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25. The enkephalins are known to be enzymatically degraded very rapidly in the brain. Their greater epileptic potency (compared to their analgesic potency) could be explained, therefore, by assuming that they have more ready access to thalamic sites mediating seizures than to midbrain sites mediating analgesia. However, we have noted (*5*) that the latencies to enkephalin-induced analgesia and seizures are, in fact, very similar.
26. We were unable to block enkephalin seizures with systemic injections of naloxone at 2 mg/kg in four rats tested (*5*), although naloxone at 10 mg/kg has subsequently proved effective (*12*). By contrast, Belluzzi *et al.* (*6*) reported that naloxone at 2 mg/kg did block enkephalin analgesia.
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28 November 1977; revised 9 February 1978

Neurotrophic Protein Regulates Muscle Acetylcholinesterase in Culture

Abstract. *Skeletal muscles lose acetylcholinesterase in culture as a result of denervation. A protein fraction isolated from peripheral nerves maintained the level of acetylcholinesterase in cultures of aneural embryonic muscle or denervated adult chicken muscle. These results indicate that trophic regulation of muscle acetylcholinesterase might be mediated by a protein produced by nerves.*

In normally innervated skeletal muscles, acetylcholinesterase (AChE) is highly localized at the motor end plate (*1*). After denervation the end plate AChE decreases rapidly (*2, 3*) and, conversely, reinnervation of the denervated muscle restores its AChE (*4*). Furthermore, AChE appears in developing and regenerating muscles in relation to innervation, in vivo as well as in vitro (*5*). Thus, the spinal motor neuron is believed to exert a trophic effect on the end plate AChE.

We do not know how the motor neuron regulates muscle AChE activity, but several mechanisms have been proposed, including muscle activity (*6*), acetylcholine (ACh) release (*7*), and neurotrophic substances (*8*). Recent evidence indicates that a neurotrophic substance transported by axoplasmic flow might be responsible for regulation of muscle AChE. Blockage of axoplasmic flow by colchicine or vinblastine causes a decrease in muscle AChE without disturbing ACh release and consequent muscle activity (*9*).

Although organ culture of adult muscles results in a loss of muscle AChE as a result of denervation (*10, 11*), addition of nerve homogenates to the nutrient medium of muscle cultures prevents the decrease in AChE activity (*10*). An aqueous extract of adult peripheral nerves increases the AChE activity of cultured aneural embryonic muscle (*12*). These results thus indicate that the trophic effect on muscle AChE is mediated by a substance produced by nerves. Recently, we isolated and partially purified a protein fraction from peripheral nerves which enhances morphological differentiation, stimulates protein synthesis, and increases the creatine kinase (CK) activity of cultured muscle cells (*13*). We now report that this protein fraction regulates AChE activity in cultured muscle.

Myogenic cells (3×10^5) were obtained from trypsin-dissociated thigh muscles of 11-day chick embryos (*14*) and were cultured in a collagen-coated plastic dish (*13*). Also, slow muscles [anterior latissimus dorsi (ALD)] and fast muscles [posterior latissimus dorsi (PLD)] were isolated aseptically from 41-week-old chickens (White Leghorn) and cultured in collagen-coated dishes. The cultures were maintained in a medium of 65 percent Dulbecco's modified Eagle's medium, 10 percent horse serum, 2.5 percent chick embryo extract (brain and spinal cord excluded), 20.5 percent Hanks balanced salt solution, and 2 percent glucose (20 percent stock solution), and fed with fresh medium every 3 days. An active peripheral nerve (PN) protein fraction was obtained from chicken sciatic nerve extracts by gel filtration on Sephadex G-200 (*15*). The AChE activity of muscle homogenates was measured by a modification (*3*) of the method of Ellman *et al.* (*16*) in the presence of $10^{-4}M$ iso-OMPA (tetraisopropylpyrophosphoramide), an inhibitor of nonspecific cholinesterase. Noncollagen protein was determined as described by Rifenberick *et al.* (*17*), using the method of Lowry *et al.* (*18*).

