Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation

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RECENT studies in invertebrates indicate that a rapid genomic response to neuronal stimulation has a critical role in long-term changes in synaptic efficacy[1]. Because several of the genes (immediately early genes; IEGs) that respond rapidly to growth factor stimulation of vertebrate cells *in vitro*[2–7] are also activated by neuronal stimulation *in vivo*[8–13], attention has focused on the possibility that they play a part in synaptic plasticity in vertebrate nervous systems. Four IEGs thought to encode transcription factors, *zif/268*[5] (also termed *Egr-1*[14], *NGFI-A*[15], *Krox 24*[16]), c-*fos*[17], c-*jun*[18], and *jun-B*[7] are rapidly induced in the brain by seizure activity[8,11,13], and we have now studied the induction of these genes in a well-characterized model of synaptic plasticity in the vertebrate brain—long-term potentiation (LTP) of the perforant path-granule cell (pp-gc) synapse *in vivo*[19]. We found that high-frequency (but not low-frequency) stimulation of the pp-gc synapse markedly increases *zif/268* messenger RNA (mRNA) levels in the ipsilateral granule cell neurons; mRNA of c-*fos*, c-*jun* and *jun-B* is less consistently increased. The stimulus frequency and intensity required to increase *zif/268* mRNA levels are similar to those required to induce LTP, which is also seen only ipsilaterally, and both responses are blocked by NMDA-receptor antagonists as well as by convergent synaptic inhibitory inputs already known to block LTP[20]. Accordingly, *zif/268* mRNA levels and LTP seem to be regulated by similar synaptic mechanisms.

Continuous low-frequency stimulation of the perforant path (pp) does not produce LTP or alter IEG mRNA levels in the dentate gyrus (Fig. 1). By contrast, brief high frequency (HF) stimulation of the pp capable of inducing LTP[21,22] markedly increases *zif/268* mRNA levels in granule cell neurons of the ipsilateral dentate gyrus 30 or 60 min after the stimulus (Fig. 1; Table 1). Whereas highly reproducible increases in *zif/268* mRNA were seen following HF stimuli of the pp (12 out of 13 animals), increases in c-*fos*, c-*jun* and *jun-B* mRNA were observed in only four, six and seven of the same thriteen animals, respectively. This relatively selective increase in *zif/268* mRNA following high-frequency stimulation of the pp differs sharply from the coordinate mRNA increases of the same IEGs induced by pharmacological[13] or maximal electroconvulsive seizures (MECS) (manuscript in preparation) and is consistent with a previous report tht the *c-fos* protein is not induced by HF stimulation of the pp[12]. In subsequent studies, we therefore focused on characterizing the *zif/268* mRNA response.

Seizures induce increases in *zif/268* mRNA throughout the dentate gyri bilaterally. Increases following high-frequency stimulation of the pp however, are strictly unilateral and appear more intense in the dorsal than the ventral hippocampus. This discrete distribution of enhanced *zif/268* mRNA levels supports the premise that it reflects pp fibre activation, because most pp fibres terminate ipsilaterally[23] and the stimulating electrode is positioned to maximize synaptic potentials in the dorsal hippocampus.

A characteristic feature of IEGs is that their mRNA levels increase rapidly and transiently after specific stimuli[3,24]. After high-frequency stimulation of the pp, *zif/268* mRNA levels displayed such a pattern, increasing within 15 min ($n=2$), attaining highest levels between 30 and 60 min, and returning to basal levels by 3 h ($n=6$).

Induction of LTP by high-frequency synaptic stimulation at many synapses, including the pp-gc synapse[25], involves stimulation of NMDA (*N*-methyl-D-aspartate) receptors[26]. Therefore, we determined whether increases in *zif/268* mRNA induced by high-frequency stimulation are also mediated by NMDA receptor activation. Intraperitoneal (i.p.) administration of (+)MK-801 ($1\ mg\ kg^{-1}$), a non-competitive NMDA receptor antagonist[27] that blocks LTP[28], blocked the increase in *zif/268* mRNA in the dentate gyrus normally induced by high-frequency stimulation of the pp, whereas (−)MK-801 ($1\ mg\ kg^{-1}$, i.p.), which

