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Increase in activin βA mRNA in rat hippocampus during long-term potentiation

Kaoru Inokuchi[a,*], Akihiko Kato[a], Keiko Hiraia, Fumio Hishinuma[a], Maki Inoue[b], Fumiko Ozawa[a]

[a]*Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194, Japan*
[b]*Department of Physiology II, Azabu University, Sagamihara, Kanagawa 229, Japan*

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Abstract We have used mRNA differential display to isolate genes that are induced by neural activity in rat hippocampus. One of these encodes activin βA subunit. Convulsive seizure caused by kainate significantly induced the expression of activin βA mRNA. Furthermore high frequency stimulation (HFS) of perforant pathway, which produced a persistent long-term potentiation (LTP) (>10 h), caused a marked increase at 3 h in the level of activin βA mRNA at the dentate gyrus of urethane-anesthetized rat. The increase was NMDA receptor-dependent. By contrast the level of inhibin α mRNA was not changed following the induction of LTP. The results suggest a role for activin in maintenance of neural plasticity in the adult brain.

Key words: mRNA differential display; Long-term potentiation; Seizure; TGF-β superfamily; Activin; Synaptic plasticity

1. Introduction

A number of behavioral and cellular studies have revealed two distinct forms of memory according to their time span, short-term memory and long-term memory. In mammals, long-term memory persists for days, weeks and even years. From a molecular point of view, there is a fundamental difference between these two forms of memory system. Long-term memory, but not short-term memory, requires de novo RNA and protein synthesis [1,2], suggesting that the neural activity associated with learning leads to the expression of various genes whose protein products play a critical role in memory acquisition and consolidation.

Activity-dependent synaptic plasticity has been thought to be a cellular basis of learning and memory. As is the case with behavioral memory, LTP in the hippocampus, one form of the synaptic plasticity well studied thus far [3], has two stages, macromolecule synthesis-independent early phase and protein and RNA synthesis-dependent late phase [4–10]. In hippocampal slices, a brief HFS induces a short-term early potentiation that persists 1–3 h, whereas repeated HFS is required to elicit late phase that lasts for up to 8 h. Similarly longer HFS is required to produce persistent LTP in vivo. Importantly in vivo experiments demonstrate that repeated HFS that produces the late phase accompanies expression of specific transcription factors, NGFI-A (also called *zif*268 or *krox*24), c-*fos* and *jun*-B [6,11–13], indicating an involvement of altered gene expression in prolonged LTP maintenance. Therefore, to understand the molecular mechanisms underlying long-lasting plastic changes in synaptic efficacy, we need to identify the various classes of genes that are regulated during the switch from short- to long-term synaptic plasticity. During the last few years attention has been focused on the identification of genes that might be involved in activity-dependent plasticity [14–21].

In this study we have differentially displayed, by means of reverse transcriptase-polymerase chain reaction (RT-PCR) [22], mRNA species isolated from the rat hippocampus in which seizure had been induced by kainate or pentylenetetrazol (PTZ) and from the hippocampus of untreated animals. We then compared radio-labeled RT-PCR banding patterns to isolate cDNAs that are differentially expressed. We found that one of the neural activity-dependent genes isolated encodes βA subunit of activin, a member of the transforming growth factor-β (TGF-β) superfamily. Activin is a multifunctional protein which causes mesoderm induction [23–26], induces differentiation of erythroid progenitor cells [27], has ability to release follicle-stimulating hormone from pituitary cells [28,29], and promotes nerve cell survival [30]. We provide an evidence that high frequency tetanic stimuli, which elicit long-lasting LTP, induce the activin βA transcript in the adult hippocampus in an NMDA receptor-dependent manner.