Figure 1 shows the change in AChE activity during differentiation of embryonic muscle cells in culture. The AChE activity increased markedly during the period of fusion (between 28 and 72 hours) (*19*). Thereafter it decreased rapidly as muscle maturation progressed. By day 8, muscle fibers exhibited cross-striations and spontaneous contractions. The AChE activity decreased moderately between 8 and 12 days in culture. Addition of an active PN protein fraction (61 μg/ml) to cross-striated mature muscle cultures prevented a further decrease in muscle AChE activity, whereas the inactive PN protein fractions (*15*) were ineffective (Fig. 1).

To determine whether this phenomenon resulted from simple enzyme induc-

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Table 1. Effects of cycloheximide and actinomycin D on AChE activity of active PN protein-treated skeletal muscle cells in culture. Activity is expressed as micromoles of acetylthiocholine hydrolyzed per hour per milligram of protein. Each value is the mean ± S.E. for six dishes.

Treatment	AChE activity (μmole/hour-mg)
Control	1.58 ± 0.17
Cycloheximide ($10^{-5}M$)	1.10 ± 0.05*
Actinomycin D (2 μg/ml)	1.99 ± 0.04†

*Significant decrease ($P < .05$) from control value. †Significant increase ($P < .05$) over control value.

tion, cycloheximide ($10^{-5}M$) or actinomycin D (2 μg/ml) was added to muscle cultures (8-day) immediately after the addition of an active PN protein fraction (Table 1). After 18 hours in the presence of the protein synthesis inhibitors, the AChE activity of muscle cells was determined. Cycloheximide caused a significant decrease in AChE levels compared to those in inhibitor-free cultures. By contrast, actinomycin D caused a "superinduction" of AChE as evidenced by an increase in AChE activity. These results seem to indicate that the maintenance of AChE levels by the active PN protein fraction is regulated by a posttranscriptional mechanism (*20*).

Since adult muscles lose AChE activity in organ culture as a result of denervation (*10, 11*), we investigated whether the active PN protein fraction would maintain the AChE activity of cultured muscles. Table 2 shows that addition of the active PN protein fraction (62 μg/ml) to the media of adult chicken ALD and PLD muscles prevented loss of muscle AChE compared to that in uncultured muscles. However, the control lost significant AChE activity during the 6 days in culture. Also, the inactive PN protein fractions (44 μg/ml) were ineffective in maintaining muscle AChE activity.

The effect of surgical denervation on muscle AChE activity in chickens is a controversial subject. Vigny *et al.* (*2*) reported that denervation caused a significant decrease in the AChE activity of both slow (ALD) and fast (PLD) muscles. The molecular weight distribution of AChE extracted from normal and denervated muscles with Triton X-100 and separated by sucrose gradient centrifugation revealed that the highest-molecular-weight form (19.5*S*), which is associated with motor end-plate regions, disappeared after denervation (*2*). Wilson and co-workers (*21*) showed that the total AChE activity of denervated muscle homogenates was three to four times higher than that of normal muscle homogenates. The increase in AChE activity not in end-plate regions coincided with the appearance of AChE in the sarcoplasm, whereas end-plate AChE disappeared after denervation (*21*).

Embryonic and adult chicken muscles lose AChE activity in culture (Fig. 1, Table 2) and in this regard are similar to muscles from newt and rat (*10, 11*). The fact that a protein fraction from peripheral nerves maintained the level of AChE in cultured muscle strongly suggests that a protein in this fraction mimics the maintenance effect of innervation on muscle AChE. Furthermore, that this maintenance effect was mediated through a simple nutritive effect is highly unlikely, since the biological activity of this fraction is greatly diminished on enzymatic treatment with trypsin or protease or oxidation by periodate (*13*).

Neural substances transported by axoplasmic flow appear to participate in trophic regulation of muscle AChE, since application of colchicine or vinblastine to sciatic nerves caused a decrease in muscle AChE without affecting release of ACh or muscle activity (*9*). Furthermore, a similar decrease in muscle AChE was observed in rats with flaccid paralysis after the injection of batrachotoxin into spinal cord (*22*). Since electrical stimulation failed to restore AChE activity in these animals (*23*), muscle activity can be ruled out as a major factor in the trophic regulation of muscle AChE. These reports, considered with the data presented here, strongly reinforce the concept that a neurohumoral substance (*24*) is essential in mediating trophic regulation of muscle AChE.

