Synaptic Vesicles: Selective Depletion in Crayfish Excitatory and Inhibitory Axons

Abstract. *Stimulation of the excitatory axon of the opener muscle of the crayfish in the presence of the metabolic inhibitor 2,4-dinitrophenol leads to depletion of synaptic vesicles in nerve terminals containing round vesicles. Stimulation of the inhibitory axon under these conditions produces depletion of vesicles in other nerve terminals containing more elongate synaptic vesicles. The experiments show that terminals with round synaptic vesicles are excitatory and that terminals with elongate synaptic vesicles are inhibitory. Replenishment of synaptic vesicles appears to require metabolic energy.*

The opener muscle of the crayfish leg is innervated by two efferent axons, one excitatory and the other inhibitory, which branch together to supply each muscle fiber with at least 50 synapses (*1, 2*). Since the crustacean neuromuscular system is often used as a model for processes within the central nervous system (*3*), identification of the ultrastructure of the excitatory and inhibitory synapses and of their relations to each other is of potential value for studies of analogous situations in central nervous systems. Here, we present a new approach to identification of excitatory and inhibitory synapses.

Previously, excitatory and inhibitory nerve terminals in crayfish muscle were identified by use of the shape of the synaptic vesicles as a criterion (*4*). Excitatory terminals were postulated to contain round synaptic vesicles, whereas inhibitory terminals were thought to contain vesicles of less regular shape that, on the average, were more elongate. In the opener muscle of the crayfish, this interpretation is supported by the finding that axoaxonal synapses occur between the two types of terminals, in which the terminal with less regular shaped vesicles forms the presynaptic member (*4, 5*). This type of synapse can account for presynaptic inhibition in the opener motor axon of the crayfish (*6*). This evidence, although suggestive, is still circumstantial, and a more direct method of identification is desirable. We found that it was possible to selectively deplete either the round synaptic vesicles or the elongate synaptic vesicles from terminals by prolonged stimulation of excitatory or inhibitory axons, respectively, in the presence of a metabolic inhibitor. The experiments provide a more direct method for identifying the excitatory and inhibitory nerve terminals and also show the effects of a metabolic inhibitor on synaptic transmission and synaptic vesicles.

When a fiber in the opener muscle of the leg of the crayfish (*Procambarus clarki*) is impaled with a glass microelectrode, a small excitatory postsynaptic potential (EPSP) appears with stimulation of the excitatory axon. All fibers in this walking leg opener muscle showed strongly facilitating EPSP's, unlike those in certain other preparations (*7*). Continuous stimulation for 20 minutes or longer at frequencies of 5 to 20 stimuli per second fails to produce any diminution of the EPSP; on the contrary, the EPSP slowly increases until by 1 hour it is three to six times its initial size (*8*).

We tested the hypothesis that impairment of metabolism would result in selective blockage of synaptic transmission of the stimulated axon. We used 0.2 to 0.7 m*M* 2,4-dinitrophenol (DNP), which penetrates nerve cells (*9*) and uncouples oxidative phosphorylation (*10*). Ten to 12 minutes after DNP in van Harreveld's solution (*11*), is perfused into the muscle during a period of prolonged stimulation, the EPSP increases greatly in amplitude, attaining at least ten times its control size. Thereafter, the amplitude of the EPSP starts to decline. About 45 minutes after addition of DNP, the EPSP disappears. Recordings at single excitatory synaptic sites with an external microelectrode (*2, 12*) showed that the initial enhancement and subsequent depletion reflect changes in the output of synaptic

