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POSTSYNAPTIC EFFECTS OF METHOCTRAMINE AT THE MOUSE NEUROMUSCULAR JUNCTION

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Abstract—Functional studies were performed to evaluate the effects of methoctramine at the neuromuscular junction of the mouse. The presynaptic control of acetylcholine release and the postsynaptic activation of the nicotinic receptor have been analysed by means of the extracellular recording with an EPC7 Patch Clamp amplifier. This electrophysiological method revealed a dose-related inhibitory effect of methoctramine on the studied parameters. The dramatic reduction of the kinetics of the quantal conductance change indicates an action at the postsynaptic level. The effects of methoctramine have been compared with those of the muscarinic agonist oxotremorine. Concentration/response curves for the two drugs were obtained and the apparent EC_{50} values calculated. The effects of oxotremorine were not antagonized by 1 μM methoctramine.

These findings suggest an interaction of some muscarinic agents on the postsynaptic receptor–ion-channel complex at the mouse neuromuscular junction.

Since Bulbring[6] first proved a facilitatory effect induced by atropine at the isolated diaphragm of rat, many studies focused their attention on the possible muscarinic control of acetylcholine (ACh) release. Although the muscarinic modulation of ACh release has been a matter of great controversy, the role of muscarinic presynaptically located receptors is now well outlined. A controversy arose in recent years as to the detection method which better demonstrates presynaptic muscarinic modulation. If biochemical (overflow) or electrophysiological (functional) studies better contribute to the knowledge of the problem. Wessler[27] has dealt with the topics that have arisen on the modulation of the ACh release.

In the context of a larger ongoing electrophysiological study on the effects of some muscarinic agonists and antagonists, we show now data related to the M_2 muscarinic antagonist methoctramine. Methoctramine is a novel polymethylene tetraamine first synthesized in 1987[16] which showed different antimuscarinic action.[17] A recent scientific work proved the presynaptic antimuscarinic effect of methoctramine in the cerebral cortex of the rat and in the guinea-pig ileum Auerbach plexus.[24] Previous data indicate that methoctramine allosterically inhibits the muscarinic actions in the heart.[8] The present work describes the results obtained with an electrophysiological technique to study any pre- or postsynaptic action of methoctramine in the neuromuscular junction. Indeed, the possibility that some postsynaptic effects could be due to a specific action of the drug seems to be noteworthy. This hypothesis is strengthened by the reported data concerning the shortening of the time constant of decay of postsynaptic events by atropine and other related muscarinic agents.[2,7,9,12]

The experiments have been carried out on a mammalian neuromuscular junction. The effects of methoctramine have been compared with those of the cholinergic agonist oxotremorine.

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Abbreviations: ACh, acetylcholine; epc, end-plate current amplitude; mepc, miniature end-plate current amplitude; mepcf, miniature end-plate current frequency; RMP, resting membrane potential; τ, miniature end-plate current decay time.

EXPERIMENTAL PROCEDURES

Preparation

Left hemidiaphragm of mouse was prepared as described previously.[19,20] Briefly, Charles River mice of either sex, 30–40 days old, were killed and the left hemidiaphragm was dissected with a short length of phrenic nerve. The preparation was bathed in Krebs' solution of the following composition (mM): NaCl (133), KCl (4.7), $MgCl_2$ (1.2), $CaCl_2$ (7.2), NaH_2PO_4 (1.3), $NaHCO_3$ (16.3), glucose (7.8), pH 7.4, gassed with 95% O_2–5% CO_2 and maintained at room temperature (18–22°C). Concentrations of $MgCl_2$ (5–15 mM) and $CaCl_2$ (0.9–2 mM) were adjusted in order to abolish the twitch of the muscle fibre. The muscle was pinned on Sylgard resin, and placed on the stage of a Leitz inverted microscope. Endplates were visible by transillumination of the preparation with an optic fibre system. The preparation was equilibrated in saline for 30 min before starting the experiments.

