# LOCALIZATION OF THE CHOLINERGIC RECEPTOR PROTEIN IN *ELECTROPHORUS* ELECTROPLAX BY HIGH RESOLUTION AUTORADIOGRAPHY

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## 1. Introduction

There is now considerable evidence that the α-toxins from the venom of a variety of snakes [1] constitute, within a given set of experimental conditions, highly specific and often irreversible reagents of the cholinergic (nicotinic) receptor site of the vertebrate neuromuscular junction and fish electric organ. This property has been used to characterize the receptor protein *in situ* [2] and in solution [3–5].

In a previous work [6], using the α-isotoxin purified from the venom of *Naja nigricollis* and an anti-serum directed against this toxin, we were able to demonstrate by immunofluorescence the almost exclusive localization of the receptor protein on the innervated face of a single electroplax from *Electrophorus electricus.* However, since this face receives several thousands of nerve terminals disposed over its entire surface, it appeared difficult to obtain a fine localization of the cholinergic receptor with the light microscope and, more particularly, to distinguish between labeling on membrane areas which underlie the nerve terminals (subsynaptic areas) from labeling on extrasynaptic areas. The problem was reinvestigated by electron microscopy.

In this paper, we present results obtained by high resolution autoradiography with a heavily tritiated α-toxin from *Naja nigricollis* (14.8 Ci/mmole) [7].

We first confirm that the [$^3$H] α-toxin binds exclusively to the innervated (caudal) side of the eel electroplax and further show that the α-toxin binds both between the synapses and under the nerve terminals. However, taking into account the particular stereology of the cytoplasmic membrane, it appears that the density of α-toxin molecules bound per unit area of actual membrane surface is approx. 100 times larger under the synapses than outside the synapses. Absolute values of this density and of the total number of receptor sites per cell are given.

## 2. Materials and methods

### 2.1. *Electric organ*

Slices of the Sachs' organ were cut off from live *Electrophorus electricus,* and single cells were dissected following the method of Schoffeniels and Nachmansohn [8].

### 2.2. *α-toxin*

Pure α-isotoxin was prepared by Boquet et al. [9, 10] from crude venom of *Naja nigricollis* by the method of Karlsson et al. [11], except that Biorex 70 was used instead of Amberlite IRC 50. The toxin was tritiated by the method of Menez et al. [7]. The labeled material, which presented the same pharmacological and biochemical properties as the native toxin, had a specific radioactivity of 14.0 Ci/mmole, (0.7 mg protein/ml) or 14.8 Ci/mmole (0.408 mg protein/ml).

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2.3. *Counting of total number of receptors per cell*

Single cells were incubated in 0.5 ml of 1.6 μg protein/ml of [$^3$H] α-toxin for 90 min in Ringer's solution (R) at room temp. and washed overnight in 100 ml of R at 4°. Two controls were run in parallel. First, cells were exposed to 10 μg protein/ml of unlabeled α-toxin for 90 min and washed for 30 min prior to incubation in 1.6 μg/ml [$^3$H] α-toxin. Second, a 50-fold excess of unlabeled toxin was added to the solution of tritiated toxin in which the cell was dipped. Labeling time and washing were the same as above. Finally, it was checked that exposure at higher concentrations of [$^3$H] α-toxin and for longer periods of time did not change the number of toxin molecules bound. The labeled cells were finally dissolved in 0.5 ml of hyamine hydroxide, supplemented, in some cases, with 0.5 ml of $H_2O_2$. The quenching by the cell was negligible.

2.4. *Autoradiography for light microscopy*

Single dissected cells were sectioned in a cryostat at −20°. The frozen sections, 16 μ thick, were collected on glass slides which were incubated at room temp. for: 1) 60 min in 1.3 μg/ml solution of tritiated α-toxin (14.0 Ci/mmole) made in 0.1 M Na phosphate buffer pH 7.1 (P), 2) 20 min in P, 3) 30 min in 0.2% neutral formaldehyde in P, 4) 20 min in P.

Before exposure to the tritiated toxin, some slides were preincubated 10 min in $10^{-4}$ M *p*-(trimethyl ammonium)benzene diazonium fluoroborate (TDF) in phosphate buffer pH 7.1, washed rapidly in R and treated by 1.0 M histidine to block unreacted TDF, and then submitted to the normal labeling procedure. After labeling, slides were covered with $L_4$ Ilford Emulsion and stored for one month at 4°. Acetylcholinesterase activity was revealed on the same slides by the method of Koelle modified by Tsuji [12].

