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Somatodendritic expression of an immediate early gene is regulated by synaptic activity

(hippocampus/neuronal plasticity/seizure/long-term potentiation/gene induction)

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ABSTRACT **Trans-synaptic activation of gene expression is linked to long-term plastic adaptations in the nervous system. To examine the molecular program induced by synaptic activity, we have employed molecular cloning techniques to identify an immediate early gene that is rapidly induced in the brain. We here report the entire nucleotide sequence of the cDNA, which encodes an open reading frame of 396 amino acids. Within the hippocampus, constitutive expression was low. Basal levels of expression in the cortex were high but can be markedly reduced by blockade of *N*-methyl-D-aspartate receptors. By contrast, synaptic activity induced by convulsive seizures increased mRNA levels in neurons of the cortex and hippocampus. High-frequency stimulation of the perforant path resulted in long-term potentiation and a spatially confined dramatic increase in the level of mRNA in the granule cells of the ipsilateral dentate gyrus. Transcripts were localized to the soma and to the dendrites of the granule cells. The dendritic localization of the transcripts offers the potential for local synthesis of the protein at activated postsynaptic sites and may underlie synapse-specific modifications during long-term plastic events.**

Activity-dependent alterations of neuronal connectivity are characteristic of plastic events in the nervous system. Plasticity is associated with physiological processes such as learning and memory as well as neuropathological states, including epilepsy. Seizure episodes set in motion a cascade of events that include gene expression, sprouting of fibers, and the establishment of new synaptic contacts (1). These long-lasting alterations are reminiscent of changes that occur during long-term potentiation (LTP) of synaptic transmission in the mammalian brain. LTP is an activity-dependent and persistent enhancement of synaptic efficacy that may underlie certain forms of long-term memory (2, 3). In both invertebrates and vertebrates, long-term memory differs from short-term memory in that it requires RNA and protein synthesis (4, 5). As is the case for memory in the intact animal, LTP is blocked by inhibitors of RNA and protein synthesis (6–9). Attention therefore has been focused on identifying activity-induced genes. In invertebrates, behavioral training elicits changes in the level of specific mRNAs in cells involved in learning (10, 11). In mammalian brain and spinal chord neurons, a variety of physiological and pathological stimuli induce rapid and transient activation of immediate early genes (IEGs) (12). Many of the IEGs encode transcription factors that may control the expression of downstream effector genes (13–15). More recent studies have identified genes that may themselves have effector function with the potential to quickly promote long-term alterations in neuronal phenotype during plastic processes, including LTP (16–20). Although the link between gene expression and neuronal plasticity seems to be well established, it remains an open question how transcriptional activation taking place in the nucleus can selectively modify stimulated synaptic sites in the distant dendritic compartment of the neuron. Such selective modifications of synapses that have experienced coincident activity are required by the Hebbian rule and might be a prerequisite for the input specificity of LTP (21).

We here identify an *a*ctivity-*r*egulated *g*ene (arg3.1)‖ in the mammalian brain and examine its spatiotemporal expression in response to natural and pathologic forms of synaptic activity, as well as stimuli that lead to the induction of LTP. Our observation that synaptic activity dramatically enriched mRNA levels of arg3.1 in dendritic laminae may, in conjunction with local protein synthesis at activated dendritic spines, provide a mechanism to specifically modify stimulated synapses.

