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NEURAGIN AS AN IMMEDIATE EARLY GENE PRODUCT *

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Abstract

Time relations and regulation of neuragin synthesis were examined with the glycine labeling method in the cerebral cortex of the rat. During activation of the somato-sensory area, 2 min. stimulation sufficed to provoke the appearance of neuragin in the neurons. The newly synthesized protein persisted in the nerve cells at least for 6 hours after the end of stimulation. Phorbol-12-monoacetate strongly enhanced, sphingosin depressed the synthesis of neuragin. It is concluded, that neuragin is an immediate early gene product, similar to proteins encoded by genes c-fos and c-jun.

Key word: neuragin.

Introduction

ROJIK and FEHÉR (1976) investigated the fate and incorporation of amino acids in situ functioning cerebral cortex. To this aim they placed on the cortical surface filter paper strips containing labeled amino acids and kept them there for varying times, typically for one hour, excised the underlying cortical tissue, fixated them and subjected the samples to light- and electron-microscopic autoradiography. This experimental paradigm proved to be suitable to decide if the applied amino acids entered the cortical tissue and were incorporated into proteins.(ROJIK and FEHÉR, 1976).

After having examined the fate and incorporation of glutamic, aspartic, gamma-aminobutyric acids and leucine, in case of glycine they observed, that this amino acid was not only incorporated into neural proteins, but this was highly dependent on the intensity of cortical function. During intensive cortical activity the incorporation of glycine was multiply higher than at rest. In presence of cycloheximide labeled glycine was not built in. An important additional finding was, that labeled glycine appeared under these circumstances only in nerve cells. In course of biochemical investigations (not yet published) it turned out, that for labeling of neurons in the stimulated neural

* This paper is dedicated to the centennial anniversary of Prof. AMBRUS ÁBRAHAM's birth.

structures a glycine rich protein (of about 200 kDa molecular weight), called by us neuragin, can be made responsible.

Since glycine autoradiography proved to be an excellent morphological indicator of neural activity, it was exploited in the physiological analysis of a good number of neural structures. Identification of neuron populations generating well defined electric signals was successfully carried out in somato-sensory, auditory, visual and motor cortices of cats and rats, in the thalamo cortical system and hippocampus of rats and in the spinal cord of frogs (ROJIK and FEHÉR, 1979, 1980, 1986a,b; ROJIK et al, 1983, 1984, 1987; TOLDI et al. 1985).

A somewhat problematic feature of this experimental paradigm was, that it has no temporal dimension, i.e. it fails to indicate the sequence of activation of different neural elements. As a first step to eliminate this failure seemed to be suitable to assess, what is the earliest time at which the glycine labeling appears, after onset of activity. Another related problem was, how long newly synthesized neuragin survives after cessation of stimulation. In this paper several observations will also be published about localization of neuragin within the neuron.

Material and Methods

Rats weighing about 250 g were anaesthetized with 1.2 g/kg urathane, intraperitoneally. The skull was opened on both sides and the cerebral hemispheres exposed. The dura was not removed, because this thin membrane did not hinder considerably the diffusion of the substances applied to the surface. The skull was fixed in a stereotaxic frame. Two steel needles were pierced into the right whisker region for activation of the left somato-sensory area. Impulses having 2/s frequency, 15 V voltage and 0.3 ms duration were capable of activating the whole barrel field and most part of the contralateral somato-sensory area. Cortical activation was in each experiment tested by recording evoked field potentials.

$[^3H]$-glycine was applied in the following manner. Solution of $[^3H]$-glycine was prepared 2x2 mm pieces of filter paper were soaked in it and dried under infrared lamp, so as to contain 3.10^6 dpm each. One piece of filter paper was placed on both somato-sensory areas. The right hemisphere serves as resting control, the left hemisphere was thrown in activity by stimulating the whisker region of the right side. After having finished the stimulation, the cortical areas, underlying to the filter papers were excised, fixed in Bouin or paraformaldehyde solution and processed for light- or electron microscopic autoradiography. Exposition lasted for 10 weeks. About further details of the procedure see ROJIK and FEHÉR (1976). Besides electric stimulation the effect of phorbol-12-acetate was also examined, as an activator of protein-kinase C (PKC).