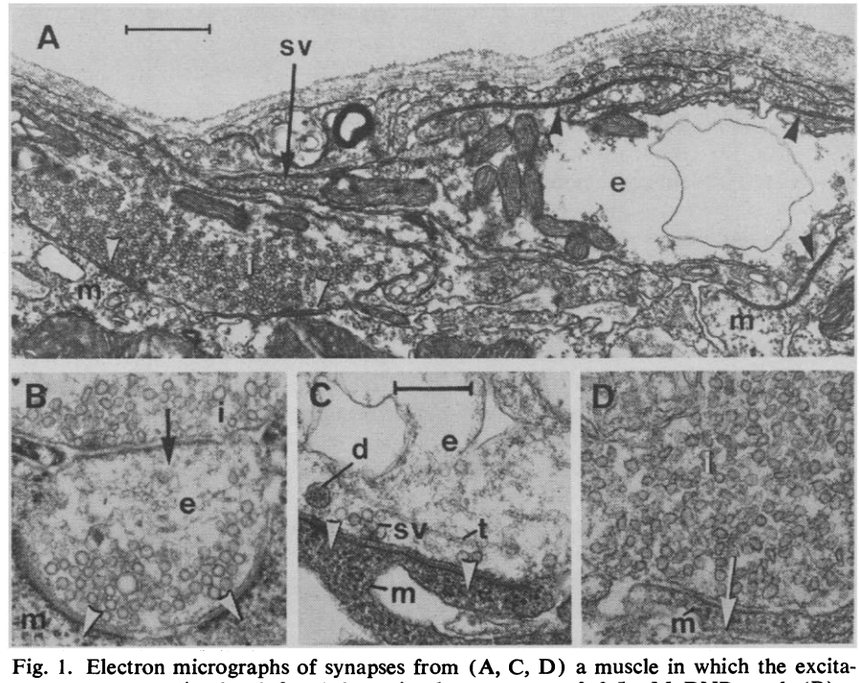

Fig. 1. Electron micrographs of synapses from (A, C, D) a muscle in which the excitatory axon was stimulated for 1 hour in the presence of 0.5 m*M* DNP, and (B) a muscle in which the excitatory axon was stimulated for 1 hour in the absence of DNP. In (A) and (B), two nerve terminals (*i* and *e*) are present. The i-terminal forms an axoaxonal synapse with the e-terminal in (B), which in turn forms neuromuscular synapses with the muscle (*m*). The arrows indicate the direction of synaptic transmission. The synaptic vesicles in the e-terminal are all round, whereas many of those in the i-terminal are elongate. In (A), adjacent i- and e-terminals are shown at the surface of a muscle fiber. Note depletion of vesicles and large vacuole in the e-terminal and many synaptic vesicles in i-terminal. The synaptic vesicles (*sv*) in the e-terminal are confined to a side branch of the axon. (C) Synapse of an e-terminal (*e*) (depleted of vesicles) with the muscle (*m*). The nerve terminal contains a few synaptic vesicles (*sv*), microtubules (*t*), a large dense-corded vesicle (*d*), and some irregular shaped vacuoles. (D) An adjacent i-terminal (*i*) in the same region, in synaptic contact with the muscle (*m*). Note the large number of synaptic vesicles and the frequent occurrence of elongate and irregular shaped vesicles. Scale mark: (A) 0.5 μm; (B–D) 0.3 μm.

Table 1. Counts of vesicles within 0.25 μm of the presynaptic membrane in nerve terminals of the crayfish.

Conditions		Terminals with round vesicles (R = 1.0 to 1.18)			Terminals with irregular vesicles (R = 1.3 to 1.45)		
Stimulation	Fixation	Terminals (No.)	Synapses (No.)	Vesicles per μm of synapse (range)	Terminals (No.)	Synapses (No.)	Vesicles per μm of synapse (range)
		Excitatory axons					
In DNP for ~ 1 hour	High Mg^{2+} (*16*)	8 1*	21 1	3–10 53	6	12	80–130
For ~ 1 hour	High Mg^{2+} (*16*)	11	25	50–120	6	13	70–125
In DNP for ~ 1 hour	Regular	9 4*	18 10	4–12 30–60	5	8	35–75
For ~ 1 hour	Regular	8	15	35–70	5	10	25–70
		Inhibitory axon					
In DNP for ~ 1 hour	Regular	5	11	25–60	4	6	4–9

* Nondepleted terminals, less than 25 percent of the total number observed in stimulated DNP-treated material, found only in a few locations.

transmitter from the nerve terminal. The quantal content of the EPSP increases at first and later decreases.

When the inhibitory axon to the DNP-soaked opener muscle is stimulated instead of the excitatory axon, the inhibitory postsynaptic potential goes through a similar sequence. However, if either the inhibitory or the excitatory axon is stimulated intermittently (for 20 seconds once every 4 minutes) rather than continuously, rapid depletion of transmission does not occur. The amplitude of the EPSP evoked by this stimulation is enhanced five to eight times by DNP treatment and remains at this level after at least 1 to 5 hours in DNP. Thus, both continuous stimulation and the presence of the metabolic inhibitor are necessary for depletion of synaptic transmission.