MATERIALS AND METHODS

Animal Preparation and *in Vivo* Electrophysiology. Adult male Sprague–Dawley or Wistar rats were used in all experiments. Cycloheximide (CHX; 120 mg/kg), MK-801 (3 mg/kg), and pentylenetetrazole (PTZ; 50 mg/kg) were administered by i.p. injection. Animals that received CHX and PTZ were injected with CHX 0.5 hr prior to PTZ. Control animals were injected with similar volumes of isotonic saline. All animals were sacrificed by guillotine decapitation at appropriate times. Freely moving male Wistar rats (8–9 weeks) were anesthetized and prepared for chronic electrophysiological recording as described (8). Electrodes were positioned under visual control while monitoring electrical activity. Stimulation electrodes were placed in the perforant path; recording electrodes were placed in the granule cell layer of the dentate gyrus. Rats were allowed 8–10 days to recover from surgery. Test potentials (40% of maximal population spike amplitude) were elicited by biphasic square-wave constant current pulses of 0.2-ms duration with a frequency of 0.2 Hz. Ten consecutive potentials were recorded in a test series and averaged on-line. LTP was induced by 3-fold tetanic stimulation of the perforant path (interburst interval, 15 min). Each burst consisted of 20 trains of 15 pulses having the same duration and intensity as the test pulses. The frequency within the trains was 200 Hz; the interval between the trains was 5 s. LTP was verified by following the time course of the population spike for the duration of the experiment. The same number of stimuli, but with a frequency of 0.2 Hz, was delivered to low-frequency

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Abbreviations: LTP, long-term potentiation; CHX, cycloheximide; PTZ, pentylenetetrazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NMDA, *N*-methyl-D-aspartate; IEG, immediate early gene.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z46925).

controls. After this treatment, the rats were sacrificed at 1 hr (n = 3 for control and LTP) and 4 hr (n = 3 for control and LTP).

Cell Line. Rat-6 fibroblasts were grown in Dulbecco's modified Eagle medium supplemented with 5% (vol/vol) fetal calf serum. Quiescent cultures were obtained by incubating cells for 48 hr in 1% fetal calf serum. For serum stimulation, quiescent cells were incubated with 20% fetal calf serum for 0.5, 1, and 4 hr (22).

Molecular Cloning. Poly(A)$^+$ RNA from hippocampi of rats that had undergone PTZ-induced seizures in the presence of CHX was isolated 3 hr posticta recovery and selected by oligo(dT)-cellulose column. The directional cloning protocol from GIBCO/BRL was used to generate a cDNA library in the pSPORT-1 vector (BRL). The library comprising 5 × 10^6 independent clones was amplified in solid state, and 2000 clones were plated for differential screening. Replica lifts were probed with ^{32}P-labeled cDNA synthesized from poly(A)$^+$-selected mRNA of control cortices and stimulated hippocampi. This protocol allowed us to search for activity-regulated and hippocampus-specific genes in the same screen. Differentially hybridizing clones were selected and further analyzed by differential Southern hybridization with ^{32}P-labeled cDNA probes from control and stimulated tissue. From this screen, we isolated arg3.1, c-fos, and one clone predominantly expressed in hippocampus but not induced by synaptic activity.

DNA Sequencing and Analysis. Both strands of a full-length arg3.1 cDNA were sequenced as double-stranded plasmid with multiple synthetic primers by the dideoxynucleotide chain termination method (23). The nucleotide sequence was analyzed using Genetics Computer Group software (University of Wisconsin, Biotechnology Center, Madison, WI).

Northern Blot Analysis. Total RNA was isolated, and Northern blot analysis was carried out as described with 5 μg of total cellular RNA per lane (16). The probe used in Northern analysis was a 3.1-kb cDNA insert of arg3.1 labeled by random priming using [α-^{32}P]dCTP. The probe used as loading control was a random-primed cDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (24). For quantification, Northern blots were analyzed on a bioimaging analyzer (Bas 2000; Fujix). Phosphorescence-stimulated luminescence signals of arg3.1, GAPDH reference, and blank areas were determined using IMAGE ANALYZE software (Fuji). After background subtraction, arg3.1 signals were normalized relative to cognate GAPDH reference signal as described (25).

***In Situ* Hybridization.** *In situ* hybridization was as described (SureSite; Novagen). Frozen control and experimental tissue was thaw-mounted on the same coated slide to ensure identical hybridization conditions. Uridine 5′-[α-[^{35}S]thio]triphosphate-labeled arg3.1 antisense RNA probe was transcribed from the SP6 promoter of a *Sal* I-linearized pSPORT-1 plasmid (BRL) containing 3.1 kb of the arg3.1 cDNA insert. A sense strand uridine 5′-[α-[^{35}S]thio]triphosphate-labeled arg3.1 probe of identical length was transcribed from the T7 promoter of the *Not* I-linearized template and used as a negative control. Selected slides were treated with photographic emulsion and counterstained with cresyl violet.