The chemicals used were: $[^3H]$-glycine (Magyar Izotop Intézet, 92.5 GBq/ mM), Phorbol-12-monoacetate, (Sigma), sphingosine sulphate (Sigma), Ilford photo-emulsion for autoradiography.

Results

For detection of earliest appearance of neuragin in the cortical neurons, the following experimental procedure was adopted. One piece of filter paper strip containing labeled glycine was laid on both hemispheres and left there for 20 min., at rest. Thereafter the right whisker area was stimulated with the above parameters for 2,

5, or 10 min., respectively, in different animals. Then the underlying cortical samples were excised and processed as usual. The introductory 20 min. period served to prevent diffusion artifacts in the sections and assure, that differences between the two sides could be ascribed only to stimulation. The long incubation time and short stimulation period excluded interferences from side of vasomotor effects. As it can be seen in Fig. 1B, already after 2 min. of stimulation significant labeling in the left side cortical sample appeared as compared with the contralateral resting control (Fig. 1A). This indicated, that synthesis of neuragin must have started with onset of cortical excitation. Such a fast activation of genes could be observed only in case of immediate early genes. At longer stimulation periods (5 or 10 min.),the labeling caused by neuragin became even more intensive (Fig. 1C). In order to clear up, whether proteinkinase C, known to be involved into the stimulus- transcription chain, is participating in regulation of neuragin synthesis, the actions of the PKC activator, phorbol-12-monoacetate (Pha) were examined. To this aim, Pha in 10 μM concentration and glycine were applied to the right hemisphere without stimulation, for one hour. On the left side only glycine was laid. As it can be seen in Fig. 2A. activation alone was able to enhance the synthesis of neuragin and the labeling was considerably higher than on the control right side, also in absence of sensory stimuli. The relatively long duration of Pha application made it sure, that the drug reached the deepest layers of the cortex. The appearance of neuragin at these depths indicated, that diffusion of Pha was complete and PKC activation was present in the whole cortical depth (Fig. 2B).

When together with the Pha also 10 μM sphingosin, an antagonist of Pha in activating PKC, was applied, the synthesis of neuragin seemed to be rather depressed and the labelling by glycine incorporation scarcely differed from that in the untreated control (Fig. 2A and C).

The next question to be answered was the lifetime of the newly synthesised neuragin in the cortex. This was examined in the following way. The usual quantities of glycine were applied to both sides, but the left hemisphere was activated by stimulating the right whisker area for one hour. Then stimulation was stopped and the brain was left at rest for 2, 4 or 6 hours respectively, in different animals. The right hemisphere was resting control also in this case. In Fig. 1D-G it is demonstrated, that after lapse of 6 hours neuragin was present in the cortex, if not very abundantly and with somewhat different localization than in samples taken out after 1 hour rest. In these, neuragin filled out the cytoplasm and was richly present in the nucleolus (Fig. 1E and 3), while the nucleus was relatively free of it. In the 6 hour samples the neuragin was concentrated in the nuclear membrane, arranged in a spoke-like manner (Fig. 1G). When explaining this change of distribution one is led to think that i) neuragin was broken down by protease, and/or ii) was transported towards peripheral extensions of the neuron. In earlier examinations the evidences were favourable for the latter possibility.