End-plate signals

Spontaneous (mepcs) and evoked end-plate currents (epcs) were recorded with a focal extracellular pipette pressed against the edge of an end-plate.[22] The pipettes from Drummond 100-μl measuring pipettes of soft glass (1.4 mm) were pulled with a Kopf 700C Puller, fire polished with a Narishighe MF83 Microforge, and had a final tip diameter

ranging from 3 to 15 μm. Following filling with physiological solution the electrode was connected to a List LM EPC7 current to voltage converter. Pipette resistances were of 100–300 KΩ and seal resistances, measured after pressing the pipette against the sarcolemma, ranged between 300 and 600 KΩ. The Loose Patch Clamp method[23] enables a good control of the series resistance throughout the experiment. The technique enables a good voltage control of the muscle fibre, virtually clamped at the resting value. Indirect stimulation of the muscle was achieved by means of a suction electrode. Supramaximal square wave pulses of 0.1 ms duration at 2 Hz were applied by a stimulator (model 300A, WPI) via a stimulus isolator unit (model 305–2R, WPI). The signals were visualized on a Tektronix 5113 dual-beam storage oscilloscope and fed to the input stage of the analogue-to-digital converter of a computer system (HP486, Hewlett-Packard) which enables a fully automated analysis of the data. The decay phase of the mepcs was analysed to calculate the decay time constant. The elaboration was carried out on the mepc decay part that fell within 10–90% of its peak amplitude. The function used was:

$$I_t = I_o e^{-t/\tau}$$

where I_t is the current at time t, I_o is the current at time zero (i.e. the peak current) and τ is the time-constant of decay. Marquardt's least-squares method was used for the fitting.

The parameters were first recorded after each 10 ml of physiological solution flowed at a constant rate of 1 ml/min and repeated until stable values were obtained. The acquisition started with the analysis of the decay time of spontaneous mepcs (12–20 signals) with the calculation of the mean τ value. The acquisition of each block proceeded with the capture of mepcs over a 60-s time period for the evaluation of the spontaneous released quanta, i.e. the mepcs frequency (mepc). The amplitudes of the mepcs captured in each 60-s block were averaged and the mean peak value (mepc) recorded. Finally, the preparation was stimulated and 200 evoked epcs were acquired and averaged to obtain a mean epcs peak value (epc). Subsequent similar blocks were performed either after the flowing of 10 ml of the tested drug at the desired concentration or after a final wash out.

The resting membrane potential (RMP) was measured during the experiment by conventional intracellular microelectrodes (3 M KCl) connected to a P16 differential amplifier (GRASS).

Statistical analysis

Given data are expressed as the means ± standard deviations of the means. The half-maximal effective concentration (EC_{50}) of each drug was obtained performing a least-squares fit to the logistic function:

$$\text{Response} = 100 \times \frac{[\text{Conc}]^n}{[\text{Conc}]^n + EC_{50}^n}$$

where Response is the percentage effect, [Conc] is the drug concentration and n is the slope of the curve, i.e. the Hill coefficient.

The statistical significance was assessed by Student's t-test. P values <0.05 were considered as significant differences.

Materials

The drugs used were methoctramine (Tetrahydrochloride) and oxotremorine (Free base, Sigma). Methoctramine was kindly supplied by Prof. Melchiorre, University of Bologna, Italy. All the assayed drugs were prepared daily and diluted before the experiment.

RESULTS

Effects of methoctramine

Table 1 reports the effects obtained with methoctramine on the parameters analysed. Methoctramine showed a very striking blocking action on the epcs amplitude. This reduction was well correlated with the inhibitory effect observed for the time constant. The action of methoctramine was dose-dependent giving apparent EC_{50}'s values of 8.3, 11.8 and 89.3 μM for epc, τ and mepc with slopes of 0.98, 1.18 and 0.58, respectively. These data, and the effect induced by methoctramine on the frequency of the spontaneous release, proved a presynaptic inhibitory modulation of the drug. The action of methoctramine in this preparation was most potent when compared to the effect of other muscarinic antagonists[21] and its inhibitory effect on neuromuscular transmission was fully complete at 20–30 μM concentrations. An example of raw data related to the effect of methoctramine on epc and mepc is given in Fig. 1. As can be seen, a complete recovery of τ and mepc parameters was obtained after the first wash out of the drug while a long lasting reduction of the epcs amplitude was constantly seen. The RMP of the postsynaptic muscle fibres was not modified by any concentrations of the drug. Its control values of 70.1 ± 2.57 mV from five fibres were modified to 69.3 ± 1.25 mV in the presence of 20 μM methoctramine. The pH of the solutions was not changed when measured in the presence of a higher concentration of methoctramine assayed.