RESULTS

We used differential cloning techniques to isolate arg3.1 and determined the entire cDNA sequence of 3018 nucleotides. The cDNA encodes a predicted open reading frame of 396 amino acids (Fig. 1). The putative 5′-noncoding sequence includes two in-frame stop codons. The open reading frame is followed by a termination codon and 1630 nucleotides of nontranslated 3′ sequence with a poly(A) addition signal (AATAAA) 12 nucleotides from the 3′-terminal poly(A) tail. The predicted protein has a calculated M_r of 45,352, has a pI of 5.3, and is hydrophilic. There are no identifiable signal sequences or hydrophobic stretches of sufficient length for a putative membrane-spanning region. A FASTA search of the European Molecular Biology Laboratory and GenBank data bases suggests that arg3.1 might be distantly related to brain α-spectrin, a major constituent of the cytoskeletal network (Fig. 1) (27, 28).

Expression of arg3.1 mRNA was assayed in Northern blot analysis (Fig. 2). The corresponding transcripts had a size of 3.1 kb and showed a low constitutive level of expression in hippocampus but were strongly induced following seizure activity after 0.5 hr and were further increased after 4 hr. Induction was independent of new protein synthesis as it occurred in the presence of the protein synthesis blocker CHX (Fig. 2 *A* and *B*). The basal level of arg3.1 mRNA was higher in cortex than in hippocampus (Fig. 2 *C* and *A*). Systemic administration of the *N*-methyl-D-aspartate (NMDA) receptor blocker MK-801 reduced basal levels of arg3.1 mRNA within 4 hr in cortex (Fig. 2 *C* and *D*). As observed in hippocampus, seizure activity led to a rapid increase in arg3.1 transcripts in cortex. However, in cortex, induction was transient and transcripts were down-regulated 4 hr after the onset of seizure (Fig. 2 *C* and *D*). Treatment with CHX blocked the down-regulation, as is typical of immediate early genes (Fig. 2 *C* and *D*) (12, 22, 29).

To further characterize arg3.1, we examined constitutive expression in several tissues. We found highest expression in the brain and very weak but detectable hybridization signals in kidney, stomach, liver, spleen, lung, muscle, and heart (data not shown). Since constitutive expression was apparent in different tissues, we were interested in determining whether arg3.1 mRNA can be induced in fibroblasts by growth factors. Fig. 2 *E* and *F* shows that in quiescent fibroblasts basal expression is below the level of detection. Serum stimulation increased transcript levels transiently after 0.5 hr and 1 hr, and transcripts returned to basal levels after 4 hr.

Using *in situ* hybridization, we examined the anatomical distribution of basal and induced levels of arg3.1 mRNA in the brain. In agreement with our Northern blot analysis, we observed a high constitutive level of arg3.1 mRNA in cortex.

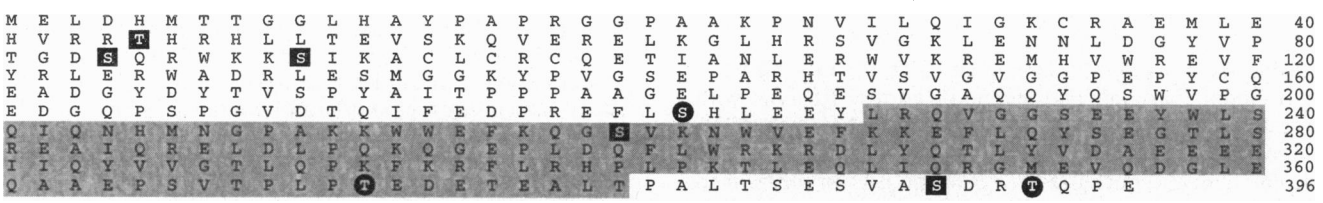

FIG. 1. Deduced amino acid sequence of the arg3.1 cDNA insert. Amino acids are given in the single-letter code and are numbered at right. Putative phosphorylation sites for protein kinase C and casein kinase II are in black boxes and circles, respectively. The 152-amino-acid C-terminal fragment of arg3.1 (positions 228–380), which shares 20% sequence identity with human and chicken brain α-spectrin (GenBank accession nos. P07751 and J05243, respectively), is shaded. The alignment scores were Int1, 45; Intn, 85; Opt, 76. arg3.1 shares a similar sequence identity with rat brain α-spectrin (20% identity; positions 228–362). Sequence comparison was performed using the FASTA program (26). The sequence data for 3018 nucleotides of the arg3.1 cDNA are available from the GenBank data base (accession number Z46925).