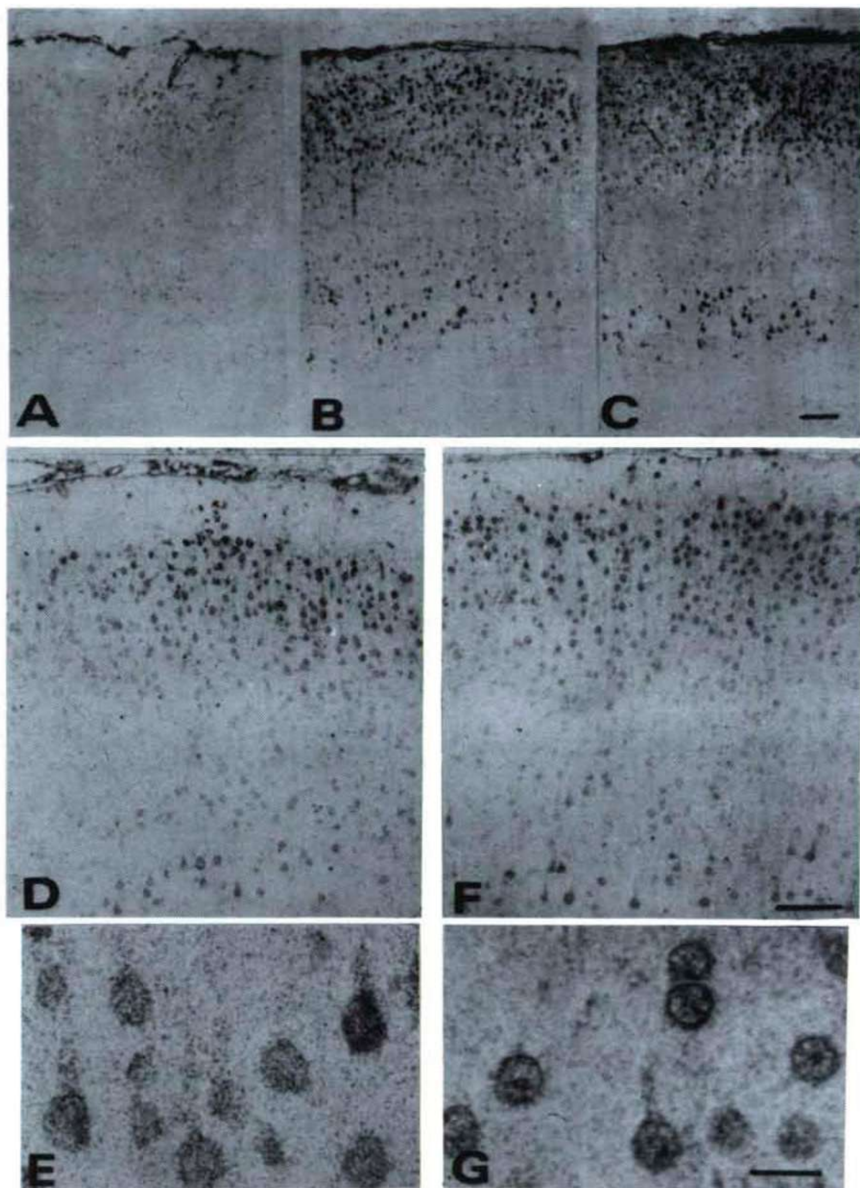

Fig. 1. Autoradiographic picture of somato-sensory cortex of rat. A. Resting control from the right side. B. Incorporation of labelled glycine after 2 min, C.after 10 min stimulation of the whisker area. D. labelling of the cortex after 1 hour rest following 1 hour peripheral stimulation, E. the same at higher magnification, F. The same after 6 hours rest following stimulation, G. The same with higher magnification. Light microscopic autoradiography, Ilford emulsion, Exposition time:10 weeks. Calibrations : 200 μm in A, B, C, D, and F; 20 μm in E and G.

Discussion

The purpose of this work was to take under examination some possible analogies between immediate early gene products and the protein, neuragin, discovered by us in the early seventies. The experimental data presented here allow some conclusions in this respect. First, the time course of initiation of synthesis shows obvious parallelisms with that of early gene products. In our experiments 2 min. of sensory stimulation was sufficient to provoke neuragin synthesis and make appear newly synthesized protein molecules of 200 kDa molecular weight. This tends to show, that the whole synthetic apparatus was in a state of readiness and awaiting only for some trigger stimulus. In the experiments of COSTA et al. (1991) after PTZ induced seizures of 20-36 second duration a significant Fos-like immunreactivity (FLI) was observable in hippocampus of the rat. Essentially the same was reported by GASS et al. (1992) after bicuculline induced seizures lasting for 15 min. BULLITT et al. (1992) applied noxious stimuli enduring from 3 s to 24 hours to rat nerves and assessed FLI in the spinal cord. After the shortest exposition to noxious stimuli FLI was present and with prolongation of stimulation periods it became more and more intensive.

