. Biochem.*, 48 (1979) 923–959.

24 Neary, J. T., Crow, T. and Alkon, D. L., Change in a specific phosphoprotein band following associative learning, *Nature (Lond.),* 293 (1981) 658–660.

25 Sutherland, E. W., Studies on the mechanisms of hormone actions, *Science,* 177 (1972) 401–408.

26 Swartz, B. E. and Woody, C. D., Correlated effects of acetylcholine and cyclic guanosine monophosphate on membrane properties of mammalian neocortical neurons, *J. Neurobiol.*, 10 (1979) 465–488.

27 West, A., Barnes, E. and Alkon, D. L., Primary changes of voltage responses during retention of associative learning, *J. Neurophysiol.*, 48 (1982) 1243–1255.

28 Woody, C. D., The electrical excitability of nerve cells as an index of learned behavior. In D. L. Alkon and J. Farley (Eds.), *Primary Neural Substrates of Learning and Behavioral Change,* Cambridge University Press, New York, in press.

29 Woody, C. D. and Black-Cleworth, P. Differences in excitability of cortical neurons as a function of motor projection in conditioned cats, *J. Neurophysiol.*, 36 (1973) 1104–1116.

30 Woody, C. D. and Brozek, G., Changes in evoked responses from facial nucleus of cat with conditioning and extinction of an eye blink, *J. Neurophysiol.*, 32 (1969) 717–726.

31 Woody, C. D., Carpenter, D. O., Gruen, E., Knipsel, J. D., Crow, T. W. and Black-Cleworth, F., Persistent increases in membrane resistance of neurons in cat motor cortex, *AFRRI Scientific Report,* (February, 1976), pp. 1–31.

32 Woody, C. D., Swartz, B. E. and Gruen, E., Effects of acetylcholine and cyclic GMP on input resistance of cortical

neurons in awake cats, *Brain Research,* 158 (1978) 373–395.
33 Woody, C. D., Yarowsky, P., Owens, J., Black-Cleworth, P. and Crow, T., Effect of lesions of cortical motor areas on acquisition of eyeblink in the cat, *J. Neurophysiol.,* 37 (1974) 385–394.