Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3047–3050, May 1987
Neurobiology

On-line visualization of dendritic release of acetylcholinesterase from mammalian substantia nigra neurons

(subcisternal release/calcium-dependent acetylcholinesterase release)

RODOLFO R. LLINÁS* AND SUSAN A. GREENFIELD†

*Department of Physiology and Biophysics, New York University Medical Center, 550 First Avenue, New York, NY 10016; and †University Department of Pharmacology, South Parks Road, Oxford OX1 3QT, United Kingdom

Contributed by Rodolfo R. Llinás, January 12, 1987

ABSTRACT **This study presents, to our knowledge, the first on-line measurement of acetylcholinesterase (AcChoEase) release from brain tissue. It is now well established that a soluble form of the enzyme is released from central nervous system neurons, and it has been proposed on indirect grounds that such release may occur not only from presynaptic terminals but also from the dendrites of dopamine-containing nigrostriatal neurons. We have used a chemiluminescent reaction to examine the real-time release of AcChoEase from the substantia nigra *in vitro* in brainstem slices. The light emission was captured by two fiber optic systems, one in direct contact with the brain slice from below and the second 4-mm above the slice, allowing simultaneous imaging of the emitted light and quantitative photometry. It was determined that the light signals are not due to the spontaneous hydrolysis of acetylcholine or the presence of free choline, but are caused by the enzymatic action of AcChoEase. Using this technique, it can be directly shown that AcChoEase is spontaneously released from the soma or dendrites of nigral neurons. The release of the enzyme, which is stored in the subcisternal dendritic compartment, is resistant to blockade of voltage-dependent sodium conductances, is calcium dependent, and can be increased by addition of potassium to the bathing solution. The procedure we describe here will make it possible to study the release of endogenous AcChoEase on a time-scale close to that over which it functions.**

Ultrastructural (1), neurochemical (2, 3), electrophysiological (4, 5), and behavioral (6) findings have suggested that acetylcholinesterase (AcChoEase) is released from nigrostriatal somata and dendrites. It has been proposed (7) that dopamine is also released from the dendrites of these neurons. This release of transmitter and protein has been shown to play a significant role in nigrostriatal cell function (8). However, a precise understanding of the cellular mechanism underlying the release of large molecules has been hampered by technical limitations making it impossible to demonstrate dendritic release in a direct manner. To date, the methods used have entailed *in vitro* (9) or *in vivo* (3) perfusion (via push–pull cannulae) followed by spectrophotometric (10) or fluorometric (11) analysis of perfusates. However, dilution of released AcChoEase by perfusate limited sensitivity of the assays and restricted time resolution to periods vastly exceeding the time course of the release mechanism. To study release of AcChoEase on a time scale corresponding more to that of true neuronal events, we report the adaptation of a chemiluminescent reaction (12) for immediate and sensitive detection of AcChoEase and the development of optical probes for both on-line visualization and also measurement of luminescence, directly on the surface of nigral tissue.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Mesencephalic slices (300 μm) were prepared from female guinea pigs (200 g) as described (5). Slices were subsequently maintained separately in a solution, constantly bubbled with 95% O_2/5% CO_2, containing 6 ml of "standard" Ringer solution (13) (pH 7.5), 20 μl of 10 mM luminol (Sigma), 20 μl of choline oxidase at 50 units/ml (Boehringer Mannheim), 20 μl of microperoxidase at 10 mg/ml (Sigma; MP II). The composition of this solution is modified from that used in a chemiluminescent reaction developed to detect acetylcholine (AcCho) (12). Choline produced by the hydrolysis of AcCho by AcChoEase is oxidized to betaine with the generation of H_2O_2. The subsequent oxidation of luminol peroxidase by H_2O_2 generates photoemission (12, 14).

The slice was continuously perfused with the above solution at a rate of 0.3 ml/min. Since we wished to measure endogenous AcChoEase, the reaction was initiated by addition of acetylcholine chloride (Sigma; final concentration 1 mM) to the perfusing solution.