As to the endurance of the immediate early gene products larger variability of data is encountered. GASS et al. (1992) claim, that KROX-2 and c-Fos returned to the base level within 8 hours, while Fos-B and Jun remained above normal until 24 hours. According to MULLER et al. (1984) and CURRAN et al. (1984) the half lifetime of the Fos protein was 2 hours. Although in our experiments was not attempted to determine the time when the newly synthesized neuragin from the neurons completely disappears, the pictures obtained at 6 hours after stimulation point to similar time course of disappearance as with Fos and Jun.

As links in the second messenger system leading to activation of the genetic apparatus, the growth factors and calcium are emphasized, there are also observations in favour for the role of phorbol esters in these events. Thus MORGAN and CURRAN (1989) found phorbol esters to be relatively weak activator of c-fos expression in PC-12 cells of the rat, but in the human cell line they were much more potent in this respect. FISCH et al. (1987) and GILMAN (1988) report about unequivocal effects of phorbol esters on immediate early gene activation.

Although to date nothing is known about regulation of neuragin synthesis, these analogies indicate that neuragin represents one of the proteins ubiquitous in the nervous system, being apparently closely involved in basic processes of the stimulus-transcription coupling. It may be noted, that some glycine rich proteins are abundant in plants and play important role in excited states and in regeneration of plant cells (KELLER et al, 1988).

Acknowledgment

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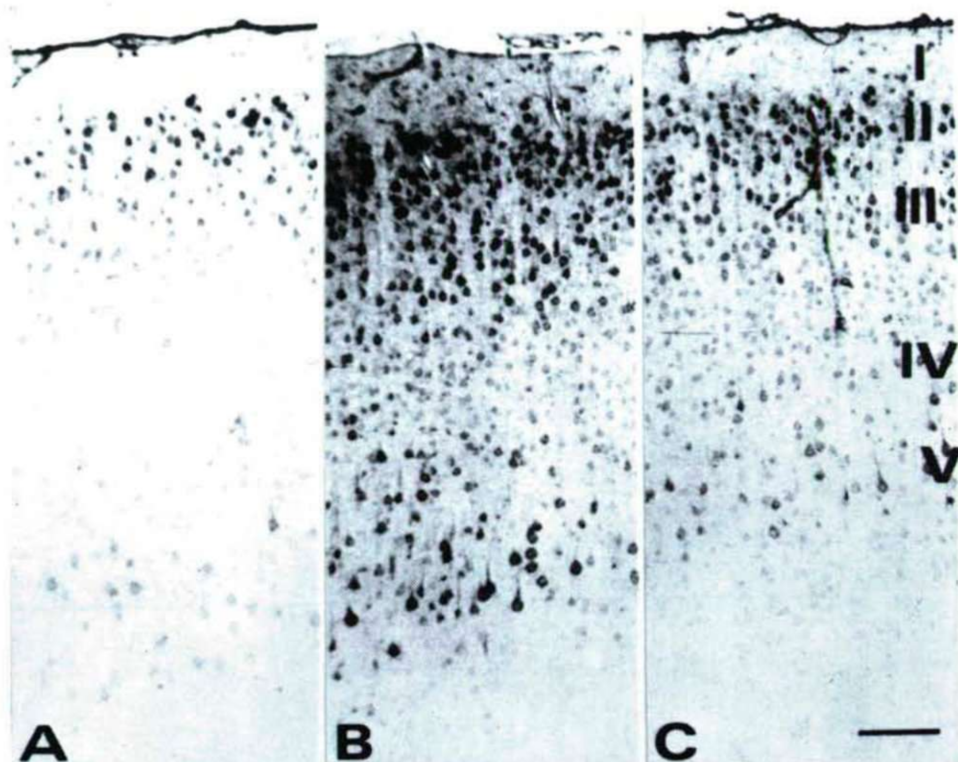

Fig. 2. Autoradiographic picture of the somato-sensory cortex of the rat. A. resting control, B.after 1 hour application of 10 μM phorbol-12-,monoacetate, C. the same in presence of 10 μM sphingosine sulphate. Roman numerals on the right denote cortical layers. Calibration:200 μm.