Effect of oxotremorine

The experimental results obtained in the presence of oxotremorine are demonstrated in Table 2. Regardless of this drug, the two opposite effects exerted at different concentration levels should be noted. A little enhancing effect on epcs amplitude was present at concentration of oxotremorine below 5 μm. A parallel increase of the mepcs frequency obtained at the same concentrations indicates that a presynaptic site may be responsible for such an effect. Moreover, a reduction of epc seems to be operative at concentrations above 5 μM with a presynaptic mechanism which could be scheduled as an inhibitory modulation. The reduction of epc, proportionately greater than the reduction of mepc at 20, 50 and 100 μM concentrations (see Table 2), demonstrates the presynaptic effects of oxotremorine. Concentration–response curves to oxotremorine gave apparent EC_{50}'s of 33.2, 51.4 and 514 μM for epc, mepc and τ with slopes of 0.99, 0.83 and 0.62, respectively. The inhibitory effect on the time constant of mepc decay was less pronounced if compared with that induced by methoctramine. Furthermore, concentrations higher than 5 μM of oxotremorine reduced the frequency of spontaneous mepcs. It should, however, be noted that doses of oxotremorine above 30 μM produced a high baseline noise level due to the activation of the

Table 1. Effects of methoctramine at the mouse neuromuscular junction

	Control values	1 μM	2 μM	5 μM	10 μM	20 μM	Wash
epc (nA)	1.06(0.46; 9)*	−15.25(2.79; 3)*	−24.38(17.33; 5)*	−29.73(41.13; 2)	−54.34(11.97; 6)*	−75.35(9.09; 5)*	−48.13(15.83; 6)*
mepc (nA)	0.29(0.12; 9)*	—	−14.63(12.07; 5)*	−13.26(20.36; 3)	−18.21(10.36; 4)*	−32.62(7.13; 5)*	−11.79(18.15; 5)
mepcf (Hz)	2.26(0.56; 7)*	−11.53(22.56; 2)	−13.64(0.00; 1)	−11.84(23.61; 4)	−46.66(18.44; 5)*	−53.86(31.41; 3)	−32.70(25.17; 6)*
τ (ms)	0.87(0.40; 9)*	−10.43(13.03; 4)	−11.77(6.83; 6)*	−26.96(8.31; 2)	−40.34(11.73; 5)*	−70.09(9.38; 4)*	−28.41(21.52; 6)*

Values show the effects of methoctramine on four parameters related to the function of the neuromuscular junction of the mouse. The amplitudes of the evoked (epc) and spontaneous (mepc) ACh release, the frequency of the spontaneous release (mepcf) and the mepcs decay time-constant (τ) have been analysed. Data are expressed as the mean percentage variation from the respective control value. Standard deviations and number of experiments are indicated in brackets.

*$P < 0.05$ with Student's t-test.

Table 2. Effects of oxotremorine at the mouse neuromuscular junction

	Control values	0.5 μM	1 μM	5 μM	10 μM	20 μM	50 μM	100 μM	Wash
epc (nA)	1.16(0.49; 11)*	+7.99(1.36; 2)	+2.18(5.71; 8)	−10.25(12.05; 11)*	−19.78(16.09; 2)	−45.51(22.69; 9)*	−59.58(17.50; 4)*	−72.61(15.08; 4)*	−35.07(18.93;10)*
mepc (nA)	0.32(0.17; 12)*	−6.05(0.00; 1)	−7.92(13.72; 9)	−11.16(20.92; 11)	−22.01(21.25; 2)	−25.34(26.55; 9)*	−54.82(10.89; 4)*	−62.01(16.04; 4)*	−36.22(31.28;10)*
mepcf (Hz)	1.79(1.1; 11)*	+5.37(19.63; 2)	+13.73(26.12; 8)	+1.28(48.66; 10)	−14.40(18.93; 2)	−22.88(36.15; 8)	−51.68(19.91; 4)*	−75.25(8.79; 3)*	−24.91(47.23; 8)
τ (ms)	0.90(0.23; 10)*	+0.44(0.09; 2)	+3.04(9.59; 10)	−2.91(10.62; 9)	—	−15.29(10.34; 8)*	−17.83(6.33; 4)*	−27.02(5.58; 2)	+2.64(11.09; 9)

Values show the effects of oxotremorine on four parameters related to the function of the neuromuscular junction of the mouse. The amplitudes of the evoked (epc) and spontaneous (mepc) ACh release, the frequency of the spontaneous release (mepcf) and the mepcs decay time-constant (τ) have been analysed. Data are expressed as the mean percentage variation from the respective control value. Standard deviations and number of experiments are indicated in brackets.