Through its action on oxidative phosphorylation, DNP depletes the energy reserves of the cell (*10*), thereby affecting sodium extrusion and all other adenosine triphosphate-utilizing reactions. The initial enhancement of the EPSP could be due to sodium accumulation within the nerve terminal, as with ouabain (*13*), or it could be due to release of Ca^{2+} from mitochondria (*14*). The subsequent depletion could be attributable to exhaustion of the store of synaptic transmitter resulting from stimulation, if an energy supply is necessary for resynthesis or replenishment of vesicles. In this case, we would expect from the vesicle hypothesis (*3*, *15*) that synaptic vesicles would diminish in numbers in the nerve terminals subjected to stimulation, but not in unstimulated nerve terminals. Alternatively, the failure of transmission could be due to progressive blockage of nerve twigs because of sodium accumulation and the depolarization resulting from interference with the "sodium pump" (*13*). Disappearance of synaptic vesicles would not be expected in the latter case.

We prepared muscles used in the above experiments for electron microscopy, using two different methods of fixation (*5*, *16*), and examined their nerve terminals. Synapses were identified in electron micrographs (*4*, *5*). We measured maximum and minimum widths of 20 to 50 clear synaptic vesicles in each synapse-bearing nerve terminal and computed the ratio of mean maximum width to mean minimum width (designated R) for each terminal. Previously, we found that this ratio ranged from 1.07 to 1.25 in terminals judged to be excitatory (postsynaptic members of axoaxonal synapses) and from 1.35 to 1.57 in suspected inhibitory terminals (presynaptic members of axoaxonal synapses) (*5*). Synaptic vesicles lying within 0.25 μm of the synaptic membrane were counted in the terminals, and the number of vesicles per micrometer of length of the synapse was computed as a measure of vesicle abundance at different synapses.

Synapses in muscles soaked for 1 hour in DNP without stimulation, or subjected to stimulation by way of the excitatory axon for 1 hour at 10 stimuli per second without DNP treatment, usually had clusters of clear synaptic vesicles at the synaptic membrane of the presynaptic nerve terminal, as in normal unstimulated preparations. The round vesicles and the less regular shaped vesicles were present in separate terminals sometimes associated in axo-axonal synapses (Fig. 1B). Neither type of nerve terminal showed marked depletion of synaptic vesicles. Values of vesicle content are given in Table 1. It is evident that preliminary treatment with Mg^{2+} resulted in higher vesicle counts, as reported by Birks (*16*).

Muscles soaked in DNP during stimulation of the excitatory axon (10 stimuli per second) until the EPSP disappeared, showed severe depletion of synaptic vesicles in many terminals containing round synaptic vesicles, with R = 1.00 to 1.18 (Fig. 1 and Table 1). Adjacent terminals containing the less regular shaped vesicles (R = 1.3 to 1.45) usually had a full complement of vesicles; indeed, many of these endings were densely packed (Fig. 1, A and D, and Table 1).

Preparations soaked in DNP while the inhibitory axon was stimulated showed no depletion in terminals with round vesicles, but adjacent terminals with higher R values usually showed marked depletion (Table 1). We conclude that excitatory and inhibitory terminals are selectively depleted by stimulation in DNP and that depleted terminals can be identified by electron microscopy.

In a few cases, undepleted nerve terminals were observed in preparations in which their axons had been stimulated in the presence of DNP (see Table 1). This may have been due to accumulation of sodium ions within terminals of low safety factor for spike propagation, resulting in depolarization and then blockade of the axon spike before depletion of vesicles had occurred.

Since stimulation does not lead to vesicle depletion unless DNP is present, it is likely that a rapid, energy-dependent replenishment of vesicles normally occurs in these nerve terminals (*17*). Probably this process is not entirely dependent on axoplasmic flow, since it is known that motor nerve terminals of the crayfish retain normal transmission for at least several weeks when separated from the rest of the neuron (*18*). In addition, it has been suggested that synaptic vesicles may be formed directly from the presynaptic terminal membrane (*19*).

The results provide further evidence

in support of the vesicle hypothesis of synaptic transmission, since they show that impaired transmission is associated with loss of vesicles.