Light emission was measured directly via a light pipe suspended 4 mm above the brain-stem slice. The light pipe was coupled to a Hamamatsu 7696 photomultiplier tube, and the output signal was amplified (Pacific Photometric Picoammeter amplifier 3A27) and displayed on a chart recorder (Fig. 1). Imaging of the light emission was obtained via the direct contact of the slice with the fiber optics system at the bottom of the chamber. This lower fiber optics bundle was coupled directly to the fiber optics input of an ITT model 4144 double-microchannel plate having maximum luminous gain of 10^6. The output of the microchannel plate was coupled directly to the fiber optics input of a charge-coupled device television camera (Fairchild model CCD 3000 F). The image from this camera was taken and averaged using a Hamamatsu video frame processor (C1440-01). Pseudocolor was obtained using a DeAnza IP8000 system (Gould). The overall imaging properties of this system are such that <15% of the light emitted by the lower surface of the brain slice is lost, due to the continuous fiber optics coupling between the preparation and the camera. The resolution depth of the device (≈1 mm) is such that the total thickness of the slice was imaged. To maintain a constant concentration of reactant in the bathing fluid over the slice, the solution was constantly perfused over the slice from a reservoir and kept in constant gentle movement by a magnetic stirrer 1 mm in diameter and 8 mm in length, coupled to a dc motor revolving at 5 revolutions per sec. Under these conditions, a steady-state light emission could be maintained for several hours. In some experiments the temperature of the slice was kept at 36°C using a heating system. All of the quantitative data reported here were obtained at 22°C.

Abbreviations: AcCho, acetylcholine; AcChoEase, acetylcholinesterase; TTX, tetrodotoxin.

