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Activator Protein-1 Complex Expressed by Magnetism in Cultured Rat Hippocampal Neurons

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Brief exposure for 15 min to static magnetic filed at 100 mT led to marked but transient potentiation of binding of a radiolabeled probe for activator protein-1 (AP1) in immature cultured rat hippocampal neurons with high expression of growth-associated protein-43. Immunoblotting and supershift analyses revealed that brief exposure to static magnetic field increased AP1 DNA binding through expression of Fra-2, c-Jun, and Jun-D proteins in immature cultured hippocampal neurons. Significantly less potent increases were seen in both intracellular free Ca^{2+} concentration and AP1 binding following the addition of *N*-methyl-D-aspartate in these immature neurons exposed to magnetism 24 h before. These results suggest that brief exposure to weak static magnetic field may lead to desensitization of NMDA receptor channels through modulation of *de novo* synthesis of particular inducible target proteins at the level of gene transcription by the AP1 complex expressed in the nucleus of immature cultured rat hippocampal neurons.

Key Words: **activator protein-1; hippocampus; static magnetic field; cell density; maturity; GAP-43; c-Jun; Jun-D; Fra-2.**

Signal propagation involves neurotransmission mediated by neurotransmitters as well as conduction mediated by electric current in neurons. Magnetic force is shown to affect a variety of physiological functions through mechanisms associated with generation of electric current induced by altered flows of different ions in the body fluids. Transcranial magnetic stimulation (TMS) has been used as a diagnostic tool in neurobiological fields due to its painless and noninvasive properties (1). In clinical studies, repetitive TMS (rTMS) is supposed to be beneficial for the treatment and therapy of selected patients with depression, bipolar affective disorder and schizophrenia as a possible alternative to electroconvulsive therapy (ECT) that is often used for the treatment of refractory depression (2–4). In healthy subjects, rTMS leads to transient mood enhancement (5, 6). Weak magnetic fields are effective in the treatment of Parkinsonism and motor complications of chronic levodopa therapy (7). In rat brain, rTMS induces expression of both c-*fos* mRNA and c-Fos protein as a consequence of stimulation of neural activity in particular brain nuclei such as the paraventricular nucleus of thalamus and the suprachiasmatic nucleus (8).

c-Fos protein family is a partner of c-Jun protein family members to compose the nuclear transcription factor activator protein-1 (AP1) that is a heterodimeric and homodimeric protein complex with high affinity for the nucleotide sequence TGACGTCA (9). Transcription factors are nuclear proteins with abilities to specifically recognize particular core nucleotide sequences located at the upstream or downstream on double-stranded DNA for modulation of the activity of RNA polymerase II responsible for the synthesis of mRNA from genomic DNA in the nucleus. Gene transcription would therefore lead to long-lasting and sometimes permanent alterations of a variety of cellular functions through consolidation of transient extracellular signals following regulation of *de novo* biosynthesis of inducible target proteins (10). Such consolidation mechanism would be operative in certain situations including neuronal plasticity and degeneration.

Accumulating evidence for expression of AP1 complex in response to neural activities *in vitro* and *in vivo* is available in the literature. In primary cultures of rat cerebellar neurons, exposure to L-glutamate (Glu) results in marked expression of both c-*fos* gene and c-Fos protein through activation of a particular ionotropic Glu receptor subtype sensitive to *N*-methyl-D-aspartate (NMDA), followed by potentiation of AP1 DNA binding (11, 12). Intraperitoneal (13) and intracerebroventricular (14) injections of NMDA express c-*fos* mRNA and c-Fos protein in the rodent brain. Punching out dissection technique on frozen sections reveals that

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the systemic NMDA induces rapid but transient potentiation of AP1 DNA binding only in the dentate granule layer of murine hippocampus without affecting that in the CA1 and CA3 pyramidal layers (15). Hippocampal dentate layers are shown to contain immature neurons that spontaneously undergo proliferation and subsequent migration in a manner sensitive to prevention by NMDA receptors even in matured brain (16, 17).

In this article, we have investigated the influence of static magnetic field on DNA binding activity and expression of nuclear AP1 complex in immature and mature cultured rat cortical and hippocampal neurons as a guidepost for possible long-lasting functional alterations by magnetism in the brain.