2. Materials and methods

2.1. mRNA differential display

Male Wistar rats (6 weeks old, 180–220 g) were injected intraperitoneally with PTZ (60 mg/kg body weight, Sigma) or kainate (15 mg/kg, Sigma) to produce a seizure. In some cases cycloheximide (100 mg/kg, Sigma) was co-injected with PTZ. Animals were sacrificed, and the hippocampus was dissected, immediately frozen on liquid nitrogen and stored at −70°C. Total cellular RNA was prepared from the hippocampus using acid-guanidinium-phenol-chloroform method [31,32]. Chromosomal DNA contamination was removed from total RNA by DNase I treatment [33].

mRNA differential display was performed as described [22,33]. Briefly, total RNA (0.5 μg) was heat-denatured for 5 min at 70°C, then reverse-transcribed with 300 units of MMLV reverse transcriptase (Gibco BRL) in a 20 μl reaction volume for 60 min at 35°C in the presence of 20 μM dNTP and 40 units of RNasin Ribonuclease Inhibitor (Promega) using 2.5 μM $T_{12}MA$, $T_{12}MC$, $T_{12}MG$ or $T_{12}MT$ (M, a mixture of A, C and G) as an anchor primer. An aliquot (0.5 μl) of the sample was then added to 4.5 μl of PCR solution containing 2 μM dNTPs, 0.5 μM [α-^{35}S]dCTP (1200 Ci/mmol, New England Nuclear), 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer),

*Corresponding author. Fax: (81) (427) 24-6314.
E-mail: kaoru@libra.ls.m-kagaku.co.jp

Abbreviations: EPSP, excitatory postsynaptic potential; G6PDH, glucose-6-phosphate dehydrogenase; HFS, high frequency stimulation; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PTZ, pentylenetetrazol; RT-PCR, reverse transcriptase-polymerase chain reaction; TGF-β, transforming growth factor-β

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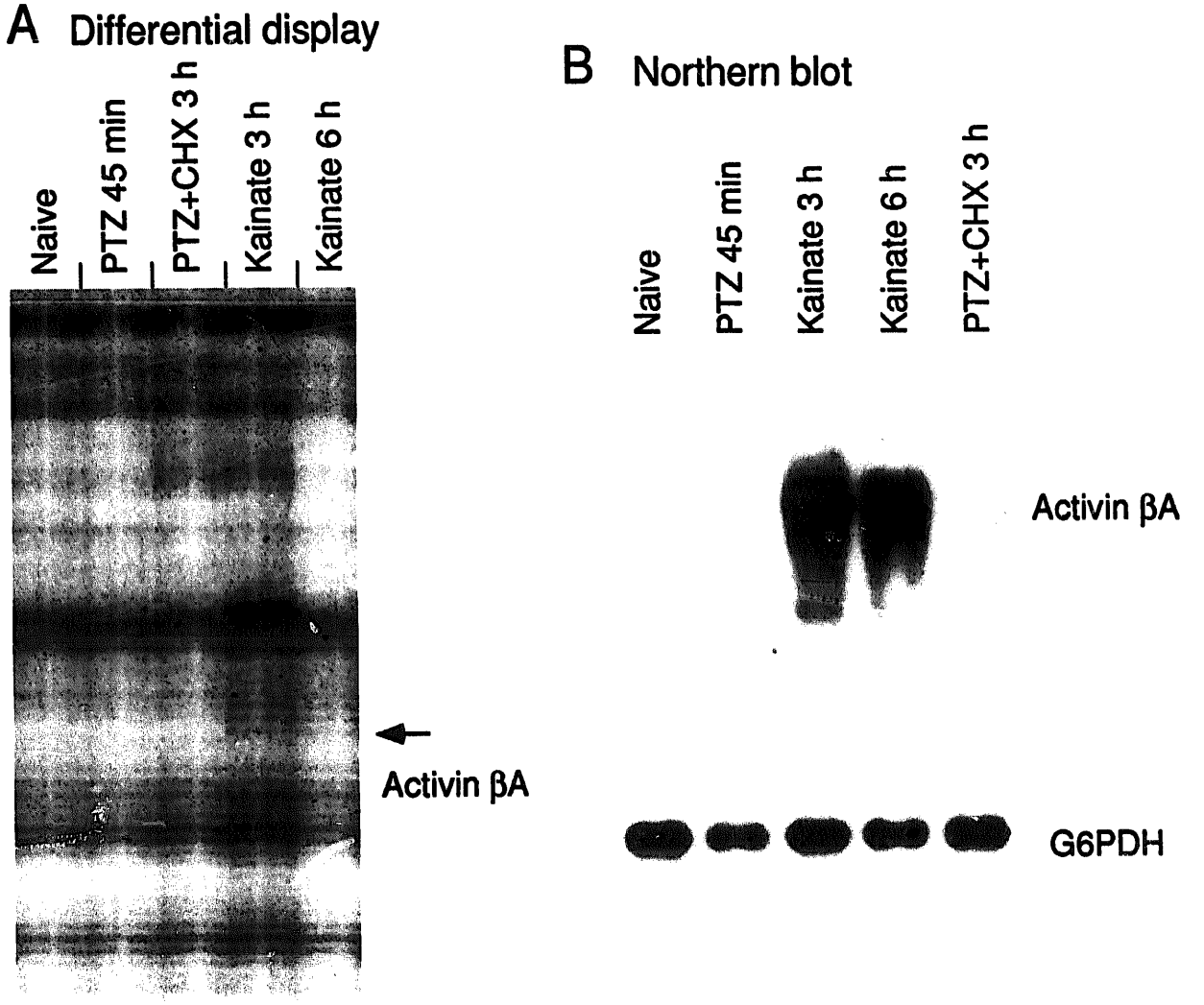