2.5. *Autoradiography for electron microscopy*

Single cells were incubated successively: 1) 90 min at room temp. in 1.6 μg/ml tritiated α-toxin (14.8 Ci/mmole) in R, 2) 30 min in R, 3) 30 min in 0.2% neutral formaldehyde in R, 4) 30 min in R.

Controls were run in parallel where, prior to exposure to the tritiated α-toxin, the cell was exposed for 60 min to a 10 μg/ml solution of unlabeled α-toxin in R medium and washed for 30 min in R. Labeled cells were then fixed in 4% glutaraldehyde post-fixed in 1% $OsO_4$ and stained in 1% uranyl acetate. Samples were dehydrated in acetone, embedded in Vestopal and cut on a Reichert ultramicrotome. Slices were stained 4 min with lead citrate and carbonated before being covered with an $L_4$ Ilford Emulsion (for detailed description of the method see [13]. The slices were stored 106 days in the dark before revelation with microdol and observation on a Siemens Elmiskop 101.

## 3. Results and discussion

3.1. *Autoradiography by light microscopy*

Fig. 1 (A and B), shows the distribution of grains observed after labeling of an electroplax slice with [$^3$H] α-toxin following the procedure described in Methods. In agreement with our previous findings [6], the grains are located almost exclusively on one face of the electroplax. This face stains for acetylcholinesterase and thus is the innervated face. That the tritiated material is indeed bound to the cholinergic receptor site is confirmed by the observation that when the slices are treated by an irreversible cholinergic antagonist, TDF [14], prior to [$^3$H] α-toxin exposure, grains are no longer detectable on the innervated side (fig. 1B). This finding confirms our earlier observations carried out *in vitro* with the same toxin and excitable membrane fragments: the α-toxin is, in the present experimental conditions and with this particular biological material, a highly specific label of the cholinergic receptor site [3, 5]. Although we did not use this method to measure the actual number of toxin molecules bound, we might nevertheless say that at least 99% of the grains did correspond to [$^3$H] α-toxin associated with the cholinergic receptor protein, i.e., that the non-specific background lay below 1%.

3.2. *Autoradiography by electron microscopy*

In fig. 1 (C and D) are presented radioautographs of the innervated face of an electroplax labeled with [$^3$H] α-toxin after exposure of the emulsion for 106 days. They clearly show that silver grains are present both between the synapses and under the nerve terminals but with a strikingly different density. Grains