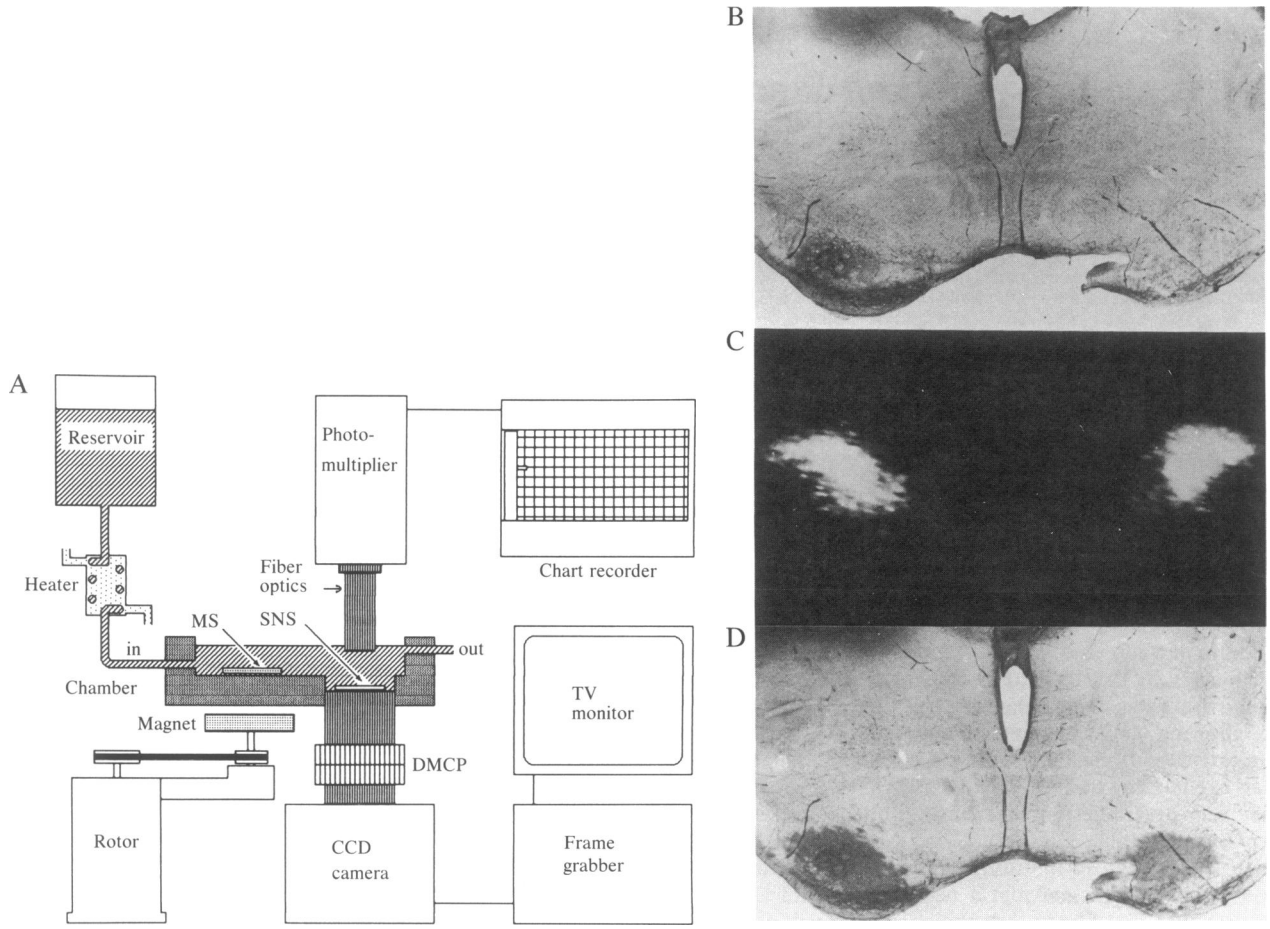

FIG. 1. (*A*) Instrumentation. Substantia nigra slice (SNS) is kept under continuous perfusion from a reservoir of thermally controlled fluid into the chamber (in) and is constantly removed (out). The fluid in the chamber is constantly stirred by a magnetic stirrer (MS) powered through the rotor and an attached magnet. The slice lies under a fiber optics column directly coupled to a double-microchannel plate (DMCP) itself coupled by fiber optics to a charge-coupled device (CCD) TV camera. The image is taken by frame grabber and displayed on the TV monitor. On the top, light emission is measured directly by fiber optics through a photomultiplier and printed on a chart recorder. (*B*) Example of substantia nigra slice showing the location of pars compacta neurons following horseradish peroxidase injection to the caudate nucleus. Note also the anterograde unilateral labeling of striatonigral terminals in pars reticulata. (*C*) Location of light emission using the charge-coupled device camera. (*D*) Superposition of *B* and *C*.

RESULTS

In the absence of AcCho, no signal was detectable. However, following addition of AcCho, a signal was detectable both qualitatively (Fig. 1*C* and 2) and quantitatively (Fig. 3) from the tissue. Using a light pipe that covered the activity of the substantia nigra at a distance of 4 mm from above, the light signal had a mean latency of 1.13 ± 0.44 min (SEM), $n = 39$, a rate of increase of 11.4 ± 3 pA/min (SEM), $n = 39$, and a mean maximum amplitude of 78 ± 11.8 pA (SEM), $n = 39$ (see Fig. 3 *A*, *B*, *C*, and *E*). The initial large signal may be due to hydrolysis of substrate by AcChoEase already released into the extracellular space. The reaction appeared to last for as long as perfusion was continued; the maximum period monitored in this study was 3 hr, within which time a mean steady-resting level was obtained at 26 ± 7.5 pA (SEM), $n = 7$. Following the initial large increase, oscillations in the signal were visible (see Fig. 3*E*). Closer analysis of five of these latter studies revealed that the oscillations occurred at ≈0.5 Hz.