Fig. 1. Increase in the activin βA mRNA following seizure activity. (A) Representative autoradiogram of mRNA differential display with an anchor primer, $T_{12}MC$, and an arbitrary primer, 5′-AGAATGTAAG-3′. RNA was isolated at different times after the injection. (B) Northern blot analysis of the activin βA mRNA. 5 μg of total RNA was loaded on each lane. G6PDH serves as loading control.

1 μM anchor primer ($T_{12}MN$), and 0.5 μM arbitrary primer. The arbitrary primers (10-mer, 40–60% GC content) were specifically designed for differential display. PCR conditions were as follows: 94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles followed by a final extension reaction at 72°C for 5 min. PCR product was analyzed on a 6% DNA sequencing gel.

Recovery and reamplification of cDNAs of interest were carried out essentially as described [22,33]. Amplified cDNAs were subcloned into the pCR™II vector (Invitrogen) via TA cloning method. Inserts were then analyzes by dideoxinucleotide sequencing method.

The primers were synthesized on a Model 380A DNA synthesizer (Applied Biosystems). They were purified by oligonucleotide purification cartridges (Applied Biosystems).

2.2. cDNA cloning

Total RNA was prepared from the rat hippocampus (6 weeks old, male) 3 h after the injection of PTZ as described above. Poly(A)$^+$ RNA was purified from total RNA with PolyATtract mRNA isolation system (Promega). A λZAPII phage cDNA library was constructed using a ZAP-cDNA synthesis kit (Stratagene). The primary library contained 6×10^6 independent clones. Plaque hybridization screening was carried out as described [34].

2.3. Northern blot analysis

Blots of the total cellular RNA were prepared after electrophoretic separation in a denaturing formaldehyde/agarose gel [34], and RNA was immobilized on Duralose membrane filters (Stratagene). RNA was visualized by autoradiography following hybridization to random-prime-labeled cDNA inserts that corresponds to the 3′-half (4.7 kb) of the activin βA mRNA [34]. The mouse glucose-6-phosphate dehydrogenase (G6PDH) gene [35] was chosen as a reference because of its constitutive expression and its lack of induction by seizure activity.

2.4. Electrophysiology

Adult male Wistar rats (10–12 weeks old, 300–350 g) were anesthetized with urethane (1.5 g/kg, i. p.) and placed on a stereotaxic apparatus. The body temperature of the rats was monitored by a rectal thermometer and maintained at 37.0 ± 0.1°C by animal blanket system MK-900 (Muromachi Kikai) throughout the experiment. The skull was exposed and electrodes were implanted through burr holes in the skull. Stimulating and recording electrodes were of 100 μm of epoxylite-coated tungsten wire. Bipolar stimulating electrode was positioned unilaterally to the perforant pathway at stereotaxic coordinates of 8 mm posterior and 4.5 mm lateral to bregma. A monopolar recording electrode was inserted ipsilaterally into the hilus of the dentate gyrus, 4 mm posterior and 2.5 mm lateral to bregma. All stimuli were constant-current, monophasic square wave pulses. The current intensity of the test pulses (0.1 ms duration) was set at 1.0 mA. Test stimuli were delivered at 20 s intervals.