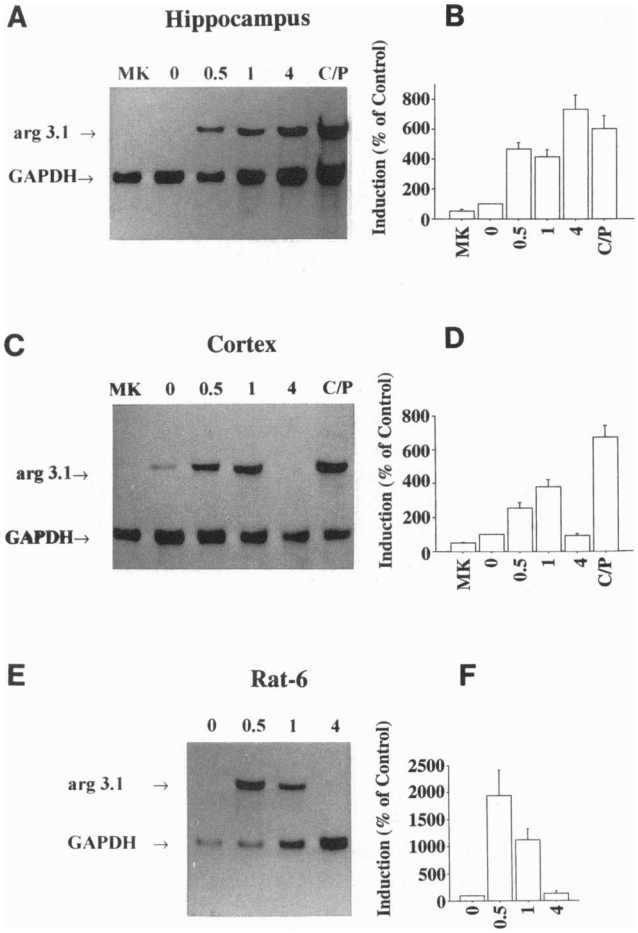

FIG. 2. Regulation of arg3.1 mRNA levels in the hippocampus, cortex, and Rat-6 fibroblasts. (*A*) Autoradiograph of Northern blot analysis of RNA extracted from hippocampus. Five micrograms of total RNA was loaded per lane. The blot was hybridized to a probe specific for arg3.1. Hybridization to a probe specific for GAPDH was used as a loading control (24). Lane MK, RNA isolated 4 hr after animals received the NMDA receptor blocker MK-801. Lane 0, RNA of saline-injected control animals. Numbers above the lanes indicate time in hours after the onset of PTZ-induced seizures at which RNA was isolated. Lane C/P, RNA isolated 4 hr after the onset of seizure in the presence of CHX. The positions of arg3.1 and GAPDH RNAs are indicated. (*B*) Quantification of hippocampus Northern blots given in bar diagrams. Error bars indicate SEMs ($n = 3$). Abbreviations are as in *A*. (*C*) Regulation of arg3.1 mRNA levels in the cortex. Autoradiograph of Northern blot analysis of RNA extracted from cortex. RNA amounts, probes, and abbreviations are as in *A*. (*D*) Quantification of cortex Northern blots given in bar diagrams ($n = 3$). (*E*) Regulation of arg3.1 mRNA levels in Rat-6 fibroblasts. Autoradiograph of Northern blot analysis of RNA extracted from Rat-6 fibroblasts. Total RNA from quiescent or serum-stimulated Rat-6 fibroblasts was used. Numbers above the lanes indicate time in hours after serum stimulation. RNA amounts and probes are as in *A*. (*F*) Quantification of Rat-6 fibroblast Northern blots given in bar diagrams ($n = 3$). Abbreviations are as in *E*.

