Neuroscience Letters, 65 (1986) 72–78
Elsevier Scientific Publishers Ireland Ltd.

NSL 03815

ACETYLCHOLINE RECEPTORS AND ACETYLCHOLINESTERASE ACTIVITY IN SOLEUS MUSCLE OF TREMBLER DYSMYELINATING MUTANT: A CYTOCHEMICAL AND BIOCHEMICAL ANALYSIS

N.A. DO THI[1], C. BON[2], H.L. KOENIG[1] and J.M. BOURRE[3]

[1]*Laboratoire de Neurobiologie du Développement, Université de Bordeaux I, Avenue des Facultés, F-33405 Talence Cedex;* [2]*Unité des venins, Institut Pasteur, 25 rue du Dr. Roux, F-75015 Paris; and* [3]*Unité de Neurotoxicologie, INSERM U.26, Hôpital F. Widal, F-75010 Paris (France)*

(Received March 14th, 1985; Revised version received December 2nd, 1985; Accepted December 4th, 1985)

Key words: trembler mutation – acetylcholinesterase – acetylcholine receptor – muscle

We performed comparative biochemical and morphological studies of trembler and control soleus muscles. In the mutant, small multiple endplates were observed on some muscle fibers. The acetylcholine receptor (AChR) concentration and the acetylcholinesterase (AChE) activity of the muscle were not modified in the mutant. Our results suggest that both AChR and AChE levels are similar in trembler and control soleus but that these molecules are localized differently in the sarcolemma of the mutant muscles.

The trembler mutation is a neuropathy affecting the peripheral nervous system. It is characterized by hypomyelination and segmental demyelination of peripheral axons [1–3, 12, 13] with an abnormal persistence of Schwann cell multiplication [15]. Motor innervation is also aberrant: multi-innervated muscle fibers exist and anarchical outgrowth of motor nerve fibers give rise to 'large, complex terminal arborizations', especially in the soleus muscle [7, 8, 10]. In addition, numerous muscle fibers display additional clusters of acetylcholine receptors (AChRs) which have been visualized by radioautography after exposure to [^{125}I]α-bungarotoxin [10].

The purpose of our study was to establish: (1) whether the additional AChR clusters, visualized by radioautography, really correspond to multiple motor endplates, in which case AChR clusters should be associated with acetylcholinesterase (AChE) activity deposits located at the same site, and (2) if the total number of AChR molecules and the total AChE activity of the muscle are modified in trembler mutant muscles, as a consequence of multiple innervation.

Morphological approach. The soleus muscles of 3 trembler mice (B6 D2/Tr) aged 3–4 months and of 3 littermates (controls) were removed immediately after sacrifice and prepared for simultaneous visualization of AChE activity and AChR clusters using a technique previously described [4]. The totality of the muscle fibers was teased, mounted in glycerol and examined with a fluorescence microscope for AChR bound to α-bungarotoxin conjugated with tetramethyl-rhodamine (Rh-α-Bgt) and a dark-field microscope for AChE activity.

In a quantitative cytomorphometric analysis, we compared the number and the diameter of muscle fibers as well as the length and surface area of the motor endplates in soleus muscles of trembler and control mice (Table I). All measures were performed with an eyepiece micrometer on the teased fibers stained for AChR. The motor endplates selected for measurement were those clearly seen in frontal view. The surface area of the motor endplates considered as an ellipse was calculated according to the formula: $S^2 = \pi a\ b$ (Documenta Geigy, Ed. J.R. Geigy S.A.) in which a and b correspond respectively to the half-length and width measured. To determine more precisely the mean number of fibers, we counted, in addition to the 3 teased muscles, the fibers of 5 more trembler and control muscles on 1% toluidine blue-stained cryostat cross-sections. Two of them corresponded to animals N4, N5, T4 and T5 used for the AChR assays (Table II).

In order to compare the AChR concentration in soleus muscles with the morphological data, we calculated the total surface area occupied by the motor endplates per muscle.