When KCl (30 mM, final concentration) was added to the solution, there was an increase of light emission of 120 ± 39% (SEM), $n = 26$, above the preceding value (see Figs. 2 and 3*B*). The presence of tetrodotoxin (TTX; final concentration, 1 μM) throughout the experiment did not appear to modify the signal; i.e., there was an initial reading of 91.5 ± 24.9 pA (SEM), $n = 10$, and a subsequent KCl-evoked increase of 125 ± 58% (SEM) above the preceding level.

In some experiments, slices were pretreated with a specific inhibitor of AcChoEase [Sigma; BW 284C51: 1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide, final concentration, 8.5 μg/ml]. This treatment led to a much smaller initial signal from the reaction [mean, 19.8 ± 5.1 pA (SEM), $n = 5$ (Fig. 3*D*)] and when added during the course of the experiments, caused a rapid decrease in the resting signal (Fig. 3 *C* and *E*) to a mean of 5.4 ± 1.5 pA (SEM), $n = 8$. Oscillations in signal were never apparent in the presence of BW 284C51 and were considerably reduced and then abolished (Fig. 3*E*) when inhibitor was added during the course of the experiment. In addition, a KCl-induced signal was never seen in the presence of the inhibitor (Fig. 3*D*).

The signal observed could not be due to the spontaneous hydrolysis of AcCho, since no reaction could be measured in the absence of tissue. Furthermore, it is unlikely that light emission was resulting from the initial oxidation of free choline within the brain slices, as the addition of the substrate AcCho was required to detect a signal. Indeed, the substantia nigra is very low in endogenous AcCho (15, 16). Hence, only when exogenous AcCho was added was there sufficient substrate for production of choline leading to the reaction for light emission. The latency observed before the signal was

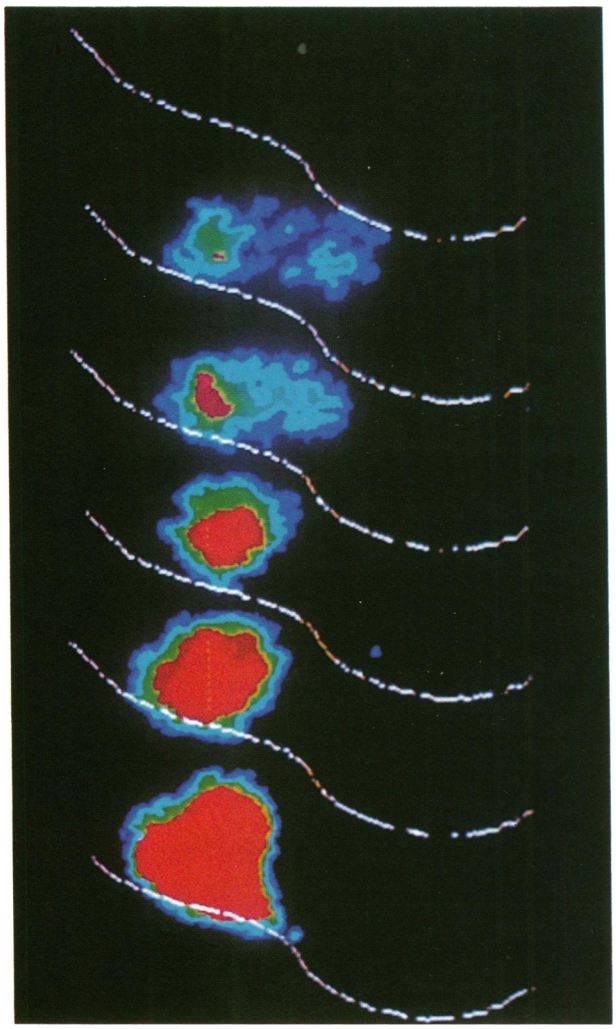

FIG. 2. Dendritic AcChoEase release as observed from a brainstem slice *in vitro*. The upper five photographs show the location and magnitude of light emission generated in substantia nigra following addition of AcCho to the bathing solution in the presence of TTX. Each picture was taken at a 1-min interval after addition of AcCho. The fifth picture represents the final steady-state light emission as the light-emitting reaction reaches a peak. The sixth picture shows increased light emission by addition of 30 mM KCl to the bathing solution.

