Eduardo De Robertis

Pioneer in the Molecular Approach to Synaptic Function

Unlike the other contributors here, I never worked personally or collaborated with the late Eduardo, but I was in a position to follow his scientific progress over much of his career and to know him as both a scientist and a personality. It is indeed a pleasure and an honour for me to provide an appraisal of his contributions to neuroscience, written from outside his own laboratory, outside his circle of collaborators, outside his own country, and from a longterm perspective.

Eduardo De Robertis was responsible for many original contributions to biochemistry, physiology, and neuroscience. I shall dwell here only on those that are on the topics of synapses and neuroreceptors, which were his major, long-lasting interests. It must be recalled that those studies started when there was universal ignorance of both of these topics. Synapses were mysterious black boxes, little more understood than in the completely classical and anatomical descriptions of Cajal and of Sherrington. Receptors were a pharmacological abstraction, of unknown composition and properties. For us today, comfortable with our powerful tools for their study (specific radioligands, cloned and expressed receptor subtypes, single-channel recordings, and so on), it is sobering to imagine ourselves back in the misty pharmacological world of the 1950s. Without such tools, false starts and mistaken pursuits in the understanding of synapses and receptors were highly probable. Most work from that era fell into such traps and has disappeared totally from sight, leaving no influence on the field. Not so with Eduardo De Robertis, who had, above all, foresight and a powerful biological imagination.

Let me illustrate this by revisiting some of his achievements:

(1) In 1953, Eduardo De Robertis (EDR) with H. S. Bennett sought and found the synaptic vesicles. These were seen, in an early application of the electron microscope (EM), as spheres of 500 Å diameter, apposed to the synaptic membrane. Four other EM laboratories of the time subsequently saw such "granules," but it was EDR who named them "synaptic vesicles" (De Robertis and Bennett, 1954) and interpreted them as the morphological basis for the quanta of transmitter that had recently been found by Fatt and Katz (1952). EDR pointed out their association with the presynaptic membrane and made the proposal (which has been pursued universally ever since) that they flow to it, fuse with it and liberate into the synaptic cleft a transmitter that they contain

(De Robertis and Bennett, 1955). EDR went on to obtain experimental support for this concept, by introducing the methods (which subsequently became standard) of depletion of the vesicles by stimulation in a frequency-dependent manner and of deafferentiation to cause the vesicles to disappear. Many, including Katz himself, initially expressed themselves as skeptical about his vesicles—a common experience for De Robertis—but soon after they became converted to his interpretation (del Castillo and Katz, 1956).

Further, the paper of EDR from the late 1950s introduced the still-valid and important concepts of the synaptic densities and the synaptic web and the variation of vesicle type with the synapse and transmitter type.

(2) In 1959 EDR introduced the technique of homogenization and subcellular fractionation of brain tissue, again an endeavor that was to provoke much skepticism. This, in fact, proved to be the most fruitful route to take with the tools of that time. By it, he moved the study of synapses from the morphological to the biochemical plane and started a new era of neurochemistry. He was seeking to see, and duly saw, his beloved vesicles in EM preparations of the fractions obtained. Further, both he and Victor Whittaker independently recognized therein isolated "pinched-off" nerve endings, in the form of the synaptosomes. EDR clearly recognized at once the potential of this, which could easily have been dismissed as an uninteresting artifact (as indeed it was dismissed, by many others). He and Whittaker brought different contributions to the exploitation of this discovery. From the start in EDR's laboratory, EM analysis was applied in a way new to biochemistry, enabling them to identify components of the nerve terminal. For example, they identified by biochemical markers, the fractions obtained in centrifugation, released the synaptic vesicles by osmotic shock, and monitored this process in the EM (De Robertis e al., 1962a). Quantitative cytochemistry was developed to interpret the organization revealed in the nerve terminal. Whittaker's group introduced isotonic negative staining to facilitate the EM identification, and determined the acetylcholine content of the vesicle purified from Topedo electric organ.

The keen competition between the two laboratories (or, more accurately, between their directors) was a prominent feature of the world of neurobiology in the 1960s and persisted long after that original excitement. It was, for example, much contested who was the first to characterize synaptosomes or to demonstrate unequivocally that their vesicles carry the neurotransmitter molecules. However, from our wider perspective this question of priority in a given year has no long-term interest. It is clear now that both laboratories made important advances. Victor Whittaker, as with EDR, certainly attracted to his laboratory over that period a talented group of scientists, most of whom later went on to make, independently, a considerable impact on one or another field of neurobiology; it would have been surprising if the initial contributions of that team had not also been excellent. The fierce and sustained competition probably, in fact, spurred both groups on even more, so that we owe a more rapid advance to it. However, we can see clearly now that the achievement of the Buenos Aires laboratory in this field was indeed major and of lasting significance. From 1957 (De Robertis and Vaz Ferreira, 1957) onward, starting with observations on the fusion of adrenal chromaffin granules with the cell membrane, it was EDR who was principally responsible for introducing the concepts of the vesicular storage of transmitters in general and their release by cell membrane fusion, i.e., the process of exocytosis. This was an original and daring theory and for a long time highly controversial: The actual events involved are still in dispute. The work of his laboratory over this period contributed substantially to the experimental basis for this concept.