Table I shows that the total number of muscle fibers is not significantly modified in the mutant mice. Most of the fibers (approximately 90%) were found to be mono-innervated, as in normal muscles; the size of the endplates was very similar to those in normal mice (Table I). On the other hand, approximately 10% of trembler soleus muscle fibers showed multiple endplates (from one to 3 supernumerary endplates). Their diameter was found to be smaller than that of the mono-innervated muscle

TABLE I

CYTOMORPHOMETRIC ANALYSIS OF MOTOR ENDPLATES IN CONTROL AND TREMBLER SOLEUS MUSCLES

Length and width were measured to obtain the surface area of motor endplates (MEPs). For multiply-innervated muscle fibers, the length and surface area of all MEPs were added to obtain the total length and surface area per fiber. The total surface area of MEP per muscle was obtained by multiplying the mean surface area of MEP by the total number of muscle fibers. For trembler muscles, we added the values obtained for single and multiply innervated fibers. Data shown are means ± S.D.

	Control muscle (single endplates)	Trembler muscle	
		Single endplates	Multiple endplates
Number of fibers per muscle	600 ± 90	540 ± 70 (90%)	60 ± 8 (10%)
Diameter of muscle fibers (μm)	36 ± 7	37 ± 8	31 ± 5.5
Total length of MEPs per fiber (μm)	40 ± 10	40 ± 10 (105%)	66 ± 14 (165%)
Total surface of MEPs per fiber (μm²)	830 ± 350	860 ± 300	1080 ± 430 (130%)
Total surface of MEPs per muscle (μm²)	498,000	464,000 529,000 (106%)	65,000


A
B
C
D

fibers (Table I). In both cases, AChE activity was colocalized with AChR clusters, suggesting that they represent functional endplates (Fig. 1).

Although the size of the individual supernumerary endplates was smaller than that of normal endplates (Fig. 1), the total length and surface area of the endplates were larger in multi-innervated fibers than in mono-innervated normal or mutant fibers, by 65% for length and 30% for surface areas (Table I). These data allowed us to calculate the total surface area of the motor endplates per muscle (Table I). This surface area was 498,000 and 529,000 μm^2 in normal and trembler, respectively, indicating that the innervated area of a trembler soleus muscle is equivalent (106%) to that of a normal muscle. This result is mainly due to the relatively low proportion of multi-innervated fibers in the mutant muscle.

The distance separating the multiple endplates on the same fiber varied from 0.17 to 0.2 mm. The multiply-innervated muscle fibers were usually grouped in small bundles comprising from 2 to 6 fibers. The small number of multiply-innervated muscle fibers (10%) and their grouping suggest that a few motor units only are affected.

Biochemical approach. We evaluated AChRs as the number of binding sites for purified [^{125}I]α-bungarotoxin in the soleus muscle of 5 trembler mice, and of 5 littermates used as controls, distributed in two series (Table II). In series 1 (control: N1, N2 and N3; trembler: T1, T2 and T3), both soleus muscles of each animal were homogenized and used for biochemical assays. In series 2 (control: N4 and N5; trembler: T4 and T5), one muscle was assayed, whereas the other was used for counting the number of muscle fibers. The muscles were weighed and homogenized in phosphate buffer with a Virtis homogenizer. Aliquots were retained for assays of protein concentration [14] and of AChE activity [5]. The bulk of the homogenate was used for AChR determination. After addition of Triton X-100 at a final concentration of 1%, the samples were incubated for 150 min at 0°C and then cenrifuged at 3000 *g* for 10 min. The supernatant was incubated with [^{125}I]α-bungarotoxin (Amersham; spec. act., 100–500 Ci/mmol) in phosphate buffer for 18 h at room temperature. The non-specific binding was measured in samples of the supernatant to which cold α-bungarotoxin was added in a 100-fold excess. The [^{125}I]α-bungarotoxin bound to AChR was measured after filtration in G-75 Sephadex columns [11]. In order to ascertain that the AChRs were totally solubilized by Triton X-100, the pellet was resuspended in phosphate buffer. Aliquots were incubated with [^{125}I]α-bungarotoxin for AChR assays as described above. The radioactivity of the pellet was measured as the difference between the radioactivity of aliquots of the solution before centrifugation and that of the supernatant obtained after centrifugation for 10 min in an Airfuge at its maximum speed (148,000 *g*).