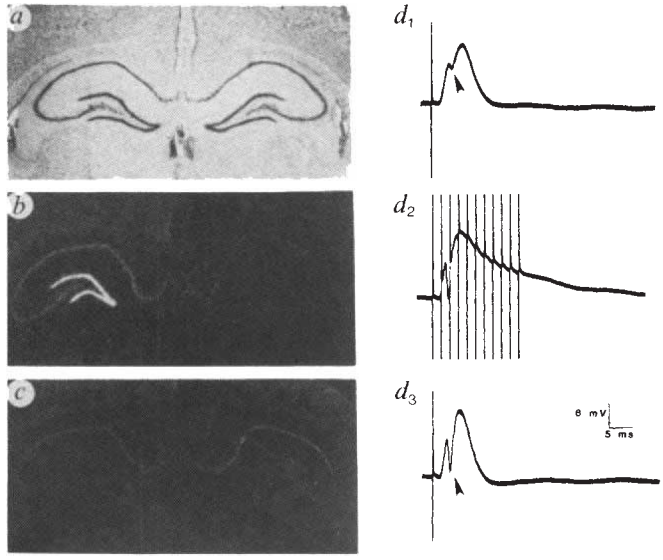

FIG. 1 High-frequency stimulation of the perforant path induces increases in *zif/268* mRNA in granule cell neurons. *a*, Rat dorsal hippocampus (×5). Tissue section stained with Cresyl violet shows the molecular and granule cell layers of the dentate gyrus. Perforant path afferents synapse on granule cell dendrites in the molecular layer. *b*, *c*, Autoradiograms of *in situ* hybridizations with ^{35}S-labelled *zif/268* riboprobe to rat dorsal hippocampus following *in vivo* orthodromic stimulation of granule cells via pp afferents. *b*, 1 h after a high-frequency pp stimulus composed of 12 20-ms bursts of monophasic pulses (50 μs) at 500 Hz, repeated at 0.1 Hz. The intensities of the individual pulses were sufficient to elicit a maximal PS response at 0.1 Hz (60 V). In 12 of 13 preparations (data pooled from animals killed 30 or 60 min after stimulation), hybridization was unilaterally increased in the dentate gyrus ipsilateral to the stimulating electrode (left side). Levels of *zif/268* mRNA induced by the high-frequency pp stimulus are similar to those induced in the dentate gyrus by pharmacological seizures[13] or MECS (Fig. 2). Low frequency stimulation. Synaptic responses to single stimuli were monitored every 10 s throughout a 1-h recording period before death. Hybridization remained at basal levels in all of nine preparations. *d*, Recordings from the dentate gyrus of the animal illustrated in *b*. d_1, Perforant path stimulation evokes a broad upgoing e.p.s.p. and superimposed PS (arrowhead). d_2, Synaptic potential induced by a high-frequency stimulus. d_3, Synaptic potential recorded 1 h after the stimulus train. The stimulus intensity is the same as in d_1. Note potentiation of the PS amplitude (arrowhead).

METHODS. Standard *in vivo* recording techniques[21,22] were used with little modification. Adult male Sprague–Dawley rats (175–225 g) were anaesthetized with chloral hydrate ($400\ mg\ kg^{-1}$, i.p.) and electrodes were placed in the dorsal dentate gyrus and ipsilateral pp at the angular bundle. Stimuli were delivered by a digital timer driving an adjustable voltage stimulus isolation unit. Electrical potentials were amplified and recorded with a digital oscilliscope. Stimulating and recording electrodes were in the left hemisphere and synaptic responses were monitored every 10 s throughout the recording period in each case (25 V, 50-μs test pulse). Because seizures[13] and kindling stimuli[11] activate IEGs, spontaneous and evoked electrical activity was monitored and no after-discharges or spontaneous bursts were observed. Animals were killed by decapitation and experimental brains were routinely co-mounted with brains from non-operated animals and from animals 30 min after MECS (UGO Basile ECT unit 7801; 85 mA, 100 Hz, 500 μsec pulses, 1 s duration) to provide stimulated and naive controls in the same histological section. *In situ* hybridization was performed as previously described[13]. To ensure specificity of *in situ* hybridization, stringent conditions were maintained during probe hybridization, and wash steps were based on empirically determined melt conditions. No hybridization of sense-strand probes was detected. RNase A pretreatment of tissue sections blocked hybridization of nick-translated cDNA probes. ^{35}S-riboprobe complementary to the 3′ non-coding region of *zif/268* gave results identical to those obtained using a full length (3.2-kilobase) antisense probe.