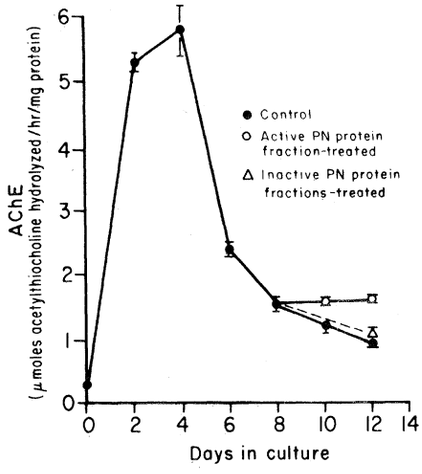

Fig. 1. Effect of PN protein fractions on AChE activity of cultured chick embryonic skeletal muscle cells during myogenesis. The value for the cultures treated with active PN protein at 12 days in vitro is significantly greater than the corresponding control value (Student's *t*-test, $P < .001$), while the value for the cultures treated with inactive PN protein is not significantly different from the control value ($P > .05$). Each point is the mean ± standard error (S.E.) for 12 dishes.

Table 2. Effects of PN protein fractions on AChE activity of chicken ALD and PLD muscles in organ culture. Chicken muscles (mean wet weight: ALD, 518 mg; PLD, 1140 mg) were cultured in the presence of active or inactive PN protein fractions. After 6 days in culture, the AChE activity (see Table 1) of the muscles was assayed. Each value is the mean ± S.E. for four muscles.

Treatment	AChE activity (μmole/hour-mg)	
	ALD	PLD
Uncultured	0.41 ± 0.02	0.28 ± 0.03
Control	0.34 ± 0.01*	0.23 ± 0.02*
Inactive PN protein fractions	0.34 ± 0.01*	0.24 ± 0.04*
Active PN protein fraction	0.41 ± 0.01	0.28 ± 0.04

*Significantly different from uncultured muscles, $P < .01$.

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hanced morphological differentiation, and increased the activities of CK and myokinase. The biologically active component appeared to be a protein, as evidenced by its lability to treatment with trypsin or protease and its stability to treatment with neuraminidase, phospholipase A_2, and concanavalin A. Polyacrylamide gel electrophoresis of the active fraction on 7.5 percent gels in tris-glycine buffer (*p*H 8.3) revealed five protein bands. The gels were negative to staining with periodic acid–Schiff reagent. In Fig. 1 and Tables 1 and 2, the term inactive peripheral nerve (PN) proteins refers to the pooled column proteins not contained in the fraction with K_{AV} = 0.54 to 0.56.
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25. We thank S. Silberberg for technical assistance and R. Ellison for preparation of the typescript. Supported in part by NSF grant BNS 76-15370 and NIH grant NS 13296-01.

15 November 1977; revised 11 January 1978

Aural Representation in the Doppler-Shifted-CF Processing Area of the Auditory Cortex of the Mustache Bat

Abstract. *In the mustache bat (*Pteronotus parnellii rubiginosus*) the frequency and amplitude of an acoustic signal are represented in the coordinates parallel to the surface of the Doppler-shifted-CF (constant frequency) processing area of the primary auditory cortex. In this area all cortical neurons studied were excited by contralateral stimuli, and almost all of them were either excited or inhibited by ipsilateral stimuli. These are called E-E (ipsilateral and contralateral excitatory) and I-E (ipsilateral inhibitory and contralateral excitatory) neurons, respectively. The I-E neurons are directionally sensitive, while the E-E neurons are not. The E-E neurons are equally sensitive to echoes between 30° contralateral and 30° ipsilateral. Of the electrode penetrations orthogonal to the Doppler-shifted-CF processing area, 57 percent were characterized by either E-E or I-E neurons. Thus, there are at least two types of binaural columns: E-E columns, mainly located in a ventral part of the Doppler-shifted-CF processing area, where neurons are tuned to weak echoes; and I-E columns, mainly distributed in a dorsal part, where neurons are tuned to moderate to intense echoes. Therefore, neurons tuned to weaker echoes integrate or even multiply faint signals from both ears for effective detection of a distant small target, while neurons tuned to moderate to intense echoes are suited for processing directional information and are stimulated when a bat approaches a target at short range. The Doppler-shifted-CF processing area may be considered to consist of two functional subdivisions.*