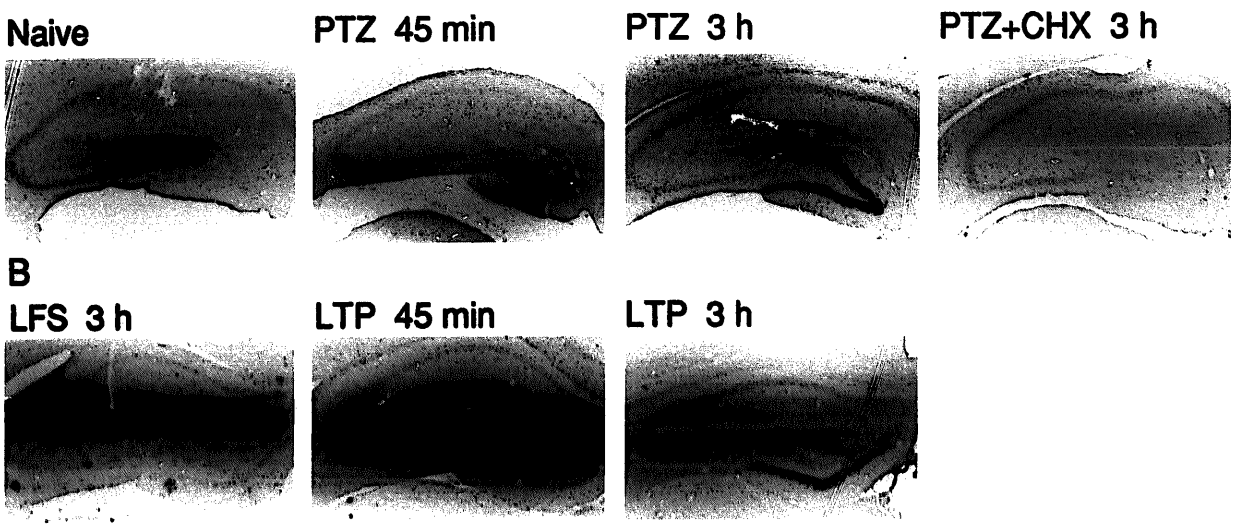

Fig. 2. In situ hybridization showing an increase in activin βA mRNA in the hippocampus following PTZ administration (A) or LTP induction (B). In (B) LTP was induced by a 1200 pulse train. CHX, cycloheximide (100 mg/kg); LFS, low frequency test stimulation delivered at 0.05 Hz.

To elicit LTP, various pulse-number of HFS (400 Hz, 0.25 ms pulse width, 1.0 mA) was delivered following a 15 min control baseline recording. High frequency trains used are: 2000 pulse train, 400 Hz for 1 s, 5 times at 2 min interval; 1200 pulse train, 400 Hz for 1 s, 3 times at 5 min interval; 400 pulse train, 400 Hz for 1 s; 100 pulse train, 400 Hz for 0.25 s; 40 pulse train, 400 Hz for 0.1 s; and 10 pulse train, 400 Hz for 0.025 s. The evoked field potentials were amplified, digitized at 50 kH and stored sequentially on computer discs (NEC PC9801 DX) for off-line analysis. Both slope of field excitatory postsynaptic potential (EPSP) and population spike amplitude were monitored as an index of LTP.

2.5. *In situ hybridization*

Rats were deeply anaesthetized with pentobarbital (50 mg/kg, i. p.), and fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4)/saline (PBS). The brains were dissected free, placed in a brain slicer and coronally sliced 3 mm thick with razor blades. They were post-fixed with 4% paraformaldehyde in PBS at 4°C for 20 h, immersed in 30% sucrose in PBS overnight at 4°C, embedded in Tissue-Tek OCT compound (Miles Inc.) and frozen in a dry ice-isopentane bath. Cryostat sections (10 μm thickness) were cut and mounted onto polylysine-coated glass slides. Sections were air-dried and stored at −80°C until use for in situ hybridization.

