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Transient increase of *raf* protein kinase-like immunoreactivity in the rat dentate gyrus during long-term potentiation

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Altered levels of cellular *raf* proteins (products of the *raf* protooncogenes) have been shown in the neurons of the dentate fascia of rats in response to high-frequency stimulation, with light microscopic immunohistochemistry by using polyclonal antibodies. No *raf*-1-like staining was seen in unstimulated tissue, while the pan-*raf* antibodies revealed immunoreactivity in the cytoplasm of neurons in the Ammon's horn and dentate fascia of rats and guinea pigs. The induction of long-term potentiation in the dentate fascia of freely-moving rats triggered the appearance of *raf*-1-like staining and increased the number of granule cells with pan-*raf*-like immunoreactivity. Since these proteins are serine/threonine-specific protein kinases, their appearance in long-term potentiation may indicate the activation of important cell membrane — nucleus transduction pathways.

Hippocampal long-term potentiation (LTP) is characterized by a long-lasting increase in the efficiency of impulse transmission, following a short train of high-frequency stimuli to any one of the monosynaptic excitatory pathways (for review, see ref. 2). The maintenance of LTP is subserved by several biochemical processes among which the activation [12] and translocation [1] of the Ca^{2+}/phospholipid-dependent protein kinase (PKC) seemed to play a substantial role. Recently, an additional serine/threonine protein kinase, encoded by *raf* protooncogenes [11] has been proposed to regulate the downstream transfer of information from the plasma membrane to the nucleus in mitotically activated cell cultures [8, 11]. Extending this proposal to

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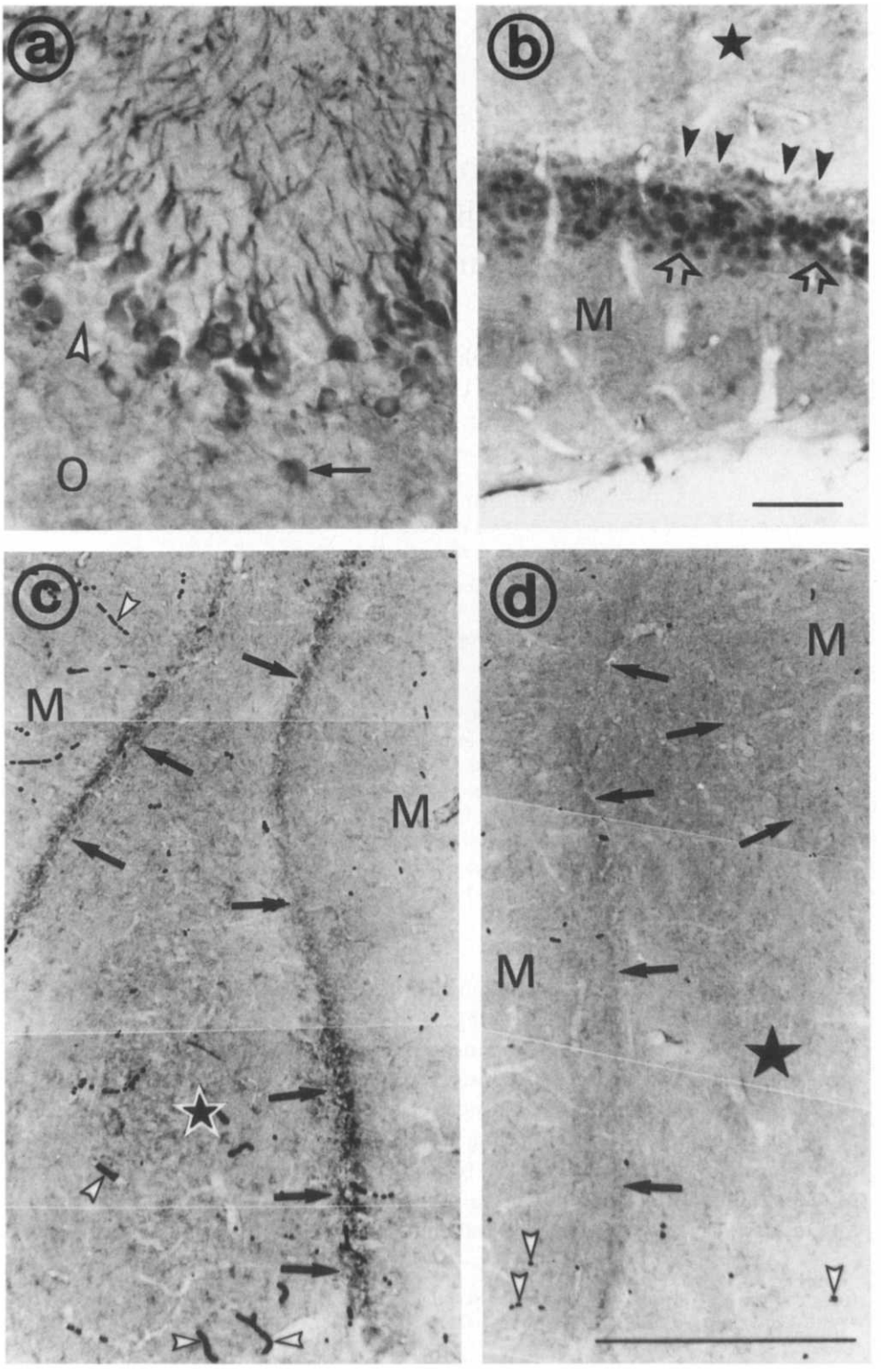
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highly differentiated nervous tissue, as a first step, the localization of the products of the *raf* protooncogenes was investigated in the hippocampus of adult rats and guinea pigs. For this purpose polyclonal antibodies against *raf* proteins were used [6, 13]. As a second step, we induced LTP in the dentate gyrus of freely moving rats and investigated the light microscopic immunohistochemical localization of *raf* proteins (the products of the *raf* protooncogenes) in the dentate gyrus.