As an example, the full description in 1962 of synaptosomes (as they were later named) by De Robertis et al. (1962b) became a citation classic. The applicability of these particles to a vast range of studies on synaptic structure, function, and pathology was found to be enormous, and to this day they remain a valuable tool for this field.

The fact that EDR initially predicted and fostered this applicability is testimony to his great biological insight.

(3) EDR extended the synaptic vesicle observations to sympathetic noradrenergic synapses (De Robertis and Pellegrino de Iraldi, 1961), and subsequently to other transmitters. His generalization of the concept of exocytosis, which later received support from detailed analysis made by others, has indisputably had a great impact on neurobiology.

Let us turn now to the studies of EDR and his colleagues on synaptic receptors. As early as 1964 he had shown the binding of cholinergic drugs to synaptic membranes. He had recognized the osmiophilic synaptic densities of the EM and showed how it was possible to isolate them. The postsynaptic membranes could be separated from these and were associated with the binding of cholinergic drugs (De Robertis et al., 1967). By his emphasis on isolating the components responsible for the binding of hexamethionum and curare he stimulated the new biochemical approach to receptor function. At that time there were only his group and two other laboratories that had begun the search for the isolated receptor. In 1968, EDR and coworkers (Wald et al., 1968) showed that an antibody prepared against the isolated brain postsynaptic membrane blocks cholinergic transmission, a further and valid stimulus to that search. Dr Robertis and Fiszer (1970) extracted *Electrophorus electroplax* membranes to remove the acetylcholinesterase, but left fully intact in the membrane the binding of the cholinergic radioligands, thereby demolishing the theory (still debated up to that time) that the ACh receptor is part of acetylcholinesterase.

From 1967 onward, EDR concentrated on the purification of the ACh receptor. From a particularly early time he postulated that the receptors have a major part of their structure traversing the membrane. Logically proceeding from this, he introduced hydrophobic chromatography to membrane protein fractionation. However, from this point his work rapidly became highly controversial. EDR placed emphasis on extracting the membranes with chloroform-methanol. This solubilization was described in 1967 (De Robertis et al., 1967), whereas solubilizations of the active receptor in aqueous nondenaturing detergents were reported by several other groups in 1970–1971. Never an investigator to follow others, EDR developed further his organic solvent approach and led his laboratory to isolate a proteolipid as the ACh receptor, and similar components as several other receptors. For about 15 years he fought a characteristically vigorous battle to defend his model of an assembly of several rod-shaped proteolipids, enclosing a channel held in the membrane by their extensive lipid attachments and their hydrophobic domains, and with a transmitter binding site on the projecting face of each. This model was in most features not basically different from that which was developed by other leading workers in this field, but history has shown that the material finally purified by EDR using an additional stage of affinity chromatography in the organic medium was not the receptor. This was a period of intense competition between a number of major groups for the purification and characterization of the ACh receptor; EDR, working in Argentina at that period under great difficulties, was isolated and handicapped in comparison.

With the benefit of hindsight, we can see that the course that EDR followed, in non-aqueous media, could not lead to a pure protein that had the properties of the native receptor in the original water-surrounded membrane. I was, at an early stage, one of those who advised him in a friendly manner that the binding and reinstitution evidence that he had reported (both on the ACh receptor and on an adrenergic receptor from spleen) showed considerable anomalies, e.g., in the low drug and toxin affinities, the retention of the bound [^{14}C]ACh in the protein peak during chromatography, the depolarization of an artificial membrane containing the receptor by curare as well as by ACh or (for the spleen product) curiously, by noradrenaline, and so on. Yet the actual observations on the ACh receptor were robust, in that they could be reproduced by other

laboratories (e.g., Izumi and Freed, 1974; Donnellan and Cattell, 1975; Wu et al., 1977).

In 1975 two scientists who had been working with EDR, Pancho Barrantes and George Lunt, went to Jean-Pierre Changeaux's laboratory in Paris to make a critical test of the "proteolipid ACh receptor" by the methods in use there; that this was done is a testimony to the scientific integrity of the Buenos Aires laboratoy. The comparison showed (Barrantes et al., 1975) that the proteolipid isolated from electroplax was not the ACh receptor as purified in detergent and characterized definitively from electroplax membranes by the Paris group. This was demonstrated by

1. The inability of an antibody to the proteolipid to recognize the detergent-purified receptor;
2. The inability of toxin to bind to the proteolipid, as measured in the standard way in aqueous detergent medium; and (most conclusively)
3. The failure of the chloroform-methanol method to extract from the membranes the receptor as revealed by its prior affinity-labeling or by the subunit content as seen in SDS/gel electrophoresis (Barrantes et al., 1975,1976).