MATERIALS AND METHODS

Materials. Oligonucleotides were all synthesized by Inter Tech Co. (Tokyo, Japan). [α-^{32}P]Deoxy-ATP (111 TBq/mmol) was supplied by NEN/DuPont (Boston, MA, USA) and Klenow fragment of DNA polymerase I by Takara Biochemicals (Kyoto, Japan), respectively. Nick column was obtained from Pharmacia (Uppsala, Sweden). Versene was supplied by Gibco BRL (Gaithersburg, MD). A monoclonal antibody against growth-associated protein-43 (GAP-43), a monoclonal antibody against microtubule-associated protein-2 (MAP-2), and a polyclonal antibody against glial fibrillary acidic protein (GFAP) were purchased from Sigma Chemicals (St. Louis, MO). Polyclonal antibodies directed against c-Fos, Fos-B, Fra-2, c-Jun, Jun-B, and Jun-D proteins were all provided by Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse and -rabbit IgG antibodies conjugated with peroxidase and ECL detection reagent were provided by Amersham Life Science (Buckinghamshire, England). Fluo-3 acetoxymethyl ester (AM) was purchased from Molecular Probes (Eugene, OR). Other chemicals used were all of the highest purity commercially available.

Neuronal cultures. Primary cortical and hippocampal neuronal cultures were obtained from 18- to 19-day-old embryonic rats according to the method of Di Porzio *et al.* (18) with minor modifications (19). In brief, cerebral cortex and hippocampus were dissected and individually incubated with Versene at room temperature for 12 min. Cells were then mechanically dissociated by using a Pasteur pipette with fire-narrowed tip in the culture medium, and plated at a density of 1.0 or 2.5 $\times$ 10^5 cells/cm^2 (diameter 35 mm, Nunc) after a dye exclusion test with Trypan Blue. Culture dishes were previously coated, sequentially with poly-L-lysine (7.5 μg/mL, Sigma) and then with 10% fetal bovine serum in DMEM. Culture medium was DMEM without fetal bovine serum but with supplements as follows: 33 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mM HEPES, 13 mM sodium bicarbonate, 50 μg/mL apotransferrin, 500 ng/mL insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/mL sodium seleniate, and 100 μM putrescine. Cells were cultured at 37°C in a 5% CO_2/95% air humidified incubator. According to immunoreactivities to MAP-2 and GFAP, more than 95% of cultured cells were derived from neurons at 3 DIV.

Western blotting. Neurons were cultured for 3, 6, and 9 DIV, and collected in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM sodium β-glycerophosphate (NaGP), 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 μg/mL of various protease inhibitors [(*p*-amidinophenyl) methanesulfonyl fluoride, leupeptin, antipain, and benzamidine], as described elsewhere (20). Buffer and other solutions used in this study were all sterilized each time before use by filtration through a nitrocellulose membrane filter with a pore size of 0.22 μm to avoid possible microbial contamination (21). Cell homogenates were mixed at a volume ratio of 4:1 with 10 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue, and 5% mercaptoethanol, followed by boiling at 100°C for 10 min. An aliquot (20 μg protein) was loaded on 10% polyacrylamide gel containing 0.1% SDS for electrophoresis at a constant current of 15 mA/plate for 2 h at room temperature, followed by blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skim milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20, the membrane was incubated with one of antibodies against MAP-2, GFAP and GAP-43 adequately diluted with the latter buffer containing 1% skim milk, followed by incubation with the anti-mouse IgG antibody conjugated with peroxidase and subsequent exposure to X-ray films for different periods to obtain immunoblots appropriate for subsequent densitometry. Densitometric determination was carried out on these X-ray films with the aid of BIO PROFIL Bio-1D densitograph (Vilber Lourmat).

As needed, nuclear extracts were also mixed at a volume ratio of 4:1 with 10 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 0.01% bromophenol blue and 5% mercaptoethanol, followed by boiling at 100°C for 10 min. An aliquot (10 μg protein) was loaded on 7.5% polyacrylamide gel containing 0.1% SDS for electrophoresis as described above.

Magnetism exposure. Primary cultures of rat cortical and hippocampal neurons were exposed for 15 min to static magnetic field at potency of 100 mT that was generated by permanent magnets placed at both sides of culture dishes in a CO_2 incubator at 37°C, where dishes were put in the center of the magnetic equipment. A control group (sham exposure) was invariably subjected to similar procedures under the same environmental influences as experimental groups. Following exposure to static magnetic field, cultured neurons were further incubated for different periods up to 60 min in a CO_2 incubator at 37°C until the time of cell harvest. At the indicated times, culture medium was aspirated and the plate was kept on ice until preparation of nuclear extracts within 5 min.