Pretreatment of the brain sections, hybridization and wash were performed essentially as described [36], except hybridization was carried out at 42°C. DIG-labeled riboprobes were prepared using T3 or T7 RNA polymerase from appropriately restricted plasmid containing cDNA insert that corresponds to 3'-half (4.7 kb) of the activin βA mRNA. Immunological detection was done using DIG nucleic acid detection kit (Boehringer Mannheim).

2.6. *Quantitative RT-PCR*

First strand cDNAs were synthesized in a 20 μl reaction volume as described above, except dT_{12-18} (5 ng/μl, Pharmacia) was used as a primer. An aliquot (1 μl) of the sample was then added to 20 μl of PCR solution containing 50 μM dNTPs, 5 units of AmpliTaq DNA polymerase (Perkin-Elmer), 0.1 μM each of primers in a 50 μl reaction volume. The cycling parameter is as follows: 95°C for 30 s, 55°C for 2 min, 72°C for 30 s. A part of the reaction mixture (5 μl) was taken at various time indicated in the text, analyzed on a native 8% polyacrylamide gel and autoradiographed on the imaging plate of a Fuji imaging analyzer (Fuji Photo Film). Quantification of the radioactivities of each cDNA was carried out according to the instruction manual.

Synthetic primers 5'-TGGTTGGATATGTTTTCATGTG-3' and 5'-GGGCATTGCTGAATACTGTCTA-3', both of which correspond to the 3'-untranslated region of the activin βA mRNA (this study), were used to detect activin βA mRNA, 5'-TCTAAAAGCACCTCGTCTCCTC-3' (1352 to 1373) and 5'-GGAAGCCTCAGACAGAAAGATG-3' (1489 to 1468) for inhibin α mRNA [37], and 5'-GGGGCCATAGATATCTCCTAAA-3' (2173 to 2194) and 5'-CTAGCTGCTGCTCTTACCTTCC-3' (2251 to 2230) for G6PDH [35]. The 5'-end of each primer was labeled with [γ-^{32}P]ATP (New England Nuclear) and T4 polynucleotide kinase.

3. Results and discussion

3.1. *mRNA differential display identifies activin βA as a neural activity-dependent gene*

We used mRNA differential display [22] to isolate cDNA clones that are regulated by neural activity in the rat hippocampus. One-hundred and four combinations of primer sets made of 4 anchor primers and a group of 26 arbitrary 10-mers were used for screening. By this means 16 independent cDNAs were isolated whose mRNA level was markedly modulated by PTZ and/or kainate in the hippocampus. DNA sequence analysis of these PCR products revealed that some of these encode previously described genes such as BDNF, *krox*20, NGFI-A, c-*fos*, and ICAM-1.

When a primer pair of T_{12}MC and an arbitrary primer 5'-AGAATGTAAG-3' was used, we detected a PCR fragment (220 bases) that was significantly induced by kainate (Fig. 1A). Since DNA sequence information obtained from the PCR product, which should correspond to the 3'-end of mRNA, showed no homology with any genes reported, we screened a kainate-induced cDNA library (see section 2), using this DNA fragment as a probe. DNA sequence analysis of a cDNA clone that had the longest insert (ca. 4.7 kb) and search of the EMBL and GeneBank databases showed that it codes for βA subunit of activin [37]. Kainate stimulated the level in the activin βA mRNA at 3 h and the increase persisted for at least 3 h, while PTZ (at 45 min) or PTZ + cycloheximide (at 3 h) had no effect on the mRNA level (Fig. 1A). In Northern blot the significant increase in the level of activin βA was seen at 3 and 6 h by kainate, confirming that the expression of activin βA mRNA was indeed regulated by kainate (Fig. 1B).