Uninduced expression was high in layers II, III, IV, and VI of the occipital, parietal, and temporal cortex. Regions with lower but detectable levels of expression included the CA1 field of the hippocampus, the caudate putamen, and the reticular thalamic nuclei (Fig. 3 *B* and *C*). Hybridization to areas of white matter, the cerebellum, and the dentate gyrus was essentially the same as in the sense control (Fig. 3 *A–C*). Seizure activity induced mRNAs for arg3.1 in several areas of the brain. Strong hybridization signals were observed after 1 hr of postictal recovery in the neocortex, the piriform cortex, and amygdala. Strongest induction was seen in the granule cell layer of the dentate gyrus of the hippocampal formation (Fig. 3 *F*). Levels of transcripts were decreased to basal levels 4 hr after seizure in all neocortical layers, except for layer II. By contrast, at the same time, mRNAs for arg3.1 were maximally increased in the dentate gyrus and were still elevated in the piriform cortex (Fig. 3 *G* and *H*). Light microscopic analysis of emulsion autoradiographs suggests a purely neuronal expression of arg3.1; e.g., staining of cell bodies was evident in the stratum pyramidale of the hippocampus proper, whereas no somata were stained in laminae containing glial cells and scarcely scattered interneurons (Fig. 3 *D*) (30). Seizure activity resulted in a dramatic increase in the density of silver grains in the dentate gyrus. Most remarkably, we found arg3.1 mRNA transcripts localized to the dendrites of the granule cells in the stratum moleculare (Fig. 3 *I*). Dark-field emulsion photomicrographs at higher magnifications show that the hybridization signals for arg3.1 in the molecular layer were not associated with Nissl-stained somata but were uniformly distributed, providing evidence for the unusual localization of arg3.1 mRNA to the granule cell dendrites (Fig. 3 *E* and *J*).

Finally, we examined long-term potentiation of synaptic transmission in the hippocampus, a primary experimental model for investigating the synaptic basis of learning and memory in vertebrates (31). To evoke LTP in granule cells, we delivered three bursts of high-frequency trains to the perforant path in freely moving rats. The high-frequency stimulation caused LTP and a marked increase in the level of arg3.1 mRNA in the soma and dendrites of the ipsilateral granule cells 1 hr after the treatment (Fig. 4 *C*). Four hours after induction of LTP, the number of transcripts returned to basal levels (Fig. 4 *D*). Delivery of the same number of stimulus pulses at low frequency, which did not evoke LTP, also did not alter the mRNA levels of arg3.1 (Fig. 4 *A* and *B*).

DISCUSSION

We cloned the IEG arg3.1. Our studies provide evidence of expression and regulation of arg3.1 mRNA in the brain, where synaptic activity markedly increased mRNA levels in discrete populations of neurons. Basal expression of arg3.1 RNA was high in cortical areas, particularly in the visual cortex. In cortex, NMDA receptors make a major contribution to normal excitatory synaptic transmission (32–34). As was previously shown for the transcription factor zif268 (35, 36), we found that blocking the NMDA receptor led to a marked reduction in the basal level of expression of arg3.1 mRNA in cortex. We suggest that the high constitutive expression of arg3.1 in cortex is driven by naturally occurring activation of the NMDA receptor—e.g., by visual experience. Markers for arg3.1 may therefore prove to be useful for monitoring synaptic activity in cortical neurons. After seizures arg3.1 mRNA was rapidly induced in cortex and hippocampus. In cortical layers, induction was transient and transcript levels were down-regulated to basal levels 4 hr after the onset of seizures. By contrast, at this time the number of transcripts was strongly elevated in the dentate gyrus. Induction in cortex and hippocampus was markedly increased in the presence of CHX, as is typical of IEGs (12, 22, 29). We cannot determine from these experiments whether the observed induction of mRNA is due to transcriptional activation or modulation of mRNA stability. The 3′-noncoding region of arg3.1 contains a single AUUUA motif at position 2999, believed to be a signal for rapid mRNA turnover (37). The apparent differences in the temporal expression of arg3.1 after seizure induction in cortex and hippocampus might be due to differential transcriptional shutoff mechanisms. Alternatively, they might reflect different propagation of epileptic discharges with postictal depression of synaptic activity in cortex (38). In line with the latter argument, we saw a solely transient induction of arg3.1 transcripts in the hippocampus during LTP. The induction of LTP and of arg3.1

