In vitro Evidence for Two Distinct Hippocampal Growth Factors: Basis of Neuronal Plasticity?

Abstract. *The rat hippocampal formation was tested for the presence of factors that would accelerate neurite extension from chick parasympathetic (ciliary ganglion) or sympathetic (lumbar chain) neurons in vitro. Two growth factors were identified in extracts of this brain region. One accelerated neurite extension from sympathetic neurons and was blocked by antiserum to nerve growth factor. The other accelerated neurite extension from parasympathetic neurons but was not affected by the antiserum. These results suggest that specific growth factors account for the specificity of neuronal sprouting.*

The rat hippocampal formation exhibits remarkably specific neuronal rearrangements after selective deafferentation (*1*). Such neuronal plasticity has been demonstrated for several hippocampal afferents that sprout only under certain conditions. In some cases the involvement of trophic or tropic factors has been suggested (*2*), and in the case of sympathetic ingrowth after cholinergic denervation the role of a factor similar to nerve growth factor (NGF) has been postulated (*3*).

A major limitation to the investigation of putative growth factors that may regulate sprouting has been the sensitivity of available bioassays. However, the discovery that neurite outgrowth from chick ciliary and lumbar sympathetic neurons can be controlled by specific additions to the culture medium allowed the development of more sensitive assays (*4*). With such an assay (*5*), it is possible to detect putative growth factors by examining their effects on the rate of neurite elongation in culture. We identified two distinct growth factors in the normal rat hippocampal formation by using this assay.

Neurons were isolated from 9-day-old White Leghorn chick embryos. Parasympathetic neurons were collected from ciliary ganglia and sympathetic neurons were collected from lumbar sympathetic chain ganglia. The neurons were plated onto culture dishes coated with a substratum factor derived from heart-conditioned medium (*4*). Forty-five minutes later the cells were exposed to medium alone (*6*) or to medium containing hippocampal extract (*7*). Some cultures in all groups received antiserum to NGF and some sympathetic cultures received medium containing commercial NGF (*8*) in the presence or absence of antiserum to NGF. Two hours after addition of the medium the cells were fixed and the total neurite length was measured for at least 30 cells in each well and averaged to obtain an index of neurite elongation. A neurite was defined as a process at least one cell body diameter in length—approximately 15 μm. Cells that had not initiated neurite elongation were not included in the analysis (*4*, *5*).

Addition of the extract to the medium accelerated the rate of neurite extension (Table 1). After 2 hours, mean neurite length for both parasympathetic and sympathetic neurons was up to five times greater in medium plus extract than in medium alone. The response of sympathetic neurons to extract plus antiserum to NGF was the same as their response to medium alone. The response of parasympathetic neurons to extract was unaffected by antiserum. The maximum response of sympathetic neurons to 2.5*S* NGF was the same as that elicited by extract and was completely blocked by antiserum. The presence of antiserum did not decrease the rate of neurite elongation from cells exposed to medium without extract.

To obtain some indication of the amount of activity present in the hippocampal formation, serial dilutions of the extract were made and added to the cells in culture. Extract diluted by as much as 1:100 still accelerated neurite elongation from parasympathetic and sympathetic neurons (Fig. 1).

The major finding in this study is the detection of substances in the adult rat hippocampal formation which will accelerate the rate of neurite elongation from chick sympathetic and parasympathetic neurons in culture. The fact that antiserum to NGF blocks the response of sympathetic, but not parasympathetic, neurons suggests the presence of at least two distinct growth-promoting factors, one of which may be NGF or a related substance. Other studies have suggested the presence of NGF-like activity in the central nervous system (*9*). The second factor is distinct from the first since its activity is not antagonized by antiserum to NGF.

The assay used in this study is not dependent on the ability of growth factors to initiate neurite extension: neurons exposed to the medium in the presence of the substratum factor extend

Table 1. Lengths of neurites extended from sympathetic (N = 50) and parasympathetic (N = 30) neurons exposed to medium alone or to medium plus hippocampal extract (dilution 1:120) in the presence or absence of antiserum to NGF (0.01 mg of antibodies per milliliter). Values are means ± standard deviations.

Neuron type	Antiserum to NGF	Neurite length (arbitrary units)*		P†
		Medium alone	Medium plus extract	
Parasympathetic (ciliary ganglion)	No	2.7 ± 0.7	5.7 ± 1.3	< .01
	Yes	2.3 ± 0.5	5.5 ± 1.6	< .01
Sympathetic (lumbar chain)	No	2.2 ± 0.9	5.6 ± 1.9	< .001
	Yes	2.7 ± 1.4	2.8 ± 1.3	

*One unit ≃ 30 μm. †Two-tailed Student's t-test with the Cochran modification for comparing means with unequal variances.

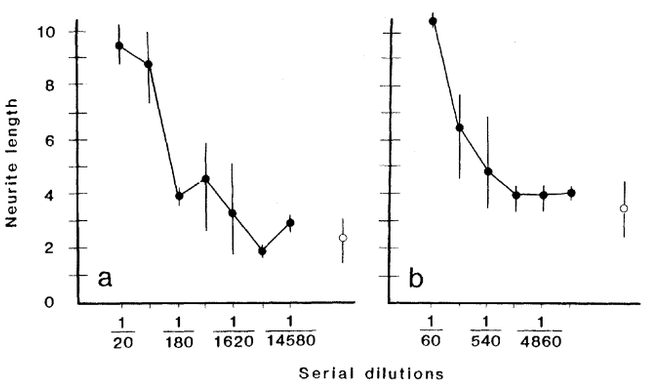

Fig. 1. Dilution curves obtained by serially diluting the extract (threefold dilutions) and adding it to parasympathetic (a) or sympathetic (b) neurons in culture. (●) Average value for three separate experiments; (○) neurite elongation from cells exposed to medium alone. Vertical lines show ranges. Neurite length is expressed as arbitrary units relative to control values (1 unit ≃ 30 μm).