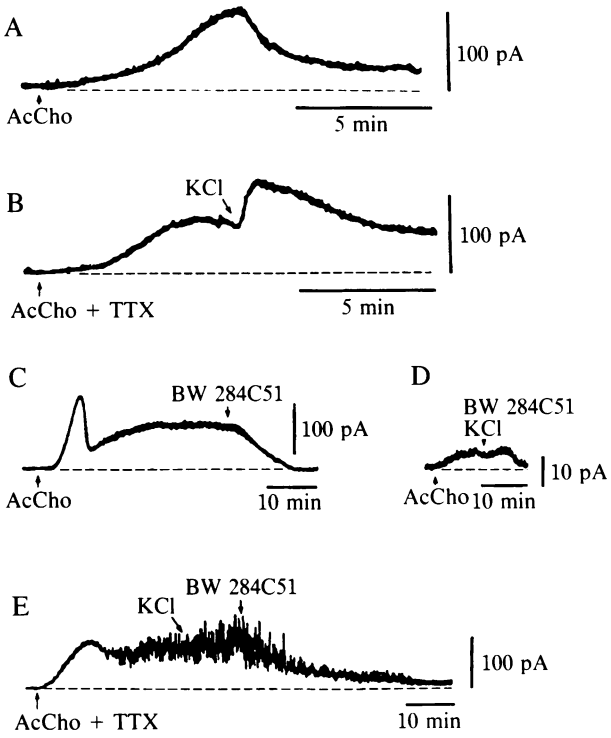

FIG. 3. (*A*) Light response measured at the onset of perfusion. The light signal is seen to increase to a peak and then come to a steady-state level. In *B* a similar example in another preparation in the presence of TTX. Perfusion with AcCho produces the initial peak. Addition of 30 mM KCl produces a fast secondary response. (*C*) Similar response as in *A* to maintain plateau following initial response. Addition of BW 284C51 produces a sharp decay of plateau level. (*D*) Response under similar conditions is shown when BW 284C51 is present from the beginning of the experiment. This represented the largest response to AcCho observed and the largest response to addition of KCl to the bathing solution under these conditions. (*E*) Similar responses as *A*, *B*, and *C* in another experiment in the presence of TTX to show marked oscillations of light emission that are increased by addition of KCl and blocked by addition of BW 284C51.

detectable was probably due to the time taken for the AcCho to diffuse evenly throughout the slice.

Although the area covered by the upper light pipe included brain tissue beyond the substantia nigra, direct visualization of light emission via the camera ensured that the signal did originate specifically from the substantia nigra. That light was not emitted by intracellular hydrolysis of AcCho but resulted from a reaction caused by extracellular AcChoEase can be safely assumed from the facts that AcCho does not penetrate the cell membrane and that the degree of conversion of uptaken choline to AcCho is very low in nigral cells (17).

The actual amount of enzyme released can be estimated as follows. The AcChoEase-reactive area of the guinea pig substantia nigra is ≈2.2-mm across and 0.8-mm deep (1). In a 300-μm slice, it is, therefore, possible to calculate the volume of extracellular fluid (occupying ≈20% of the total tissue volume) (C. Nicholson, personal communication) as being ≈0.1 μl. Calibration with exogenous AcChoEase (Sigma; type V-S, electric eel) showed that 26 pA, the mean resting level, corresponded to an AcChoEase concentration of 140 milliunits/ml; i.e., the absolute amount of enzyme in the extracellular space would be ≈0.014 milliunit, if the signal were indeed derived from released enzyme. When this amount was diluted in the perfusate normally used in push–pull cannulae (20 μl/min) (3), the final concentration of AcChoEase would be 0.7 milliunit/ml. This value corresponds closely to that reported with *in vivo* (3) and *in vitro* (9) perfusions of the substantia nigra by conventional methods.