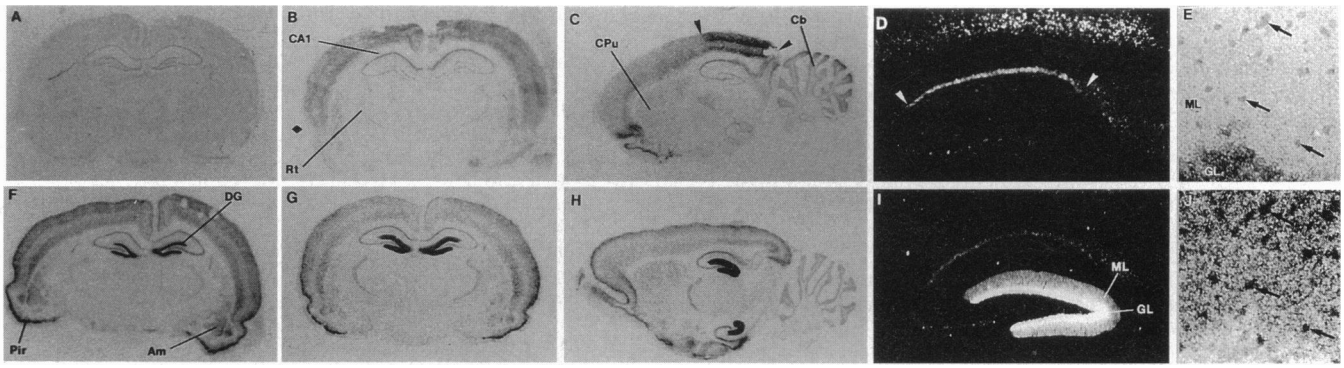

FIG. 3. Spatiotemporal expression of arg3.1 mRNA in brain after seizure. Coronal (*A*, *B*, *F*, and *G*) and sagittal sections (*C*, *D*, *H*, *I*, and *J*) were assayed for arg3.1 mRNA using *in situ* hybridization with sense (*A*) and antisense probes (*B–J*). (*B–D*) Control animals. (*F*) Animal 1 hr after seizure. (*G–J* and *E*) Animals 4 hr after seizure. (*A–C* and *F–H*) Autoradiographs. (*D*, *I*, and *J*) Dark-field emulsion photomicrographs. (*E*) Bright-field photomicrograph corresponding to *J*. Hybridization in the molecular layer is uniformly distributed and not associated with Nissl-stained somata (arrows). Am, amygdaloid complex; CA1, CA1 field of the hippocampus; Cb, cerebellum; CPu, caudate putamen; DG, dentate gyrus; GL, granular layer; ML, molecular layer; Pir, piriform cortex; Rt, reticular thalamic nucleus; diamond demarcates boundary between temporal and perirhinal cortex; black arrowheads indicate boundaries of occipital cortex; white arrowheads show the borders of the CA1 subfield; arrows point to somata in the molecular layer.

mRNA was correlated and spatially confined to the granule cells of the ipsilateral dentate gyrus. Most strikingly, after LTP and seizure activity, we observed strong hybridization signals in the stratum moleculare of the dentate gyrus. The staining pattern suggests that arg3.1 mRNA is localized to the dendrites of the granule cells. To date only a few mRNAs have been identified to be prominent in the dendritic laminae (39). To our knowledge, arg3.1 represents the first example of a gene whose mRNA occurs in the dendrites and that is regulated by synaptic activity.