*$P < 0.05$ with Student's t-test.

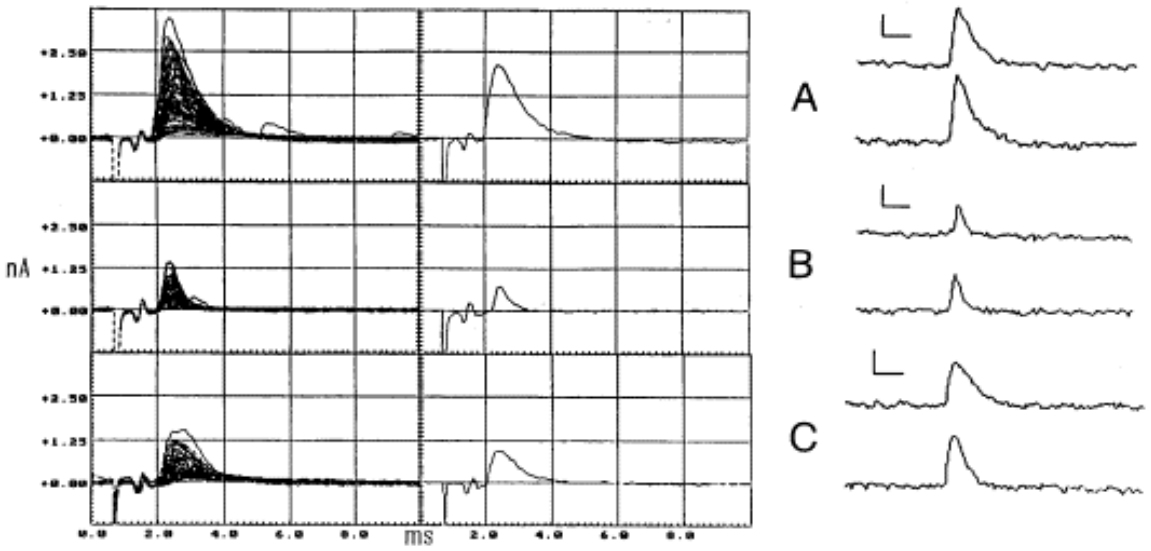

Fig. 1. Superimposed and single evoked end-plate currents (left) and spontaneous miniature end-plate currents (right) at the mouse neuromuscular junction. The figure shows digitized raw data related to the Control (A), to the treatment with methoctramine 10 μM (B) and to the final Wash Out. Right panel calibration; vertical bar = 0.2 nA, horizontal bar = 1 ms. Note the stimulus artifact and the biphasic action potential which precede the evoked signals.

postsynaptic receptor–ion channel complex. This condition disturbed the detection of the mepc, and any interpretation of the mepcf parameter could be hazardous. In Fig. 2 a small increase of the noise level could be seen at 20 μM oxotremorine (B, right) as compared to the control (A, right).

The RMP of the postsynaptic muscle fibres was reduced by concentrations of the drug higher than 50 μM. The control values of 70.6 ± 4.4 mV from eight fibres were reduced at 64.3 ± 2.93 mV and at 60.5 ± 4.97 by 50 and 100 μM oxotremorine, respectively.

Effect of oxotremorine in the presence of methoctramine

With the aim to better characterize the proposed M_2 presynaptic site responsible for the inhibitory effect of oxotremorine,[5,25] the same drug has been assayed in the presence of the novel cardioselective M_2 antagonist methoctramine.[18] In this preparation methoctramine did not antagonize either the presyn-