Table II shows that the fresh weight of the soleus muscles and the concentration of protein per milligram of muscle were similar in trembler and control mice. The

←

Fig. 1. Multiple innervated fibers in soleus muscle of Trembler mutant mouse. A: AChRs are visualized with Rh-α-Bgt. B and C: simultaneous visualization of AChRs (B) by Rh-α-Bgt and AChE activity by cytochemistry (C), dark-field microscopy [4]. D: AChE activity revealed by Koelle's method, dark-field microscopy. ×300.

TABLE II

BIOCHEMICAL ANALYSIS OF TREMBLER (T) AND CONTROL (N) SOLEUS MUSCLES

Data shown are means ± S.D. Serie 1: two soleus muscles were used for each experiment. Values were calculated for one soleus muscle. Serie 2: one soleus muscle was used for each experiment. The heterogeneous results for supernatant AChR concentration obtained in series 1 and 2 are probably due to slight differences in homogenization.

Samples	Weight (mg)	Protein		AChR				AChE	
		conc. (mg/mg muscle)	Total (mg)	Pellet		Supernatant		spec.act. (nmol/ min × mg)	Total (nmol/min)
				spec. act. (fmol/mg prot.)	Total (fmol)	spec.act. (fmol/mg prot.)	Total (fmol)		
Serie 1									
N1	7.5	0.10	0.75	0.26	0.20	10.13	7.6	–	–
N2	7.0	0.10	0.70	–	–	10.04	7.0	9.4	6.58
N3	5.5	0.11	0.61	–	–	11.13	6.8	14.2	8.66
Serie 2									
N4	6.0	0.12	0.72	0.13	0.10	7.5	5.4	–	–
N5	5.0	0.12	0.60	0.16	0.10	7.5	4.5	–	–
Mean	6.2 ± 0.9	0.10 ± 0.01	0.68 ± 0.07	0.18 ± 0.05	0.15 ± 0.05	9.3 ± 1.5	6.3 ± 1	11.8 ± 2.4	7.62 ± 1.0
Serie 1									
T1	6.0	0.10	0.60	0.17	0.11	11.00	6.6	–	–
T2	7.5	0.10	0.75	–	–	11.24	8.4	11.6	8.7
T3	6.5	0.10	0.65	–	–	–	–	13.5	8.8
Serie 2									
T4	5.5	0.11	0.61	0.16	0.10	8.6	5.25	–	–
T5	5.6	0.11	0.62	0.16	0.10	8.6	5.3	–	–
Mean	6.2 ± 0.7	0.10 ± 0.01	0.65 ± 0.06	0.16 ± 0.05	0.11 ± 0.01	9.9 ± 1.3	6.4 ± 1.3	12.6 ± 0.95	8.8 ± 0.1

majority of AChR extracted after homogenization and exposure to Triton X-100 was recovered in the supernatant (97–98%). Thus, only a negligible proportion of non-extracted AChR remained in the pellet (Table II). No significant difference in the AChR concentration, determined by the concentration of [^{125}I]α-bungarotoxin binding sites, was observed in trembler and control muscles (Table II). This result is consistent with the fact that the total surface area of motor endplates, measured on AChR-stained preparations, was similar in trembler and control soleus muscles (Table I). Our observations suggest that the formation of supernumerary endplates could result from either an increase in the number of AChR molecules or a redistribution of AChR molecules.

The AChE activity of the soleus muscle did not differ significantly in trembler and control mice (Table II). As previously reported [10], the relative proportions of AChE asymmetric molecular forms were also similar (47–48%) in mutant and control. The AChE activity which is detected at the supernumerary endplates probably corresponds to a modification in the distribution of the enzymatic molecules.

In conclusion, our results indicate that the AChR concentration and AChE activity are not significantly affected in trembler soleus muscle. The regulation process, which dispatches both molecules towards the supernumerary motor endplates to where they concentrate, is similar. As only few motor units are affected by the mutation, it was not possible, from our data, to ascertain whether AChR and AChE molecules in the supernumerary endplates correspond to a redistribution or to an increased synthesis in multiply-innervated muscle fibers. Because of the lack of sensitivity of AChR level measurements (S.E.M. = ± 15%), significant variations of AChR synthesis in only 10% of the soleus fibers would remain undetected.