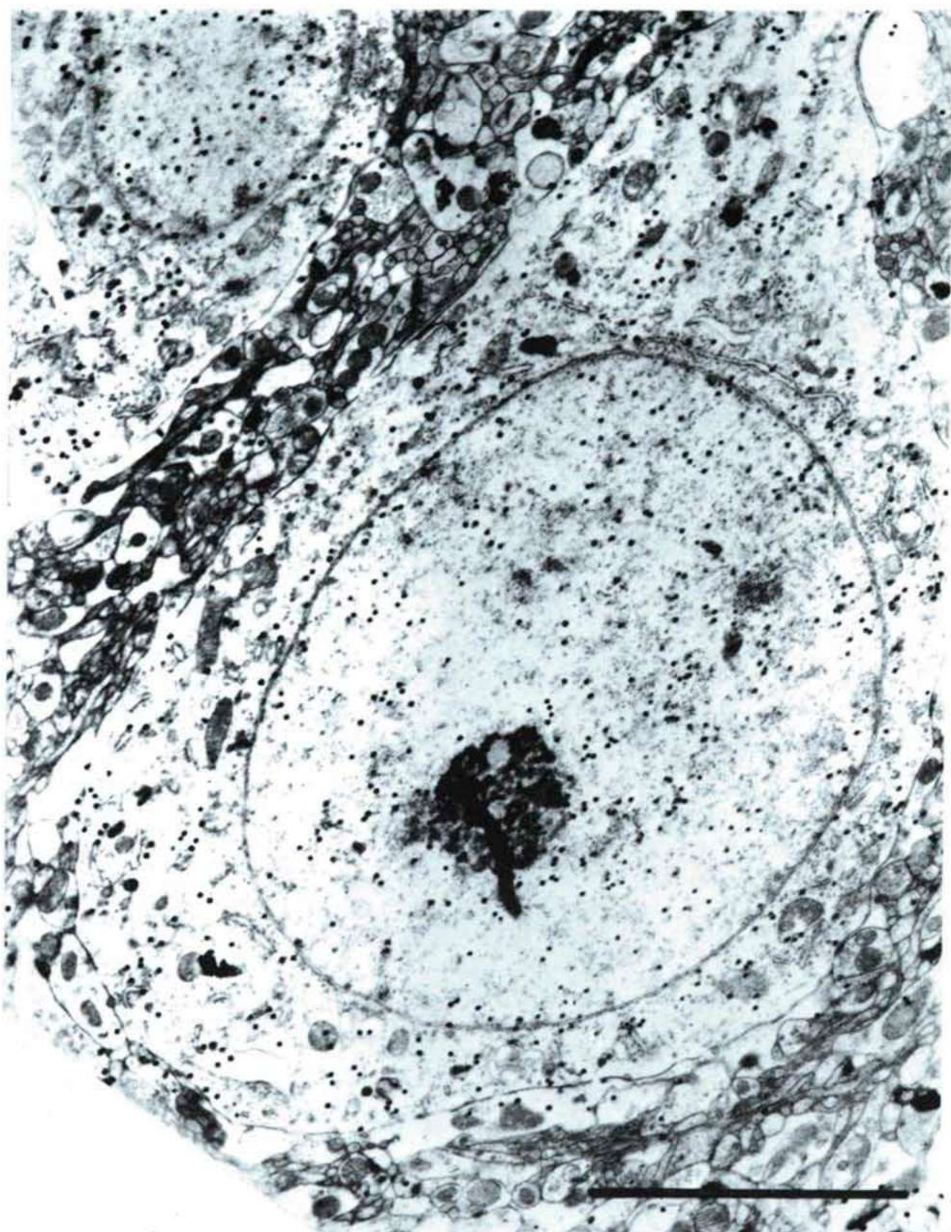

Fig. 3. Electron microscopic autoradiography of somato-sensory cortex of rat, treated with labelled glycine, after 1 hour stimulation. Small pyramid from layer III. Exposition time: 10 weeks, developed with Phenidon. Calibration: 5 µm.

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