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is relatively inactive at the NMDA receptor[27], did not (Fig. 2). Additionally, the competitive antagonist CGS-19755[29] (10 mg kg^{-1}, i.p.), which blocks neuronal responses mediated by NMDA receptors *in vivo*[30], blocked the effect of high-frequency stimulation of the pp on *zif/268* expression. Neither (+)MK-801 (10 mg kg^{-1}) nor CGS-19755 (15 mg kg^{-1}) blocked increases in *zif/268* mRNA induced by MECS (Fig. 2). These results indicate that NMDA rceptor activation is necessary for high-frequency stimulation of the pp to induce increases in *zif/268* mRNA, and emphasize differences between IEG responses to such stimuli and to seizures.

LTP of the pp-granule cell synapse can be blocked by a crossed inhibitory input through activation of the contralateral dentate hilus[20]. Stimulation of this pathway immediately before, but not after, stimulation of the pp-granule cell synapse blocks LTP, presumably by reducing activation of postsynaptic NMDA receptors. We thefore assessed whether convergent inhibitory inputs also alter mRNA levels of *zif/268*. Results indicate that mRNA levels of *zif/268* and LTP are either induced or blocked in parallel (Fig. 3). This demonstrates that specific synaptic pathways control *zif/268* mRNA levels, presumably through modulation of events mediated by NMDA receptors.

Although NMDA-receptor activation seems to be critical in triggering both LTP and increases in *zif/268* mRNA, these responses may require quantitatively different degrees of NMDA-receptor activation. To induce LTP reliably the intensity of a train of high-frequency stimuli must exceed a critical threshold[21,22]. This threshold is thought to produce the minimum activation of the NMDA receptor necessary to induce LTP[26] and is identified as the intensity required for a single pulse to elicit a minimum population spike (PS). Therefore, we administered high-frequency stimuli to the pp composed of pulses either at or below this electrophysiological threshold. We found that increased expression of *zif/268* is induced more frequently with increased stimulus intensity and that the minimum stimulus intensity required to induce LTP is similar to that required to produce an increase in *zif/268* mRNA (Table 1).

TABLE 1 Effect of stimulus type on *zif/268* mRNA and LTP

Stimulus type	Number of animals	Number of animals showing: Increased *zif/268* mRNA	LTP	LTP and increased *zif/268* mRNA
Low frequency (0.1 Hz)	9	0	0	0
Subthreshold (500 Hz)	6	1	2	0
Threshold (500 Hz)	13	6	8	5
Maximal (500 Hz)	13	12	11	10

Relationship of stimulus frequency and intensity to increases in *zif/268* mRNA. The low frequency group is described in Fig. 1. HF (frequency parameters, see Fig. 1) stimulus trains of three different intensities were defined by the amplitude of the PS. For subthreshold stimulation, the voltage was adjusted to just below that at which individual pulses at 0.1 Hz elicit an excitatory postsynaptic potential (e.p.s.p.) without a PS. For the threshold-intensity group, the stimulation voltage was adjusted to elicit <25% of the maximal PS. The maximal PS group is described in Fig. 1. Animals were killed 30–60 min after the stimulation. Just before death, presence of LTP was determined by comparing the PS amplitude and e.p.s.p. slope at submaximal stimulus levels. Animals that displayed >50% potentiation of submaximal PS amplitude or >30% increase in e.p.s.p. slope were scored positive for LTP. To score *in situ* hybridization results, optical densities (OD) of autoradiograms were measured over the granule cell layers (Loats Image Analysis System). An increase in OD of ≥30% between the stimulated dentate and the contralateral side in at least two sections was scored as positive for an increase in *zif/268* mRNA. In the low frequency group, differences in OD never exceeded 5%. Statistical analysis of these data demonstrated that more intense stimuli induce *zif/268* mRNA more frequently ($P=0.0018$; logistic regression analysis), and indicate that threshold stimuli are sufficient to induce *zif/268* mRNA ($P<0.05$; χ^2 comparison of threshold stimulus with subthreshold and low frequency stimulus groups).