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Unseparated Rare Earth Cobalt Oxides as Auto Exhaust Catalysts

Since Meadowcraft (*1*) reported that strontium-doped $LaCoO_3$ was comparable to platinum as an oxygen electrode, the testing of the catalytic activity of $LaCoO_3$ on certain gas systems has been pursued by our group (*2*) and others (*3, 4*). In our study, this particular rare earth cobalt oxide again had activity which rivaled platinum. Also, earlier work by Broyde (*5*) showed that certain rare earth tungsten bronzes were active as fuel cell catalysts. Consequently, the compound was suggested as a candidate for auto exhaust catalysis. As a next step, cobalt oxides with other rare earths have been prepared, and their catalytic activities have been compared. Little difference between them has been found. This result suggested that a catalyst, just as effective, could be made from the unseparated rare earths with substantial cost savings. Tests with the rare earth mixture as mined did give equally satisfactory results.

The tests were made with a gas mixture of hydrogen (98.82 percent by volume) and *cis*-2-butene (1.18 percent by volume) at a total pressure of 1 atm. The compounds were prepared by firing in air at 1000°C for approximately 24 hours and then were ground in a ball mill. To be certain that the firing process was complete, we checked the x-ray diffraction patterns of the products for significant peaks from the reactants. Samples (1 to 4 g) of the powders were supported on a glass wool plug in a Pyrex tube (0.8 cm in diameter) and subjected to a flow rate of the test gas at some 15 cm^3/min. Various temperatures were used beginning at 100°C and progressing to 400°C; the composition of the emergent gas was determined on a 6-m gas chromatographic column (β,β'-oxydipropionitrile) with a flame ionization detector. The surface areas of the powders before catalysis were measured by the BET (Brunauer-Emmet-Teller) method with N_2.

The data obtained are given in Table 1. For each rare earth cobalt oxide the temperatures (°C), weight of sample (g), surface area (m^2/g), and contact time (seconds) are shown. The samples were freshly oxidized in air.

Hightower (*4*), using a "reduced" $LaCoO_3$ which had been heated in H_2 at 420°C for several hours, found little methane even at 419°C, the main products being the isomers and *n*-butane. Our earlier results (*2*) were similar. In our tests, water was observed to form at temperatures 300°C and above, so some reduction was occurring. Thus it seems that the strong hydrogenolysis reaction to produce methane, ethane, and propane which begins at about 300°C probably is associated with the oxidized state of the catalyst. Further investigation is needed to clarify this point. In the proposed use as the oxidation catalyst for auto exhaust this effect should be minor.

Our main conclusion is that the three

Table 1. Catalytic activity of rare earth cobalt oxides. The test gas was H_2 with 1.2 percent (by volume) *cis*-2-butene; the pressure was 1 atmosphere. The composition of the hydrocarbon was: 96.7 percent *cis*-2-butene (c-2); 3.1 percent *trans*-2-butene (t-2); 0.1 percent 1-butene (b-1); 0.1 percent *n*-butane (n-b).

T (C°)	Percentage of feed hydrocarbon converted to product							
	Δ c-2	Δ t-2	Δ b-1	Δ n-b	Δ propane	Δ ethane	Δ methane	Δ other
	$LaCoO_3$—1.23 g of 2.86 m^2/g; 5.0 seconds contact							
400	−95.8	−3.1	−0.1	0.9	0.1	0	97.7	
300	−94.3	−2.9	0	12.9	1.7	3.8	78.3	
200	− 3.5	1.7	0.8	0.7	+	+	0.1	*
	$NdCoO_3$—1.36 g of 1.4 m^2/g; 7.3 seconds contact							
400	−95.6	−3.1	−0.1	2.8	0	0	95.8	
300	−87.1	−0.5	0.6	11.1	4.2	+	71.7	†
200	− 4.3	2.3	1.0	0.9	0	0	0	
	$DyCoO_3$—1.91 g of 0.52 m^2/g; 7.0 seconds contact							
400	−95.8	−3.0	−0.1	0.8	0	0	97.8	
300	−94.4	−2.4	0	10.1	1.5	5.3	80.4	†‡
200	− 9.7	3.8	1.5	4.2	0	0	0	
	$RCoO_3$—1.17 g of 171 m^2/g; 4.0 seconds contact							
400	−95.3	−3.0	−0.1	0.2	0	0	96.8	†
300	−92.1	−1.6	0.4	3.8	0.8	3.7	83.5	*‡
200	− 3.3	1.5	0.4	1.0	+	+	0.1	

* Trace C_2H_4. † Trace *n*-pentane. ‡ Trace isobutane.