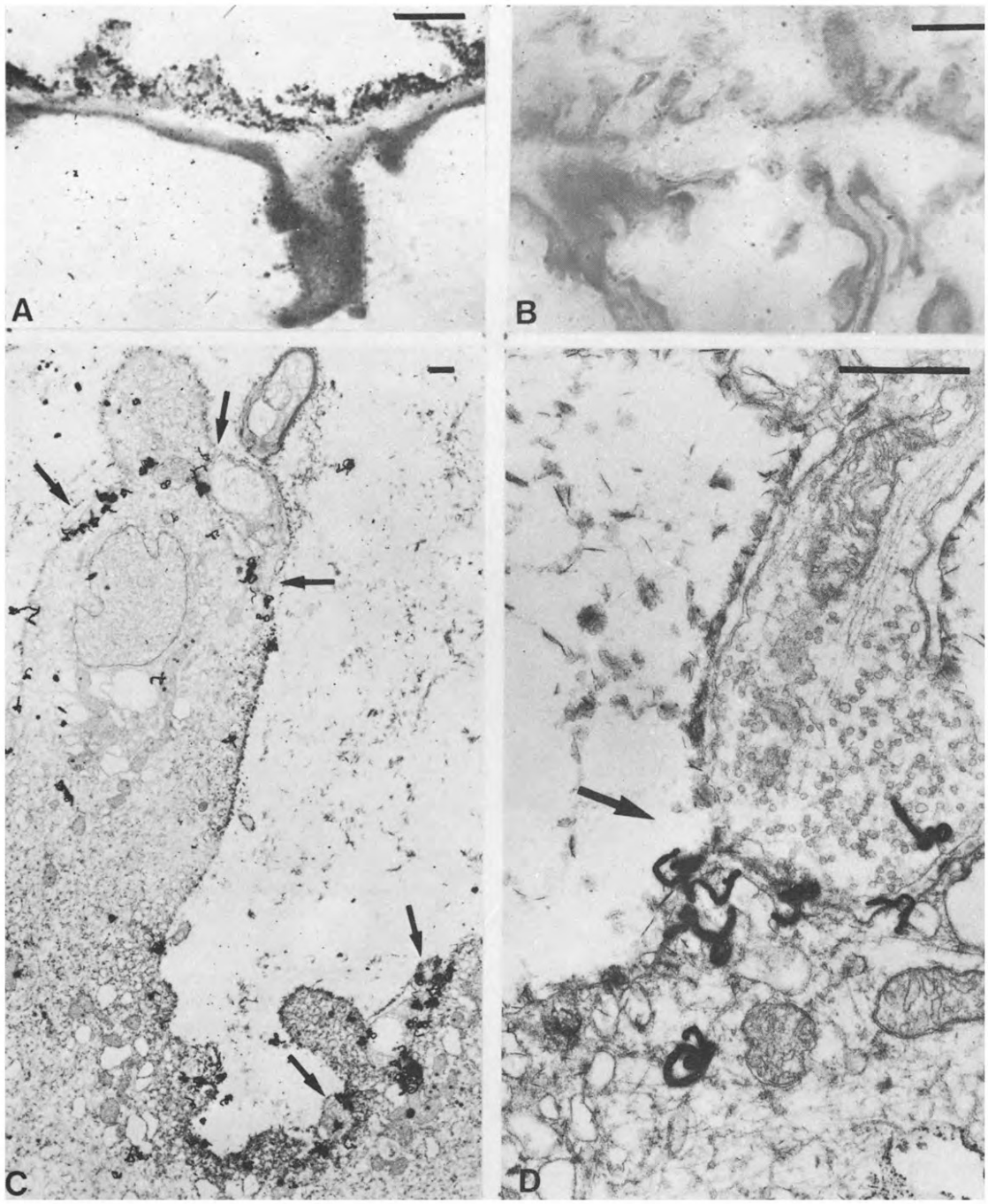


Fig. 1. Autoradiography of an electroplax from *Electrophorus electricus* labeled by [$^3$H] α-toxin from *Naja nigricollis*. Light microscopy; B) control of A): prior to the incubation with the tritiated toxin the slide was treated by an irreversible cholinergic antagonist: TDF. Sections were oriented in the same manner after acetylcholinesterase was revealed by the Koelle's method. The bar measures 20 μm. C) and D) Electron microscopy on the innervated membrane. The bar measures 1 μm. The arrows indicate the synaptic areas in A), the nerve terminals in B).

Table 1
Number of [$^3$H]α-toxin binding sites per surface area of cytoplasmic membrane.

| | | Grains per $\mu$ of cell section* | [$^3$H]α-toxin molecules bound per $\mu^2$ of membrane surface** |
|---|---|---|---|
| Innervated face | Subsynaptic | 3.10 ± 0.9 | 33,000 ± 10,000 |
| | Extrasynaptic | 0.34 ± 0.3 | 400 ± 300 |
| Non-innervated face | | 0.09 ± 0.08 | 39 ± 20 |

* Uncorrected for microvillosities.
** Taking into account an increase of cell surface by the microvillosities of 1.15-fold in the subsynaptic areas, of 10-fold in the extra-synaptic areas of the innervated face, and of 30-fold on the non-innervated one.

are more numerous in the subsynaptic areas than in the extrasynaptic ones. Similar autoradiographs made with the non-innervated face of the same electroplax showed, as expected from the results obtained by light microscopy, very few grains.

In order to obtain quantitative information on the absolute number of toxin molecules bound per cell and per area of excitable membrane, a statistical distribution of the grains on the surface of the cytoplasmic membrane was made. 943 silver grains were counted on a series of 15 photographs from two different cells labeled under the same conditions.

The stereology of the electroplax is particularly complex. On large villosities or papillae, which protrude approx. 10 $\mu$ to 20 $\mu$ on the surface of the innervated face and two or three times more on the non-innervated face, are superimposed microvillosities which consist of cylinders 0.18 $\mu$ in diameter and 1 to 4 $\mu$ in depth (average: 1.7 $\mu$). The density of microvillosities in extra-synaptic areas lies around 10 per $\mu^2$ of cell external surface. Under the nerve terminals, in contrast with what is seen at the neuromuscular junction, few microvillosities open into the synaptic cleft and the subsynaptic membrane follows closely the presynaptic membrane. Starting from a cell of average size (for instance of 6 mm × 2 mm), observed from its innervated side, the increase of surface on that side due to large villosities is approx. 3 ± 1-fold, the increase due to the microvillosities approx. 10 ± 2-fold in extrasynaptic areas and 1.15-fold in subsynaptic ones (taking 1.7 $\mu$ as mean length of the microvillosities), yielding a total average surface increase of 30 ± 10-fold. On the non-innervated side the surface increase due to microvillosities lies around 30-fold.