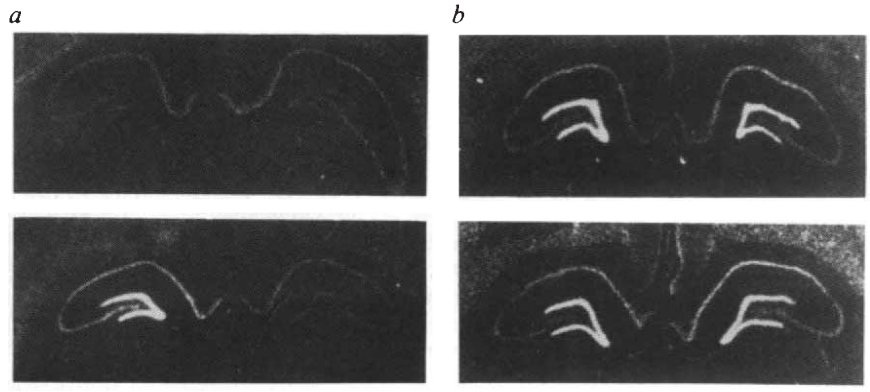

FIG. 2 NMDA antagonists block *zif/268* mRNA increases induced by high-frequency stimuli; *zif/268 in situ* autoradiograms. *a*, 30 min after a high-frequency pp stimulus as in Fig. 1. Animals were pretreated 1 h before the pp stimulus with either (+)MK-801 (upper panel) or (−)MK-801 (lower panel) (1 mg kg^{-1}, i.p.). (+)MK-801 blocked the increase in *zif/268* probe hybridization in five of six preparations whereas (−)MK-801 failed to block the effect in each of two preparations. Low-frequency synaptic potentials appeared normal in these preparations. *b*, 30 min after MECS. Animals were pretreated for 1 h with either (+)MK-801 (10 mg kg^{-1}) (upper panel) or an identical volume of solvent (ethanol) (lower panel). (+)MK-801 failed to block MECS induced increases in *zif/268* mRNA ($n=3$).

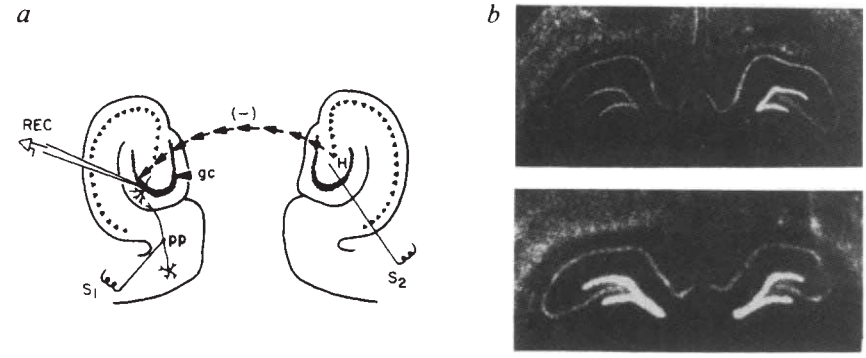

FIG. 3 Inhibitory projections from the contralateral dentate hilus block high-frequency pp stimulus-induced increases in *zif/268* mRNA in the dentate gyrus. *a*, Electrode positions and crossed inhibitory input. Standard stimulating (S_1) and recording (REC) electrodes were positioned as described in Fig. 1. The intensity of the S_1 stimulus was adjusted to evoke a maximum PS response at 0.1 Hz (60 V). A second bipolar stimulating electrode (S_2) was placed in the contralateral dentate hilus and the intensity adjusted so that an S_2 stimulus delivered 10 ms before S_1 blocked the orthodromically evoked PS (60–80 V). At these defined intensities, high-frequency stimulus trains (as described in Fig. 1*a*) were delivered to both S_1 and S_2. LTP is blocked if S_2 immediately precedes S_1 but not if their order is reversed[20]. *b*, *zif/268 in situ* autoradiograms. In three of four preparations in which S_2 preceded S_1, *zif/268* mRNA in the orthodromically stimulated dentate gyrus remained at basal levels (top panel; left dentate gyrus). By contrast, if S_1 preceded S_2, typical increases in *zif/268* mRNA were induced by the HF pp stimulus (*bottom panel*; left dentate gyrus, $n=2$). Stimulation of the dentate hilus directly with the S_2 electrode enhanced *zif/268* mRNA levels in the ipsilateral dentate gyrus in all preparations (*top and bottom panels*; right dentate gyri). However, stimulation of S_2 alone did not change *zif/268* mRNA levels in the contralateral (left) dentate gyrus ($n=2$).

In most animals, LTP and an increase in *zif/268* mRNA coincide; in a few, however, we noted a dissociation between these responses. Although both responses seem to require similar degrees of NMDA-receptor activation, this dissociation may represent differential activation of other steps in the cascades leading to the electrophysiological and mRNA responses. Alternatively, it may reflect technical limitations in detecting localized or small increases in *zif/268* mRNA.