The mustache bat, *Pteronotus parnellii rubiginosus*, emits orientation sounds which consist of a long constant-frequency (CF) component followed by a short frequency-modulated (FM) component. The long CF sound is an ideal signal for Doppler-shift measurement—that is, for the measurement of the relative velocity of a target—and it is also a good signal for target detection. The short FM sound, on the other hand, is suited for ranging, localization, and characterization of the target. In the orientation sound, the second harmonic is always predominant and its CF component is about 61 kHz (*1–4*). The mustache bat apparently uses this signal for detection of a moving target because it adjusts the frequency of the emitted CF component to receive a Doppler-shifted echo at a certain preferred frequency (61 to 62 kHz), to which the auditory system is sharply tuned. This interesting acoustic behavior is called Doppler-shift compensation (*2*).

Peripheral auditory neurons with a best frequency (BF) between 60 and 63 kHz show unusually sharp tuning (or threshold) curves. These sharply tuned neurons are apparently specialized for detection and frequency analysis of the CF component in echoes. The primary auditory cortex of this animal reflects this peripheral specialization by devoting a disproportionately large area to processing the CF component of Doppler-shifted echoes (Fig. 2A) (*5*). In this Doppler-shifted-CF processing area, the BF and best stimulus amplitude (BA) for the maximum excitation of a single neuron vary systematically with the location of the neuron in the cortical plane. The iso-BF contour lines are eccentric: neurons sensitive to 61 kHz are at the center and those sensitive to 62 or 63 kHz are at the circumference. The iso-BA contour lines are radial: neurons tuned to weaker sounds are in the ventral part and those tuned to intense sounds are in the dorsal part. These tonotopic and amplitopic representations are apparently related to those of the relative velocity and subtended angle (or cross-sectional area) of a target. The origin of the coordinates representing frequency and amplitude is off-center in the Doppler-shifted-CF processing area, so that both representations disproportionately express an acoustic signal of 61.5 to 62.0 kHz and 30 to 50 dB SPL (sound pressure level) over a larger cortical area (Fig. 2B) (*6*). These disproportionate tonotopic and amplitopic representations are apparently related to the predominant parameters of the acoustic signals used for echolocation.

To investigate the cortical organization related to the localization of a potential target, we studied how sounds stimulating the left and right ears are represented in the Doppler-shifted-CF processing area, and how this aural representation is related to the tonotopic and amplitopic representations. As described below, we found that this area is organized in a very interesting way in terms of aural representation. That is, it consists of two functional subdivisions: one specialized for target detection by integrating excitatory signals from both ears, and the other for target localization by assembling excitatory signals from the contralateral ear and inhibitory ones from the ipsilateral ear.

The experiments were performed with 27 mustache bats, *P. parnellii rubiginosus* (body weight, 20 to 25 g) from Panama. A bat was anesthetized with sodium pentobarbital (30 mg per kilogram of body weight) and ether when necessary, and the flat head of a nail (1.8 cm long) was mounted on the posterodorsal surface of its skull with glue and cement. Then the bat was placed in a soundproof room heated to 33° to 34°C. To immobilize the bat's head, the shank of the nail was locked into a hollow metal rod with a setscrew. The skull covering the Doppler-shifted-CF processing area was removed. A tungsten wire electrode with a tip diameter of 5 to 15 μm was orthogonally inserted into the exposed cortical area to record action potentials from a single neuron or a cluster of a few neurons at depths between 200 and 1000 μm. To stimulate the left or the right ear

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