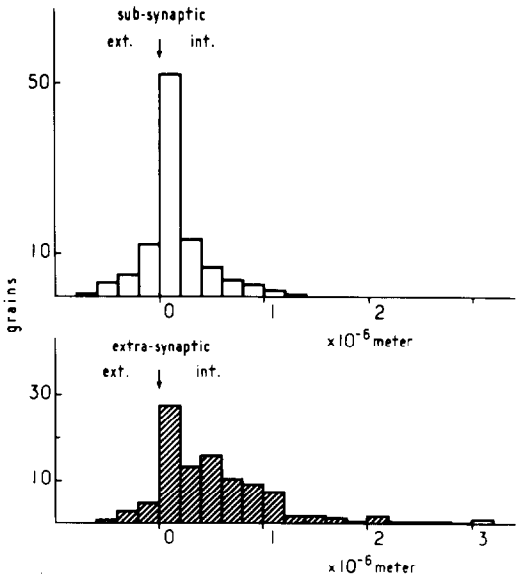


Fig. 2. Distribution of silver grains as a function of distance from the basement membrane in subsynaptic and extrasynaptic areas.

In order to make objective measurements with the smallest number of stereological assumptions, we counted the grains in a band of about 1600 × 2 $\mu$ on both sides of the main external contour of the electroplax underlined by the basement membrane. The data summarized in table 1 are expressed as grains per length of contour section measured with a curvimeter on the photographs. In other words,

the surface increase due to the large villosities was taken into account but not that due to the microvillosities. The number of toxin molecules per surface area was calculated from the number of grains in the following manner. First, from the thickness of the section (0.05 $\mu$), from the exposure time (106 days) and from the grain counts, a number of grains per min and per $\mu^2$ was estimated. Then, in separate experiments, the number of grains corresponding to a given number of dpm was measured with tritium labeled bacteria counted both by autoradiography and by direct scintillation counting. In agreement with Caro's results [13], the yield of grains to [$^3$H]dpm was close to 20%. Then an absolute number of molecules was calculated taking 14.8 Ci/mmole as the specific radioactivity of the bound [$^3$H]$\alpha$-toxin. Granted these assumptions and the ones previously made on the stereology, then we found 33,000 ± 10,000 [$^3$H]$\alpha$-toxin molecules per $\mu^2$ of subsynaptic membrane and approx. 100 times less between the synapses, the density on the non-innervated side being still 10 times smaller (table 1).

To confirm that grains counted in the extrasynaptic areas correspond to toxin molecules bound to the entire surface of the cytoplasmic membrane including microvillosities, a distribution of grains as a function of distance from the external contour or basement membrane was established. It shows (fig. 2) that the barycenter of the grain distribution lies approx. 0.5 $\mu$ inside the main contour of the cell section while this distribution is perfectly symmetrical across the boundary of the subsynaptic membrane. In the extra-synaptic areas the toxin seems thus to bind to the whole surface of the cytoplasmic membrane microvillosities included.

In the electroplax, as in muscle [15, 18], there exists a marked difference in receptor density per $\mu^2$ between extra- and subsynaptic areas indicating an heterogeneity of the cytoplasmic membrane in the innervated surface. We therefore tried to see if such a heterogeneity could be demonstrated *in vitro* on homogenates of electric organ. Fig. 3 shows the results of the centrifugation of an homogenate in a sucrose gradient where two characteristic properties of the innervated membrane were followed: toxin binding and acetylcholinesterase activity. Interestingly, membrane fragments which preferentially bind the $\alpha$-toxin appear to have, under the present experimental conditions, a density higher than those which contain the highest amounts of acetylcholinesterase activity. Further studies are in progress to clarify the relationships between acetylcholinesterase and the cholinergic receptor protein in these membrane fragments.