Preparation of nuclear extracts. Nuclear fractions were prepared according to the method of Schreiber *et al.* (22), with minor modifications (23). Cells were harvested with a rubber policeman and homogenized in 500 μL of 10 mM HEPES-NaOH buffer (pH 7.9) containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol (DTT), 10 mM NaF, 10 mM NaGP and 1 μg/mL of following protease inhibitors: (*p*-amidinophenyl) methanesulfonyl fluoride, benzamidine, leupeptin and antipain. Following the addition of 10% Nonidet P-40 to make a final concentration of 0.6%, homogenates were centrifuged at 20,000*g* for 5 min. Pellets were suspended in 20 μL of 50 mM Tris-HCl buffer (pH 7.5) containing 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 10% (v/v) glycerol, 10 mM NaF, 10 mM NaGP and the aforementioned protease inhibitors, followed by centrifugation at 20,000*g* for 5 min. Supernatants thus obtained were stored at -80°C as nuclear extracts. Protein concentration was determined by Bio-Rad Protein assay kit (Bio-Rad, CA) and adjusted at 3 μg in 8 μl of incubation buffer.

Preparation of probe. The probe for determination of AP1 binding was double-stranded oligonucleotides with 22-mer (5′-CTAGTGATGAGTCAGCCGGATC-3′/3′-GATCACTACTCAGTCGGCCTAG-5′) that was radiolabeled with [α-^{32}P]deoxy-ATP using Klenow fragment of DNA polymerase I in 10 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 50 μM deoxy-GTP, deoxy-CTP, and deoxy-TTP at 25°C for 30 min, followed by purification with gel filtration chromatography on a Nick column (14). The term "AP1 DNA binding" was used to refer to binding of a radiolabeled probe for AP1 complex throughout the paper.

Gel retardation electrophoresis. An aliquot of nuclear extracts (3 μg protein in 8 μL) was mixed with 10 μL of 0.1 mg/ml poly(dI-dC), 50 mM Tris-HCl buffer (pH 7.5), 20 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 10% glycerol, and 1 μg/mL protease inhibitors,

followed by incubation with 2 μL of 20 fmol of radiolabeled probe (0.5–5.0 $\times$ 10^6 cpm/pmol) at 25°C for 30 min (14). Bound and free probes were separated by electrophoresis on a 6% polyacrylamide gel in 50 mM Tris-HCl (pH 8.5), 0.38 M glycine and 2 mM EDTA at a constant voltage of 11 V/cm for 1.5 h in an ice bath. Gels were fixed, dried and exposed to an X-ray film for autoradiography. Gels were always exposed to X-ray films for different periods to obtain autoradiograms adequate for quantitative densitometric analysis.

Supershift assays. Prior to incubation of nuclear fractions with the radiolabeled probe, one μL of each antibody against the individual family member proteins of AP1 complex was added into 8 μL of incubation mixture, followed by the antigen-antibody reaction at 4°C for 18 h. Antibodies used were directed against each protein as follows: c-Fos, Fos-B, Fra-2, c-Jun, Jun-B, and Jun-D. Samples were then subjected to gel retardation electrophoresis and subsequent autoradiography for determination of the mobility shift on the gel as described previously.

Measurement of intracellular free Ca^{2+} concentration. Cultured neurons were washed with recording medium containing 129 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 4.2 mM glucose, and 10 mM HEPES (pH 7.4) once and incubated at 37°C for 1 h in recording medium containing 30 nM Pluronic F-127 and 3 μM fluo-3 AM that is a membrane-permeable form of the Ca^{2+}-sensitive dye. Culture dishes were then washed with recording medium twice and settled for at least 1 h in the recording medium. Medium was changed once more, followed by the cumulative addition of NMDA at concentrations of 1 to 100 μM in the absence of $MgCl_2$ and subsequent determination of the number of fluorescent neurons every 2 min later. Cells were invariably used within 1–5 h after these procedures for observation with a confocal laser-scanning microscope. Drugs were prepared in recording medium immediately before each use.