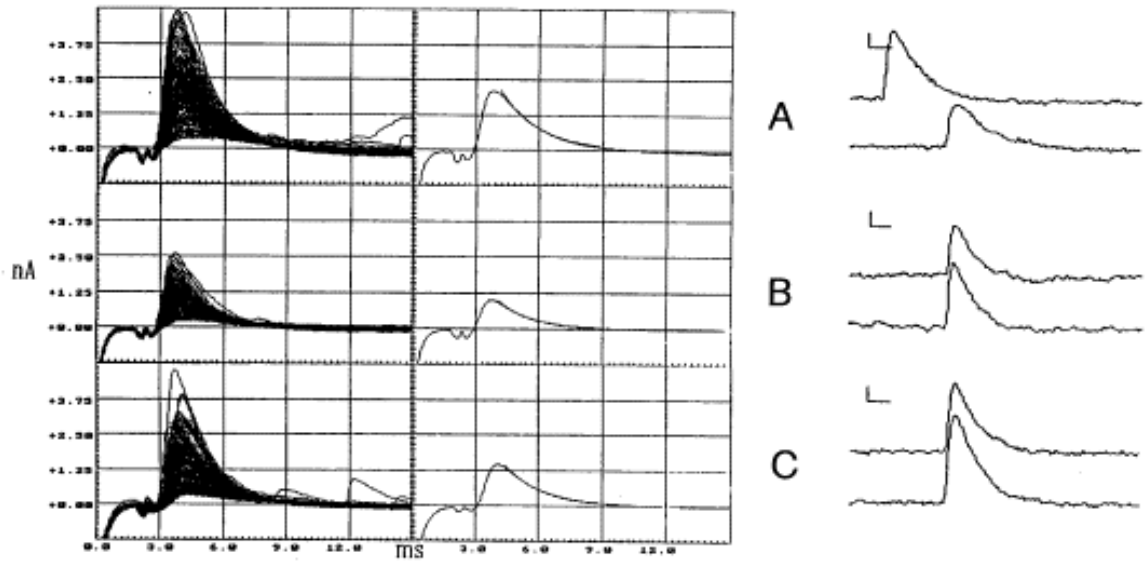

Fig. 2. Superimposed and single evoked end-plate currents (left) and spontaneous miniature end-plate currents (right)at the mouse neuromuscular junction. The figure shows digitized raw data related to the Control (A), to the treatment with oxotremorine 20 μM (B) and to the final Wash Out. Right panel calibration; vertical bar = 0.2 nA, horizontal bar = 1 ms. Note the stimulus artifact, the biphasic action potential which precede the evoked signals and the different noise level of the baseline in the right B panel.

Table 3. Effects of oxotremorine in the presence of 1 μM methoctramine at the mouse neuromuscular junction

	Control values	5 μM	10 μM	20 μM	50 μM	100 μM	Wash
epc (nA)	1.14(0.60; 4)*	−38.21(18.26; 3)*	−58.19(31.98; 3)*	—	−74.99(13.52; 3)*	—	−67.57(15.99; 3)*
mepc (nA)	0.34(0.16; 4)*	+18.92(30.37; 3)	−14.85(15.84; 3)	−22.47(25.56; 2)	−77.58(1.13; 2)*	−72.97(0.00; 1)	+2.98(83.38; 4)
mepcf (Hz)	1.83(0.79; 4)*	−40.37(16.66; 3)*	−51.04(27.24; 2)	−51.84(0.00; 1)	—	—	−21.82(51.37; 3)
τ (ms)	0.74(0.29; 4)*	−6.45(7.34; 3)	−11.02(4.52; 3)*	−13.53(0.00; 1)	—	—	−11.09(14.34; 4)

Values show the effects of oxotremorine in the presence of 1 μM methoctramine on four parameters related to the function of the neuromuscular junction of the mouse. The amplitudes of the evoked (epc) and spontaneous (mepc) ACh release, the frequency of the spontaneous release (mepcf) and the mepcs decay time-constant (τ) have been analysed. Data are expressed as the mean percentage variation from the respective control value. Standard deviations and number of experiments are indicated in brackets.

*$P < 0.05$ with Student's t-test.

aptic or the postsynaptic inhibitory effect of oxotremorine. The experimental data are summarized in Table 3. The fitting of the concentration–response curves to oxotremorine in the presence of methoctramine 1 μM gave apparent EC_{50}'s of 8.3 and 604 μM for epc and τ with slopes of 0.67 and 0.54, respectively.