Also, H. Loh and coworkers in 1977–1978 showed that in the fraction purified by EDR triphosphoinositol becomes concentrated along with some protein and that this lipid in pure form can reproduce the binding behavior that had been observed, with the same relative ligand affinities (Wu et al., 1977; Cho et al., 1978).

It is easy to understand now how EDR came to be misled by his observations. None of us in the receptor field can say that we never drew a wrong conclusion from our experimental observations. He showed all of the evidence, sufficiently so that at a later stage it can clearly be seen where the problems arose. Undeterred, he went on with his coworkers to make a series of studies on the GABA/benzodiazepine receptors in the brain, on their heterogeneity and regulation, which remain valuable in the field. This included evidence for the existence in the brain of natural regulators of the benzodiazepine site, supported recently by the findings of others: This work is reviewed by J. H. Medina, elsewhere in this volume.

In assessing this latter part of his work now we should discern through the controversies several enduring features. He pursued from the very earliest stage the goal of obtaining a pure receptor molecule. He pushed ahead with this endeavor before really adequate methods were available. He introduced the system of reconstituting an isolated receptor in an artificial bilayer membrane and measuring conductance changes evoked by agonists (Parisi et al., 1971); this approach was of great importance in the later characterization by others of the ACh and other receptors. EDR predicted endogenous modulatory compounds for the GABA/benzodiazepine receptor to exist and to be important in anxiety and memory, and encourage the program that identified benzodiazepines and β-carbolines in the brain, a question that is still an open one of worldwide interest.

In conclusion, Eduardo De Robertis is now seen as a pioneer in neurobiology. Some of his initiatives led to enduring achievements and had a tremendous influence on the field as it is today. He was not to be successful in all of his endeavors, but we must remember that he worked in the face of tremendous difficulties. His tremendous energy and tenacity were legendary. The political and personal pressures under the Peron regime and other troubled times in Argentina are difficult for us, in our privileged situations elsewhere, to truly comprehend. He had to work with poor financial and physical resources. Both geography and politics together led to the isolation of his laboratory from the mainstream of neurobiology. Despite all of this, he became a major figure in that field. Despite it all, he was able to create a school of Argentinean neuroscientists—many of whom had to leave their homeland—whose accomplishments will testify to that influence. In remembering him here, our thoughts are drawn automatically to the sentiment of Ecclesiasticus:

"Let us now praise famous men..."

References

Barrantes, F. J., Changeux, J.-P., Lunt, G. G. and Sobel, A. (1975) *Nature* **256,** 325–327.

Barrantes, F. J., Changeux, J.-P., Lunt, G. G. and Sobel, A. (1976) *Nature* **259,** 605–606.

Cho, T. M., Cho, J. W. and Loh, H. H. (1978) *Proc. Natl Acad. Sci. USA* **75,** 784–788.

del Castillo, J. and Katz, B. (1956) *Prog. Biophys.* **6,** 122–170.

De Robertis, E. and Bennett, S. H. (1954) *Fed. Proc.* **13,** 35.

De Robertis, E. and Bennett, S. H. (1955) *J. Biophys Biochem. Cytol.* **1,** 47–58.

De Robertis, E. and Fiszer, W. (1970) *Biochim. et Biophys. Acta* **219,** 388–397.

De Robertis, E. and Pellegrino de Iraldi, A. (1961) *Anat. Rec.* **139,** 299.

De Robertis, E. and Vaz Ferrieira, A. (1957) *E:cp. Cen Res.* **12,** 568–574.

De Robertis, E., Azcurra, J. M. and Fiszer, S. (1967) *Brain Res.,* **5,** 45–56.

De Robertis, E., Rodriguez de Lores Arnaiz G., and Pellegrino de Iraldi, A. (1962a) *Nature* **194,** 794–795.

De Robertis, E., Pellegrino de Iraldi, A., Rodriguez de Lores Arnaiz, G., and Salganicof, L (1962b) *J. Neurochem.* **9,** 23–35.

Donellan, J. F. and Cattell, D. (1975) *Biochem. Soc. Trans.* **3,** 106–109.

Fatt, P. and Katz, B. (1952) *J. Physiol.* **117,** 109–128.

Izumi, F. and Freed, S. (1974) *FEBS Lett.* **42,** 319–322.

Parisi, M., Rivas, E., and De Robertis, E. (1971) *Science* **172,** 56.

Wald, F., Mazzuchelli, A., Lapetina, P. G. and De Robertis, E. (1968) *Exp. Neurol.* **21,** 336–345.

Wu, Y. C., Cho, T. M. and Loh, H. H. (1977) *J. Neurochem.* **29,** 589–592.

Eric A. Barnard
MRC Molecular Neurobiology Unit,
MRC Centre,
Hills Road, Cambridge CM2 2QH, UK
Present Address:
Molecular NeurobiologyUnit,
Division of Basic Medical Sciences,
Royal Free Hospital School of Medicine,
Rowland Hill Street, London NW3 2PF