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neurites (*4*). The effect of the extract on parasympathetic and sympathetic neurons is an acceleration of the rate of neurite elongation (*5*). This response cannot be due to enhanced survival of the neurons, since the cells do not normally die within 2 hours under these conditions.

The presence of at least two distinct growth factors in the rat hippocampal formation suggests that such factors are involved in development and in the specificity of neuronal sprouting after damage or deafferentation. Such specificity might be due in part to the release or "unmasking" of growth factors specific to the denervation. Different affinities of afferent fibers for such factors could provide selectivity in sprouting responses. For example, the fact that sympathetic fibers can be diverted into the central nervous system by intracerebral injections of NGF and the observation that radioactive NGF is selectively transported by septal perikarya led to the hypothesis that NGF, or a similar factor, might be involved in sympathetic sprouting after septal lesions (*3*). This hypothesis is strengthened by our finding of an NGF-like factor in hippocampal extracts.

Since sympathetic fibers invade only brain regions whose cholinergic fibers have been removed, it is possible that central cholinergic fibers normally respond to or control the availability of an NGF-like factor. This hypothesis is supported by the observation that NGF (*10*) or hippocampal extracts (*11*) increase cholinergic activity in fetal telencephalic neurons in vitro. Whether or not the substances identified in this study are actually involved in the development of the central nervous system or in denervation-induced plasticity must be addressed in the future. The detection of such substances, however, provides a basis for designing further experiments and hypotheses.

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6. We used Ham's F-12 medium supplemented with 10 percent fetal calf serum.
7. The hippocampal formation was rapidly dissected from the fresh brains of female hooded rats (150 to 200 g) and homogenized immediately or frozen for later use. A single hippocampal formation (50 mg wet weight) was homogenized in 2.5 ml of Hanks balanced salt solution and centrifuged at 100,000*g* for 30 minutes. The supernatant was saved and diluted with Hanks balanced salt solution before being added to the culture medium.
8. Commercially prepared 2.5*S* NGF and antiserum were purchased from LAREF (Cadempino, Switzerland).
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Autoradiographic Identification of Ornithine Decarboxylase in Mouse Kidney by Means of α-[5-^{14}C]Difluoromethylornithine

Abstract. *α-Difluoromethylornithine is an enzyme-activated irreversible inhibitor of ornithine decarboxylase that forms a covalent bond with the enzyme. When α-[5-^{14}C]difluoromethylornithine was administered to androgen-treated mice, only ornithine decarboxylase became labeled. Autoradiographic examination of kidney, liver, and brain indicated much more extensive incorporation of labeled difluoromethylornithine into kidney protein than into the protein of the other tissues. Such incorporation was greatly reduced by prior treatment of the mice with cycloheximide. These results correlate with the presence of ornithine decarboxylase activity which is much higher in the kidney than in the other tissues and is lost rapidly when protein synthesis is inhibited. The binding of this drug in vivo, therefore, is useful for determining the distribution of ornithine decarboxylase. The enzyme was predominantly located in the proximal tubule cells of the kidney in androgen-treated mice.*

Ornithine decarboxylase is the first enzyme in the polyamine biosynthetic pathway and is known to be highly inducible in response to a wide variety of trophic stimuli including hormones, mitogens, drugs, and tissue damage (*1*). The importance of this enzyme in cell growth has been confirmed by experiments with specific inhibitors which reduce polyamine production and inhibit cell growth (*2*). One of the most useful inhibitors is α-difluoromethylornithine (DFMO), which acts as an enzyme-activated, irreversible inhibitor (*3*). In agreement with this concept, we have observed that radioactivity becomes incorporated into the enzyme as it is inactivated by incubation with ^{14}C-labeled DFMO in vitro (*4*). Such incorporation might provide a means to selectively label the enzyme in vivo and could provide a method by which the cellular and subcellular distribution of the enzyme could be investigated. Many of the target tissues in which ornithine decarboxylase has been shown to be induced are complex mixtures of different cell types and it would be of interest to examine the relative extent to which the enzyme is affected in each one. To test whether the radioactive drug can be used in this way we have examined the enzyme in the androgen-treated mouse kidney. Ornithine decarboxylase is highly androgen responsive in the mouse, and the amount present increases substantially when castrated mice are treated with large doses of testosterone (*5*). The results indicate that [^{14}C]DFMO exclusively labels the enzyme in vivo and that the enzyme is predominantly located in the proximal tubule cells of the mouse kidney.

Androgen-treated mice were injected with [^{14}C]DFMO at a dose of 1 mg/kg and killed 60 minutes later. This treatment led to the loss of 90 percent of the ornithine decarboxylase activity in tissue extracts. When extracts from the kidneys of these mice were prepared and subjected to polyacrylamide gel electrophoresis only one labeled protein band (molecular weight 55,000) was observed. This band corresponded to the position of authentic, labeled ornithine decarboxylase (*4*). Autoradiographs of the kidney sections indicated that there was substantial radioactivity present in the area of the cortex but much less in the medulla (Fig. 1A). The labeling of the kidney was much greater and more regionally localized than that in the liver or brain (Fig. 1, C and D). These results correlate well with the ornithine decarboxylase activities in the tissues (Table 1). As an additional confirmation that the labeling did represent ornithine decarboxylase, similar autoradiographs were prepared from mice treated with cycloheximide 6 hours before they were killed. As expected from the rapid turnover of the enzyme (*1*) this treatment greatly re-

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