Two male Wistar rats (130 g) and 2 male guinea pigs (650 g) were anesthetized with pentobarbital (40 mg/kg) and perfused transcardially with 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Forty-μm-thick coronal sections were cut with Vibratome, washed in phosphate-buffered saline (PBS, pH 7.4) and treated with 0.5% sodium borohydride in PBS [16]. Consecutive sections were incubated with polyclonal *raf*-1 [13] and pan-*raf* antibodies [6]. The *raf*-1 antibodies were raised against the synthetic SP 63 peptide [13] which is the C-terminal sequence (12 amino acids) of the *raf*-1 protein [3]. The pan-*raf* antibodies were raised against a larger (approx. 30 kDa) C-terminal protein expressed in *E. coli*, by inserting the v-*raf* oncogene into the bacterial DNA [6]. The antibodies were diluted to 1:500, 1:1000 and 1:2000, the sections were incubated free-floating for 24 h at 4°C, labelled by means of the avidin-biotin technique [4], and developed with diaminobenzidine-HCl as a substrate. Control sections were incubated without the primary or secondary antibodies.

In order to test the effects of stimulation and LTP, 20 Wistar rats (160–170 g males) were chronically implanted with a pair of stimulating electrodes in the medial entorhinal cortex and a recording electrode in the dentate gyrus, as described previously [7]. The animals were conditioned without signs of seizures or afterdischarges until a pronounced LTP could be observed after biphasic stimulation (a train of 300 impulses in groups of 15 with 5 s intervals and 200 Hz). LTP was induced in 10 animals. The controls (10 animals) were stimulated with constant current square wave pulses (0.1 ms, 100-400 μA, 0.2 Hz). Altogether, 16 animals in pentobarbital anesthesia (40 mg/kg) were transcardially perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at different times (5, 30, 120 and 240 min) following the control and the high-frequency stimulations (2-2 animals at each time). Coronal plane serial frozen sections (40 μm) were cut from the whole hippocampus between the electrodes, washed in PBS for 12–16 h and incubated free-floating with the two antisera — both diluted to 1:3000 — for 60 h at 4°C. The other steps of the reaction were identical with that described above. On average 30 sections per brain

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Fig. 1. Immunohistochemical detection of RPI with pan-*raf* (a,b) and *raf*-1 (c,d) antibodies in the rat hippocampus (M, molecular layer; asterisk, hilus of the dentate fascia). a: CA3 pyramidal cells containing RPI. Arrow point to interneuron in stratum oriens (O). The neurons at the arrowhead are not immunoreactive. b: granule cells of the dentate fascia containing RPI (arrows). Arrowheads point to unstained neurons. Bar for a and b = 100 μm. c: composit photograph of the dentate fascia 120 min after the induction of LTP. Strong immunostaining can be observed in the granular layer (arrows). Arrowheads point to red blood cells. d: no immunoreactivity can be seen on the contralateral side of the same section (arrows: granular layer; arrowheads: red blood cells). Bar for c and d = 0.5 cm.

were investigated. The immunostained cell bodies in the granular layer of the dentate gyrus were plotted at a 100× magnification with the help of an *xy* plotter, then a 1 mm^2 rectangle with one side parallel to the interhemispheric fissure was layed over the dentate fascia and the immunostained cells of the granular layer were counted. Since the electrodes were implanted stereotaxically, the serial frozen sections between the electrode tracks represented comparable series. The counting has been done on parallel sections, i.e. on sections bearing the same serial number. From each animal 5 sections were used for counting. In the case of *raf*-1-like immunoreactivity, no counting was performed.