The observation that the light emission often "oscillated" in amplitude suggests that the signal was not caused by membrane-bound AcChoEase, but that the enzyme was being released in a pulsatile fashion. Since these oscillations were detectable at various times and amplitudes throughout the experiments, it would seem that this pulsatile signal was a physiological rather than a reaction-related phenomenon. Neurons in other areas of the central nervous system have already been reported to have oscillatory properties when monitored electrophysiologically (18).

Furthermore, the light-emission signal was due almost exclusively to the enzymatic action of AcChoEase since there were marked decreases in the signal following the addition of the AcChoEase inhibitor. This observation also establishes that the reaction was indeed extracellular as BW 284C51 does not penetrate the cell membrane (19). The levels of nonspecific cholinesterase that constitute the residue signal are lower than those reported in the rat (3), cat (20), and rabbit (21) and agree with ultrastructural studies (1) showing that the guinea pig substantia nigra is particularly poor in

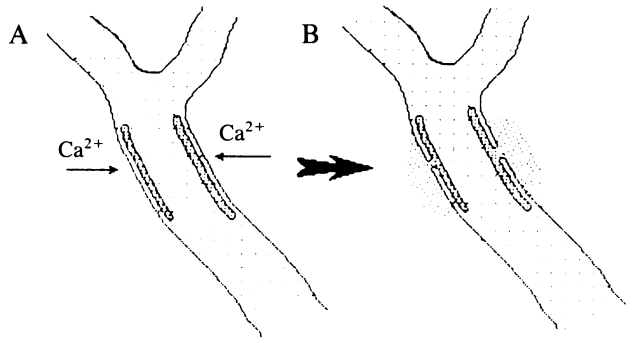

FIG. 4. Hypothesis related to dendritic release from substantia nigra pars compacta neurons. Depolarization of the dendrite allows calcium entry into the dendrite. Calcium entry is seen as the trigger for exocytosis of AcChoEase from subcisternal systems known to contain AcChoEase in these neurons.

nonspecific cholinesterase. This study also demonstrates directly that only AcChoEase specifically is released by the addition of KCl (cf. ref. 22). In addition, since oscillations in signal were never observed following treatment with BW 284C51, it would seem that only AcChoEase can be released in a pulsatile fashion.

The marked increases induced by addition of KCl show that AcChoEase may be released from the substantia nigra upon depolarization, as indicated indirectly by the push–pull cannulae experiments (3). Since TTX was inefficacious in modifying the signal, it is likely that dendritic release of the enzyme is unrelated to somatic action potentials (cf. ref. 22) and may be governed by the local and independent entry of calcium into the dendrite (cf. ref. 5).

Regarding site of release, the actual location of light emission using the double-microchannel plate charge-coupled device system demonstrates that the release of AcChoEase actually occurs from dendrites of substantia nigra pars compacta neurons: they are the only parts of the cell penetrating deep into the pars reticulata having the particular spatial distribution demonstrated and are known to contain AcChoEase along their total length (1). Indeed, the distribution of the light emission indicates that the release occurs from the more dorsal parts of substantia nigra (close to the bodies of pars compacta neurons) to the actual ventral border of the brain stem, close to the point of termination of the tips of the dendrites of the above neurons. Also directly observed was that the release is not continuous. The oscillations as well as the subgrouping of the release from these neurons indicates that the cells may release as subpopulations (cf. ref. 8), probably related to the presence of electrical coupling (23) between these cells and to possible multiple inhibition (4) produced by the release of AcChoEase.