In summary, these results indicate that the amount of *zif/268* mRNA is rapidly increased by synaptic mechanisms that involve activation of NMDA receptors. This increase shares several features with LTP, a well-characterized NMDA-dependent phenomenon, including induction by high but not low-frequency stimuli, blockade by NMDA-receptor antagonists and by convergent inhibitory inputs, and similar stimulus-intensity thresholds. Because *zif/268* encodes a presumed transcription regulatory factor, it could be important in coordinating changes in gene expression that maintain synaptic plasticity. Studies of the transcriptional regulation of *zif/268* could provide insight into the transduction processes leading from NMDA-receptor activation to gene expression. □

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High-frequency nanometre-scale vibration in 'quiescent' flagellar axonemes

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THE movement of cilia and flagella is based on the interaction between dynein arms and microtubules coupled with ATP hydrolysis. Although it is established that dynein arms cause adjacent microtubules to slide, little is known about the elementary process underlying the force production. To look more closely at the mechano-chemical conversion mechanism, we recently developed an optical method for measuring a nanometre-scale displacement with a time-resolution better than 1 ms. We now report the detection of high frequency (~300 Hz) vibration of sub-nanometre amplitude in non-beating flagellar axonemes. This vibration could reflect the movement of individual activated dynein arms.

The ATPase activities of dynein can be activated by the presence of microtubules[1–3]. Thus dynein has higher ATPase activities when contained within an axoneme than when isolated. Such ATPase activation is a prerequisite for a dynein–microtubule system to work efficiently[4]. Curiously, however, activation takes place even in axonemes that are rendered non-motile. We suspected that an uncoordinated force would be produced in such quiescent axonemes with activated ATPase activities, and therefore looked for structural fluctuations in the axoneme.

Flagellar axonemes of sea urchin sperm had their membranes removed and were fragmented and placed between a glass slide and a coverslip. Polystyrene microbeads were attached to the axonemes (Fig. 1), and the specimen was perfused with a reactivation solution. These axonemes did not beat, despite the presence of ATP, because they were detached from heads. The

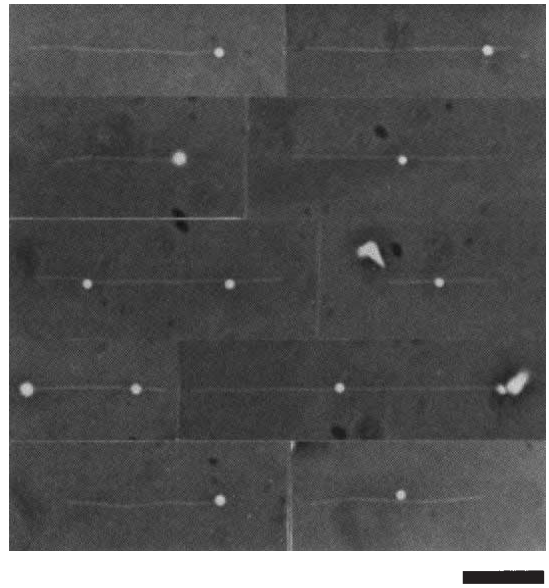

FIG. 1 Phase-contrast images of polystyrene microbeads attached to fragmented flagellar axonemes. Scale bar, 10 μm.
METHODS. Sea urchin (*Hemicentrotus pulcherrimus*) spermatozoa were demembranated at 4 °C with a solution containing 0.04% (w/v) Triton X-100, 10 mM Tris-HCl (pH 8.0), 150 mM KCl, 2 mM $MgSO_4$, 1 mM EDTA and 1 mM dithiothreitol (DTT). After being kept in this solution for 30–60 s, the axonemes were transferred to a standard buffer solution containing 20 mM Tris-HCl (pH 8.0), 200 mM potassium acetate, 2 mM $MgSO_4$, 1 mM EGTA, 0.5 mM EDTA and 1 mM DTT, and fragmented with a Teflon homogenizer. The fragmented axonemes were introduced between a glass slide and a coverslip held 0.8 mm apart with a pair of coverslip strips. After standing for 2–3 min, axonemes that did not attach to the glass surface were washed out by perfusion with the standard buffer solution. Polystyrene microbeads (Polybead-Carboxylate Monodisperse Microbeads, Polyscience Co.) of 0.9 μm diameter were attached to the axonemes by perfusing the sample with a 0.025% (w/v) suspension in the standard buffer. ATPase activity of the axoneme preparation was measured in the presence of 1 mM ATP after sperm heads were removed by centrifugation[1]. The liberated phosphate and the protein concentrations were determined by the methods of Anner and Moosmayer[23] and of Lowry *et al.*[24], respectively.

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