The *raf* protein-like immunoreactivity (RPI) could be detected in neuronal cell bodies and dendrites in every subfield of the unstimulated hippocampal formation both in guinea pigs and rats, with the help of the pan-*raf* antibodies (Fig. 1). In the dentate gyrus the staining was mainly confined to neuronal cell bodies and sometimes neuronal cell nuclei of the granular layer (Fig. 1). Although most of the immunoreactive neurons were located in the pyramidal and granular layers, not all neurons of these layers contained RPI (Fig. 1). No attempts were made to estimate the ratio of stained:unstained neurons. Increasing dilutions of the antiserum resulted in a decrease of the staining intensity, indicating the specificity of the method [10]. The control sections incubated without the primary or secondary antibodies did not stain (except for some red blood cells). No RPI could be detected in the normal, unstimulated hippocampuses with the help of the *raf*-1 antibodies.

The overall pattern of RPI did not change after the different stimulation paradigms. No increase of staining was observed in the molecular layer of the dentate gyrus containing the nerve terminals of the perforant path [9]. However, stronger RPI was seen in the granular layer 120 min after the induction of LTP. The increase of staining was due to the increased number of granule cells containing RPI (Fig. 2). Interestingly enough, the number of immunostained cell bodies decreased thereafter, suggesting that the increase of RPI was transient (Fig. 2). No significant changes in the number of immunoreactive granule cells were seen in the contralateral, non-stimulated hippocampuses and following control stimulation (Fig. 2).

No RPI could be detected in the hippocampuses stimulated with control stimuli with the help of the *raf*-1 antibodies. However, strong immunostaining appeared 120 min after the induction of LTP, on the side of stimulation (Fig. 1). The staining outlined the granular layer in the otherwise unstained hippocampal formation (Fig. 1) and was due to a population of granule cells. The staining also disappeared at 240 min and was not seen on the contralateral side (Fig. 1).

Our immunohistochemical experiments for the first time localized *raf* proteins in the highly differentiated, adult nervous tissue. The immunoreactivity was mainly confined to the cytoplasm of the neurons which finding is in agreement with recent biochemical data [8, 14], and immunohistochemical localization studies performed on NIH 3T3 fibroblast cultures [11]. Our experiments with tetanized animals suggested the elevation of neuronal *raf* protein levels during the maintenance of LTP. The transient elevation was very obvious in the case of pan-*raf* antibodies, but not so with *raf*-1-like staining, since the latter could not be detected in non-stimulated neurons.

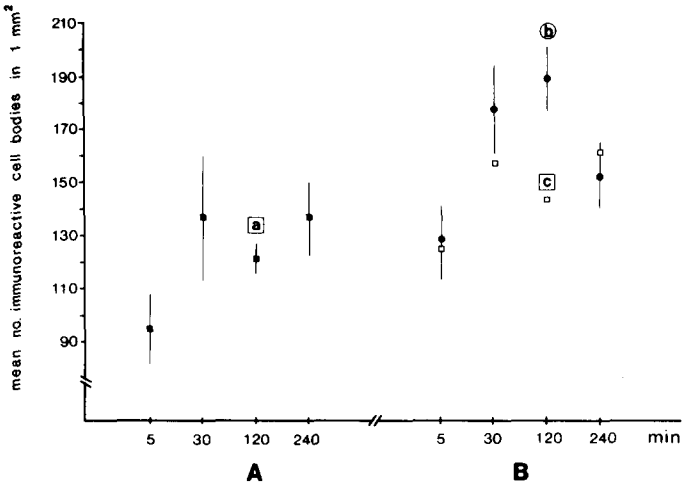

Fig. 2 Graph showing the average number of cell bodies containing pan-*raf*-like staining in the granular layer of the dentate fascia. Each point is the average of 10 measurements. A shows the counts in different time intervals after control, low frequency stimulation (■). B contains the counts after tetanization (●). The counts taken from the contralateral, unstimulated side are also depicted without the standard deviations (□). Differences between a and b ($t=8.5$; $P=0.001$, and b and c ($t=3.2$; $P=0.05$) are significant.

The RNA expression patterns of *raf*-1 in the mouse brain showed the highest level in the cerebellum, and only moderate (just above the detectable) levels in other parts of the brain [15]. This might explain the fact that RPI was not seen in the normal hippocampus. The *raf*-1 antibodies are very specific, since the SP 63 peptide is not present in other proteins of the *raf* family [5, 11]. Our results obtained in tetanized animals suggested the increase of *raf*-1 protein levels in the dentate gyrus. Due to this increase the RPI became detectable in our experiments.

The pan-*raf* antibodies, on the other hand, reacted not only with the *raf*-1 protein [14], but also with the B-*raf* protein [5] which should be a par excellence brain-related protein as shown by the RNA expression pattern [15]. On the basis of this and the similarities of the amino acid sequences [5], we think that the RPI detected with pan-*raf* antibodies should mainly be the B-*raf*-like staining. However, this has to be corroborated by reacting the tissue with specific B-*raf* protein antibodies. This work is in progress in our laboratory.

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