DISCUSSION

The above results lead us to conclude that the AcChoEase that is contained in subcisternal systems of substantia nigra may be released from these dendrites (see Fig. 2) by local calcium following the activation of voltage-sensitive calcium conductances in these processes (5). Because such release can be evoked by KCl in the presence of TTX, one can, therefore, conclude that, as shown in Fig. 2, local entry of calcium may produce exocytosis from subcisternal systems (24). The present results indicate, therefore, that calcium-dependent release in neurons is not limited to the presynaptic terminal or to conventional synaptic transmission in dendrites (25), but rather that a second calcium-dependent exocytosis mechanism may be at work. Indeed, rather than utilizing synaptic vesicles, the data suggest that release may occur directly from the larger intracellular reservoirs known as subcisternal systems that could fuse with plasma lemmal membrane (Fig. 4) during calcium entry into the dendrites as proposed (5, 8, 24). This rather slow release of protein of high molecular weight may then be an intermediate mechanism between the very rapid vesicular release of transmitter from presynaptic boutons and the release from large vesicles of the massive granules observed in non-neuronal secretory systems (Fig. 4).

We thank Dr. Paul May for performing the injections of horseradish peroxidase. This research was supported by grants from the National Institute of Neurological and Communicable Diseases and Stroke (NS13742) to R.R.L. and the Medical Research Council (G8319613N) to S.A.G.

1. Henderson, Z. & Greenfield, S. A. (1984) *J. Comp. Neurol.* **230,** 278–286.
2. Chubb, I. W., Goodman, S. & Smith, A. D. (1976) *Neuroscience* **1,** 57–62.
3. Greenfield, S. A., Grunewald, R. A., Foley, P. & Shaw, S. G. (1983) *J. Comp. Neurol.* **214,** 87–92.
4. Greenfield, S. A., Stein, J., Hodgson, A. J. & Chubb, I. W. (1981) *Neuroscience* **6,** 2287–2295.
5. Llinás, R., Greenfield, S. A. & Jahnsen, H. J. (1984) *Brain Res.* **294,** 127–132.
6. Greenfield, S. A., Chubb, I. W., Grunewald, R. A., Henderson, Z., May, P., Portnoy, S., Weston, J. & Wright, M. C. (1984) *Exp. Brain Res.* **54,** 513–520.
7. Nieoullon, A., Cheramy, A. & Glowinski, J. (1977) *Nature (London)* **266,** 375–377.
8. Greenfield, S. A. (1985) *Neurochem. Int.* **7,** 887–901.
9. Cuello, A. C., Romero, E. & Smith, A. D. (1981) *J. Physiol. (London)* **312,** 14P.
10. Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, K. M. (1961) *Biochem. Pharmacol.* **7,** 88–95.
11. Pavari, R., Pecht, I. & Soreq, H. (1983) *Anal. Biochem.* **133,** 450–456.
12. Israel, M. & Lesbats, B. (1981) *Neurochem. Int.* **3,** 81–90.
13. Llinás, R. & Sugimori, M. (1980) *J. Physiol. (London)* **305,** 171–195.
14. Roswell, D. F. & White, E. H. (1978) *Methods Enzymol.* **57,** 409–423.
15. Greenfield, S. A. (1984) *Trends Neurosci.* **7,** 364–368.
16. Silver, A. (1974) *The Biology of the Cholinesterases* (North–Holland, Amsterdam), pp. 355–388.
17. Massey, S. & James, T. A. (1978) *Life Sci.* **23,** 345–350.
18. Llinás, R. & Yarom, Y. (1986) *J. Physiol. (London)* **376,** 163–182.
19. Rotundo, R. L. & Fambrough, D. M. (1980) *Cell* **22,** 583–594.
20. Greenfield, S. A., Cheramy, A., Leviel, V. & Glowinski, J. (1980) *Nature (London)* **284,** 355–357.
21. Greenfield, S. A. & Shaw, S. G. (1982) *Neuroscience* **7,** 2883–2893.
22. Weston, J. & Greenfield, S. A. (1986) *Neuroscience* **17,** 1079–1088.
23. Grace, A. A. & Bunney, B. S. (1982) *Neuroscience* **10,** 333–348.
24. Llinás, R. (1979) in *The Neurosciences: Fourth Study Program*, eds. Schmitt, F. O. & Worden, F. G. (MIT Press, Cambridge, MA), pp. 555–571.
25. Shepherd, G. M. (1971) *Brain Res.* **32,** 212–217.