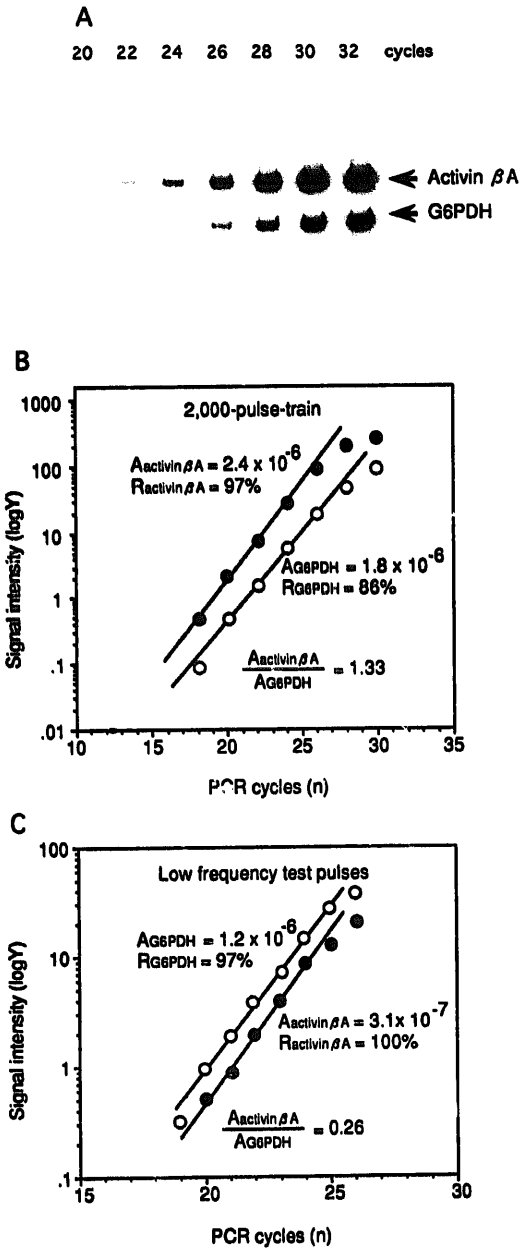

Fig. 3. Estimation of the activin βA mRNA following dentate gyrus LTP. (A) Representative autoradiogram of quantitative RT-PCR. Total RNA was isolated from the hippocampus ipsilateral to the recording electrode 3 h after HFS (2000 pulse train). (B) Estimation of the relative amount of mRNA in the hippocampus 3 h after the 2000 pulse train, measured by counting the radioactivity in the fragments visualized in (A). The extent of amplification (Y) is given by the formula: $Y=A(1+R)^n$, where A is the initial mRNA level, R the efficiency of PCR amplification and n the number of cycles. Thus relative amount of activin βA vs. that of G6PDH is calculated from the following formula: $A_{activin\beta A}/A_{G6PDH} = Y_{activin\beta A}/(1+R_{G6PDH})^n / Y_{G6PDH}(1+R_{activin\beta A})^n$. ●, activin βA; ○, G6PDH. (C) Same plot as in (B) but total RNA was prepared from control hippocampus in which low frequency test pulses (20 s interval) were given for 3 h. Symbols are the same as in (B).

On the other hand neither PTZ (at 45 min) nor PTZ + cycloheximide (at 3 h) stimulated the mRNA level, which is consistent with the result of differential display.

We next examined a spatial and temporal expression pattern of the activin βA mRNA in the hippocampus following the PTZ administration (Fig. 2A). The induction of activin βA mRNA was mainly observed in granule cells of the dentate gyrus 3 h after the onset of seizure, while no or very weak induction in the CA1 and CA3 region. The induction appears to require de novo protein synthesis, because we detected no induction at 3 h in the presence of cycloheximide. Furthermore we observed no induction in the hippocampus 45 min after the PTZ injection, suggesting that activin βA represents one of the late effector genes.

3.2. *Expression of activin βA was induced by hippocampal LTP in vivo*

Our next question is whether the expression of activin βA is regulated by synaptic activity such as hippocampal LTP. Under the urethane anesthesia, repeated HFS (1200 pulse train) given to the perforant pathway elicited a long-lasting (>10 h) enhancement of synaptic transmission measured from the dentate gyrus either by EPSP slope or an amplitude of population spike (not shown, $n=9$). This tetanic stimulation caused a drastic increase in activin βA mRNA in granule cells of the dentate gyrus ipsilateral to the stimulating electrode 3 h after the delivery (Fig. 2B). No induction was observed at 45 min, suggesting again that activin βA is one of the late effector genes.