As previously suggested, the focalization of AChR [6] and AChE [9] is probably induced by axonal contacts. In trembler soleus muscle, it has actually been shown that terminal and subterminal sprouting is present [8, 10]. In fact, Gale et al. [7] reported that the multiple motor endplates observed on some trembler soleus muscle fibers are associated with axonal sprouts. It would be of interest to analyse if other molecules usually associated with synapses, such as basal lamina and cytoskeletal components, are simultaneously modified at the supernumerary synaptic sites in the mutant muscles.

We would like to thank Dr. J. Massoulié for helpful discussions and assistance; Ms. P. Cloup (Université P. et M. Curie, Paris) for iconography and Ms. A. Paviot (Université de Bordeaux I) for typing. This work was supported by INSERM Grant 806006.

1 Ayers, M.M. and Anderson, R.McD., Onion bulb neuropathy in the Trembler mouse: a model of hypertrophic interstital neuropathy (Dejerine-Sottas) in man, Acta Neuropath. (Berl.), 25 (1973) 54–70.

2 Ayers, M.M. and Anderson, R.McD., Development of onion bulb neuropathy in the Trembler mouse. Comparison with normal nerve maturation, Acta Neuropath. (Berl.), 32 (1975) 43–59.

3 Ayers, M.M. and Anderson, R.McD., Development of onion bulb neuropathy in the Trembler mouse. Morphometric study, Acta Neuropath. (Berl.), 36 (1976) 137–152.

4 Do Thi, N.A., De la Porte, S. and Koenig, J., Simultaneous visualization of acetylcholinesterase activity and acetylcholine receptor clusters at neuromuscular synapses in vivo and in vitro, Biol. Cell., 50 (1984) 99–102.
5 Ellman, G.L., Courtney, K.D., Andres, V.I. and Featherstone, R.M., A new and rapid colorimetric determination of acetylcholinesterase activity, Pharmacology, 7 (1961) 88–95.
6 Fambrough, D., Harzell, H.C., Rash, J.E. and Ritchie, A.K., Receptor properties of developing muscle, Ann. N.Y. Acad. Sci., 228 (1974) 47–62.
7 Gale, N.A., Gomez, S. and Duchen, L.W., Changes produced by hypomyelinating neuropathy in muscle and its innervation morphological and physiological studies in the Trembler mouse, Brain, 105 (1982) 373–393.
8 Koenig, H.L., Lefaix, J.L. and Bourre, J.M., Abnormal innervation in Trembler mutant muscles. In N. Baumann (Ed.), Neurological Mutations Affecting Myelination, Elsevier/North Holland, 1980, pp. 537–542.
9 Koenig, J., Formation and maturation of nerve–muscle contacts in cultured rat embryo cells, Biol. Cell., 35 (1979) 147–152.
10 Lefaix, J.L., Koenig, H.L., Vigny, M. and Bourre, J.M., Innervation motrice de la souris Trembler, Reprod. Nutr. Develop., 22 (1B) (1982) 275–282.
11 Li, Z.-Y. and Bon, C., Presence of membrane-bound acetylcholinesterase form in a nerve endings preparation from *Torpedo marmorata* electric organ, J. Neurochem., 40 (1983) 338–349.
12 Low, P., Hereditary hypertrophic neuropathy in the Trembler mouse. Part 2. Histological studies: electron microscopy, J. Neurol. Sci., 30 (1976) 343–368.
13 Low, P.A., The evolution of 'onion bulb' in the hereditary hypertrophic neuropathy of the Trembler mouse, Neuropath. Appl. Neurobiol., 3 (1977) 81–92.
14 Lowry, O.H., Rosebrough, N.J., Farr, L.A. and Randall, R.J., Protein measurement with the Folin phenol reagent, J. Biol. Chem., 193 (1951) 265–275.
15 Perkins, C.S. Aguayo, A.J. and Bray, G.M., Schwann cell multiplication in Trembler mice, Neuropath. Appl. Neurobiol., 7 (1981) 115–126.