DISCUSSION

Wessler *et al.*[26] stated that the existence of both negative and positive muscarinic feedback mechanisms provides unexpected complexities to the investigations of neurorelease. The present study demonstrates further complexities which could arise from the possible presence of some allosteric muscarinic sites on the postsynaptic membrane. The postsynaptic muscarinic action at the neuromuscular junction was first described for the antimuscarinic racemic atropine. Earlier studies showed that high concentrations of atropine reduced the end-plate potential evoked by iontophoretically applied ACh, i.e. at postsynaptic level.[4] Later, the suggestion that atropine could block the open state of the nicotinic receptor ion channel complex has been proved.[11] Furthermore, the effects of atropine described on the neuromuscular junction[9] and on the ganglionic nicotinic receptor–channel complex[7] proved the existence of a postsynaptically located interaction site for some muscarinic agents. Moreover, this effect was reported to be different from that of local anaesthetics[13] which modify the quantal decay from a mono- to bi-exponential decay. The latter result suggests the presence of an action site different from that proposed from anaesthetic drugs, which could not necessarily be located within the end-plate receptor channel complex.[1]

In the present study, carried out with an electrophysiological technique, a possible postsynaptic binding site seems to be present for the antimuscarinic agent methoctramine as well, which showed a very potent dose-related inhibitory effect on the duration of the quantal conductance change. Indeed, micromolar doses of methoctramine reduced the rate of the activated nicotinic receptor-ion channel conformational change. However, also assuming that any effect on τ reflects a modification of the cholinergic postsynaptic channel lifetime,[14,15] the effect on the epcs amplitude cannot be ascribed entirely to a pure postsynaptic action. In agreement, the mepcs amplitude was less affected than epcs amplitude by methoctramine, giving a quite different slope of the dose/response curve. Furthermore, the same drug reduced, presynaptically, the evoked and spontaneous ACh release, suggesting the presence of both pre- and postsynaptic action sites to methoctramine.

The agonist oxotremorine showed a very complicated pattern with different presynaptic effects. Our functional study proves the existence of opposite presynaptic muscarinic feedback mechanisms such as

have been described previously for the phrenic nerve[26] using biochemical studies. The same drug is also known to produce some postsynaptic effects. While Ganguly and Das[10] denied any postjunctional effect of oxotremorine on the diaphragmatic muscle, the present functional study demonstrates a postsynaptic blocking effect which takes place in the concentration range above 20 μm. With the same technique and in the magnesium-paralysed frog sartorius muscle, Arenson[3] showed a depolarizing effect of the drug blocked by D-tubocurarine. Nevertheless, the depolarizing effect and the high baseline noise level also induced in this preparation prove the postsynaptic activation of the cholinergic nicotinic receptor.

CONCLUSION

The experimental data of the present work indicate that the two muscarinic cholinergic drugs have almost two distinct sites of action at the postsynaptic level. Moreover, the effects observed in this preparation indicate an action at the presynaptic level both for the agonist and for the antagonist, suggesting a synergy of these muscarinic agents, probably mediated at different presynaptic sites. This hypothesis is further supported by the observation that 1 μM methoctramine did not antagonize the presynaptic effect of oxotremorine in this preparation, indicating the presence of a different presynaptic muscarinic subtype receptor. In other preparations the presynaptic activation of the M_1 subtype muscarinic receptor increases the transmitter release, whereas the M_2 subtype has been proposed to inhibit release.[5,25] Wessler *et al.*,[26] studying the release of ACh in the rat hemidiaphragm using a biochemical approach, proposed the involvement of a M_1-receptor for the presynaptic muscarinic feedback on transmitter release. Other reports showed that methoctramine fails to differentiate between the cortical and the ileal presynaptic muscarinic receptor,[24] suggesting a different M_2 muscarinic receptor subtype. The present experimental data, obtained at a low stimulation frequency, are quite similar to other published data and strengthen the hypothesis of a biphasic presynaptic muscarinic control of the ACh release in this preparation. Nevertheless, a relevant result of the present study is the very potent postsynaptic action of methoctramine, similar but more selective if compared to the same action described for the racemic atropine. In agreement with many of the considerations about the criticism related to the functional study as the better tool for the evaluation of any presynaptic modulating effect of the neurorelease, we believe, on the other hand, that only electrophysiological studies could better demonstrate any postsynaptic action.

The very complex pattern of this peculiar synapse, i.e. the neuromuscular junction, is far from being completely outlined, but we think functional (electrophysiological) and biochemical (overflow) studies could well complement each other to facilitate the knowledge of the problem.

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