We employed a quantitative RT-PCR [38] to estimate the levels of activin βA mRNA following various HFS that consists of different number of pulses (Fig. 3 and 4). The 2000 pulse train caused a marked increase at 3 h in the level of activin βA mRNA (11 ± 5-fold increase compared to test pulse, ± SEM, $n=7$), but not at 45 min (1.1 ± 0.3, $n=5$). Both LTP and the increase in activin βA mRNA were completely blocked by preinjection of the NMDA receptor an-

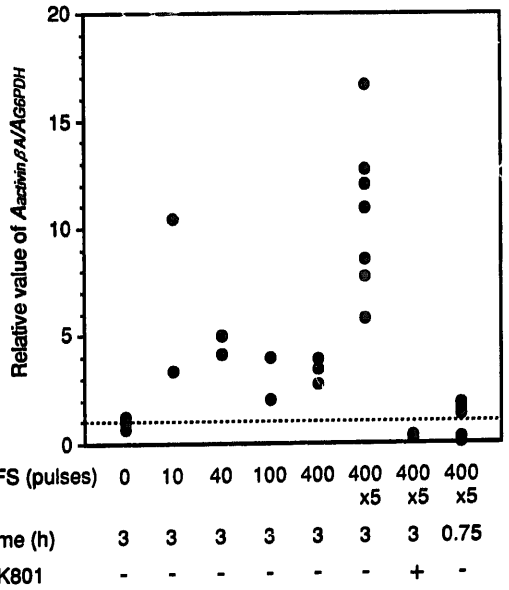

Fig. 4. Increase in activin βA transcripts following HFS. The graph shows the relative $A_{activin\beta A}/A_{G6PDH}$ value when normalized against the averaged $A_{activin\beta A}/A_{G6PDH}$ value obtained from control animals that received low frequency test stimulation alone. $A_{activin\beta A}/A_{G6PDH}$ value was obtained by RT-PCR (see Fig. 3. for calculation). Each point represents a relative value from an individual rat. +, MK801 (15 mg/kg body weight, i.p.) was preinjected 45 min before the first tetanus.

tagonist, MK801 (Fig. 4, $n=2$), indicating that the induction of activin βA transcript requires the activation of NMDA receptor.

If the induction of activin βA mRNA is required for the maintenance of LTP, threshold for the mRNA induction by HFS should have relation to the persistence of LTP. Although the degree of increase was less prominent, all the high frequency pulse-trains tested (see section 2) stimulated activin βA transcript at 3 h (Fig. 4). In addition, all these trains produced persistent LTP in the dentate gyrus that lasted for at least 10 h in anesthetized rats (not shown). Thus HFS used in this study, all of which elicited long-lasting LTP, always results in the induction of activin βA expression. Following the 2000 pulse train the activin βA response was considerably greater than those after the other HFS. This may reflect the correspondence between the persistence of LTP and the degree of activin βA response, i.e. the 2000 pulse train may elicit a longer-lasting LTP than the shorter HFS.

Activin is a dimer composed of β:β subunits (either βA or βB). A related protein, inhibin (α:β heterodimer), shares common β subunits with the activin. We measured the inhibin α transcript in the hippocampus 3 h after the 2000 pulse train by quantitative RT-PCR and found that inhibin α was constitutively expressed (not shown, $n=3$), suggesting that the inhibin is not related to the maintenance of LTP.

We described here that two forms of neural activity (seizure and LTP) lead to the expression of activin βA mRNA in the hippocampus. These results suggest a role for activin in the maintenance of neuronal function in the adult brain in addition to its well-established functions. In fact activin receptors were found to be expressed in the adult brain, specifically highly expressed in the hippocampus and the amygdala [39]. Further studies with antibodies and antisense DNA against activin βA will clarify how this protein contributes to the maintenance of LTP and perhaps to the consolidation of behavioral memory.

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