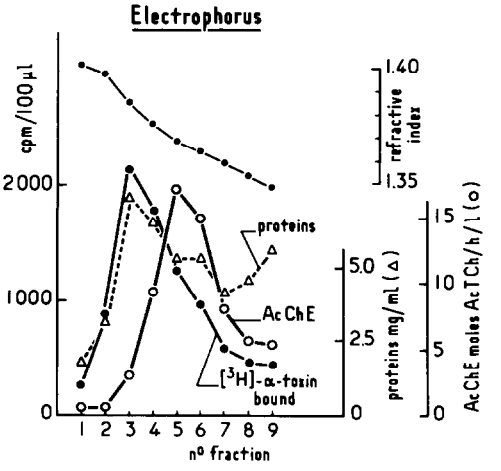


Fig. 3. Compared distribution of [$^3$H]$\alpha$-toxin and acetylcholinesterase in a sucrose density gradient after ultracentrifugation of an homogenate of *Electrophorus electricus* electric organ. The gradient contained from the bottom to the top: 1.0 ml of 1.6 M, 1.4 M, 1.1 M and 0.4 M sucrose and 12 ml of low speed $S_1$ supernatant prepared as previously described [20] except that the homogeneization was done in the distilled water instead than in 0.2 M sucrose. Labeling was done in the crude extract by adding 0.4 $\mu$g of [$^3$H]$\alpha$-toxin to approx. 70 ml of crude homogenate 30 min before high speed centrifugation. The tube was centrifuged 4.5 hr at 25,000 rpm in a SW25/3 rotor of a Beckman Model L ultracentrifuge.

### 3.3. *The number of [$^3$H]$\alpha$-toxin binding sites per cell*

Assuming a 10-fold increase of surface by microvillosities, the area of cytoplasmic membrane under the nerve terminals constitutes approx. 1.6 ± 1% of the total surface of the innervated face cytoplasmic membrane. Thus approx. 60% of the total number of toxin molecules bound to the innervated face bind to the subsynaptic areas and 40% to the extra-synaptic ones. The total number of toxin molecules per cell of an apparent surface of 12 mm$^2$ (with the naked eye) and a wet weight of 10 to 30 mg is then calculated from these numbers to be $(3 \pm 2) \times 10^{11}$. Interestingly, this value falls exactly in the range of num

Table 2
Total number of [$^3$H]α-toxin binding sites per single isolated electroplax.

| | Wet weight (mg) | (cpm) | Number of [$^3$H]α-toxin molecules bound specifically |
|---|---|---|---|
| Controls | | | |
| Exposure to 10 μg/ml native α-toxin before labeling | 9.2 | 339 | - |
| | 18 | 437 | - |
| Labeling in [$^3$H]α-toxin diluted with native α-toxin | 10 | 240 | - |
| Experiment | 14 | 1804 | $1.62 \times 10^{11}$ |
| | 18.4 | 2794 | $2.7 \times 10^{11}$ |
| | 20 | 2740 | $2.64 \times 10^{11}$ |

The labeling procedure is described in "Methods".

bers found by counting directly the whole cell after dipping in a solution of tritiated toxin (see methods and table 2). These values are in agreement with those presented by Karlin et al. [19] with electroplax labeled with a cholinergic affinity reagent.

3.4. *Comparison with other systems*

The particularly high density of toxin binding sites in subsynaptic areas as opposed to extrasynaptic ones has already been found by various authors in muscle [15–18]. The absolute densities of α-toxin binding sites per $\mu^2$ in subsynaptic areas fall in the same range as ours, although the number proposed by Miledi and Potter [16] (100,000 per $\mu^2$ in frog muscle) appears significantly higher, and those given by Barnard et al. (12,000 per $\mu^2$ in mouse diaphragm) somewhat smaller. The numbers found *in vitro* by Kasai and Changeux [20] in excitable microsacs fall in the range of those measured by autoradiography in extrasynaptic areas of the innervated surface. It is very likely that the membrane fragments they used came from these areas which, as mentioned above, constitute in *Electrophorus* approx. 98% of the total surface of the electroplax innervated membrane. If we assume now that the smallest unit of the receptor protein to which the α-toxin binds has a molecular weight of 50,000 [5, 19] and a density of 1.37, then the maximal number of receptor protein molecules which can fit in a single layer is approx. 40,000 per $\mu^2$, a number close to the 33,000 per $\mu^2$ we found in subsynaptic areas, but smaller than the 100,000 reported by Miledi and Potter [16]. This would mean that in *Electrophorus* electroplax the subsynaptic membrane surface is occupied almost exclusively by the receptor protein. As already mentioned by Barnard [21], there would be little room left for acetylcholinesterase unless one assumes that the enzyme is not integrated in the membrane framework in the same manner as the cholinergic receptor [22–24]. Another alternative, at least in the case of the electric organs, would be that the stoichiometry of acetylcholinesterase to the receptor is not the same under the synapses and outside the synapses. Finally it should be emphasized that some of the assumptions made for the calculations might have to be revised.

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