Several IEGs in the brain encode transcription factors (12, 40). The localization of arg3.1 mRNA to the dendrites of neurons suggests that arg3.1 is a member of a family of IEGs that have other functions. Recently tissue plasminogen activator, cyclooxygenase (Cox-2), and a ras-related gene (rheb) were reported to be rapidly regulated by seizures and LTP-inducing stimuli (16, 18, 20). These genes represent early effector genes that might quickly promote changes in the activated neurons. arg3.1 might be distantly related to brain α-spectrin (27). Spectrin is the major constituent of the cytoskeletal network underlying the plasma membrane (28). The mammalian brain contains at least two isoforms of spectrin, one located primarily in the axons and the other in the cell bodies and dendrites (41, 42). The processing of brain spectrin by calcium-dependent proteases at the postsynaptic membrane has been postulated to be one of the central molecular mechanisms underlying LTP (43, 44). The similarity between arg3.1 and spectrin has to be considered speculative at present. A conclusive determination of the identity of arg3.1 has to await further analysis.

arg3.1 mRNA is expressed at low levels in nonneuronal cells. Expression is virtually undetectable in quiescent fibroblasts but is strongly induced by growth factors. The finding that arg3.1 mRNA is serum inducible suggests a role for arg3.1 in phenomena of growth. In the brain, stimuli that evoke seizures or LTP are capable of inducing structural changes (1, 45–47). A hallmark of long-term memory and LTP is the requirement for transcription and translation (6, 7, 9, 48). However, LTP is spatially restricted to the postsynaptic dendrites directly opposed to the terminals of the activated fibers, whereas transcriptional changes take place in the nucleus of the cell. How synaptic activity-induced transcription can rearrange the com-

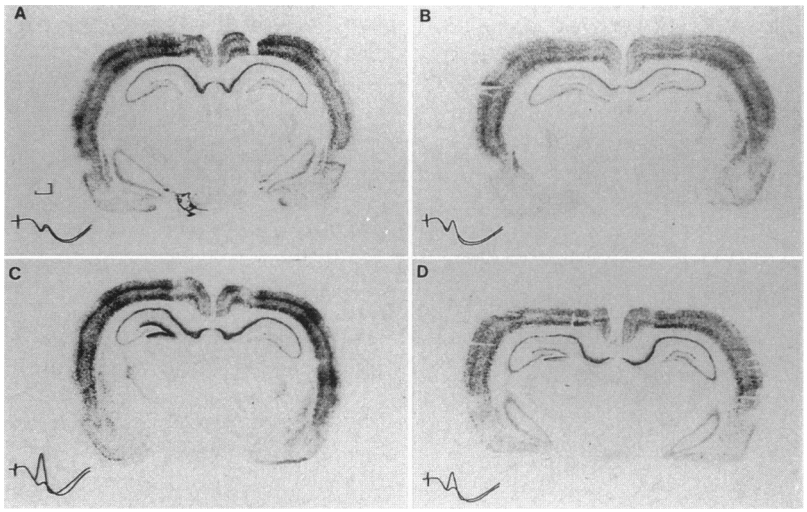

FIG. 4. Induction of LTP and arg3.1 mRNA in the hippocampal granule cell neurons in freely moving rats. Coronal sections were assayed for arg3.1 mRNA using *in situ* hybridization with antisense probe. Representative autoradiographs are shown. (*A*) One hour after unilateral application of low-frequency stimulus pulses. (*B*) Four hours after unilateral application of low-frequency stimulus pulses. (*C*) One hour after unilateral application of high-frequency stimulus pulses. (*D*) Four hours after unilateral application of high-frequency stimulus pulses. (*Insets*) Evidence that high-frequency stimulation evokes LTP in freely moving rats, whereas low frequency stimulation does not. Shown are superimposed field potentials before and 1 hr or 4 hr after low- or high-frequency stimulation. (Scale bar in *A* = 5 mV/2 ms.)

plex molecular architecture of the activated synaptic sites distant to the nucleus is not known. Recent experiments demonstrate that protein synthesis takes place within dendrites where it can be stimulated by NMDA receptor-dependent afferent synaptic activity (49, 50). The differential localization of arg3.1 mRNA to dendrites offers the potential for the local synthesis of the protein at activated postsynaptic sites and may underlie synapse-specific modifications during long-term neuronal plasticity.

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