Dye-loaded neurons were monitored for fluorescence visualized with a confocal laser-scanning microscope equipped with an argon laser. Images were obtained by using objective lens with numeral apertures of 0.5 (Plan-Neufluar) for 20-fold magnification. Fluorescence images labeled with fluo-3 AM were collected using an excitation wavelength of 488 nm. The parameters of illumination and detection were digitally controlled to keep the same settings throughout the experiments. Two successive digital images were usually collected at 512 $\times$ 512 pixels in the same visual field for evaluation as described previously (26).

Lactate dehydrogenase assay. Culture medium was replaced with new medium before exposure and collected 24 or 72 h after the transient exposure for 15 min to static magnetic field, followed by immediate measurement of the release of lactate dehydrogenase (LDH) into culture medium according to the protocol described by Koh and Choi (24). Usually, 0.5 mL of culture medium was added to 2 mL mixture of 0.1 mM β-NADH and 0.1 M potassium dihydrogen phosphate (pH 7.5). The activity of LDH was determined as the difference in the oxidation rate of NADH monitored by absorbance at 340 nm before and after the addition of 1 mM sodium pyruvate.

Data analyses. Quantitative densitometric data are expressed as the mean $\pm$ S.E. and the statistical significance was determined by the two-tailed Student *t* test.

RESULTS

Culturing Periods

Both cortical and hippocampal neurons were cultured at a low density of 1.0 $\times$ 10^5 cells/cm^2 for 3 or 9 DIV, followed by brief exposure to static magnetic field for 15 min and subsequent harvest 15 to 60 min after exposure for preparation of nuclear extracts to measure AP1 DNA binding on gel retardation electrophoresis. Brief exposure to static magnetic field did not significantly affect AP1 DNA binding in cortical cells cultured for 3 and 9 DIV irrespective of the time after exposure up to 60 min (Fig. 1a). In hippocampal neurons cultured for 3 DIV, magnetic force doubled AP1 DNA binding only when determined 30 min after brief exposure (Fig. 1b). In hippocampal cells cultured for 9 DIV, brief exposure to magnetism failed to significantly modulate AP1 DNA binding in a manner independently of the time after exposure.

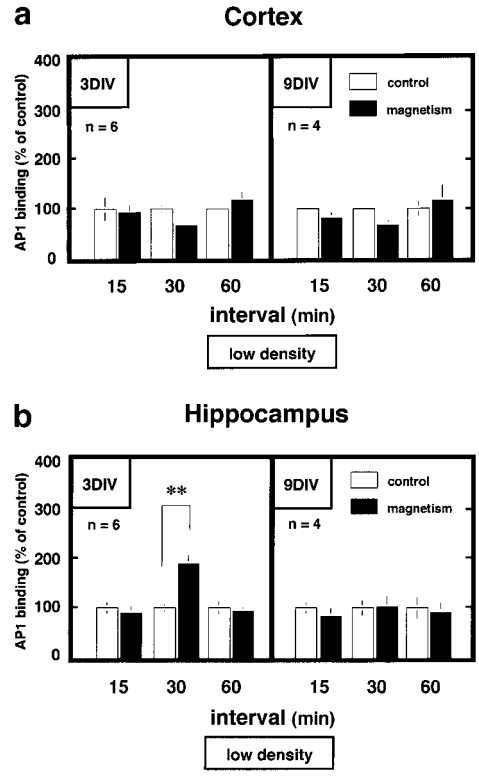

FIG. 1. AP1 DNA binding in (a) cortical and (b) hippocampal neurons cultured at low density for different periods. Rat cortical and hippocampal neurons were plated at a density of 1.0 $\times$ 10^5 cells/cm^2 and cultured for 3 or 9 DIV. Cultured neurons were exposed to magnetic field at 100 mT for 15 min, followed by harvest different times from 15 to 60 min later and subsequent preparation of nuclear extracts for determination of AP1 DNA binding. Each value represents the mean $\pm$ S.E. obtained in 4 to 6 independent experiments. $^{**}P < 0.01$, significantly different from each control value obtained in cultured neurons not exposed to magnetic field.

In hippocampal neurons cultured at a low density, expression of both MAP-2 and GFAP increased in proportion to prolongation of the duration of culture between 3 and 9 DIV, while GAP-43 was highly expressed in hippocampal neurons cultured for 3 to 6 DIV with a marked decline within 9 DIV (Fig. 2).

Culturing Densities

Hippocampal neurons were cultured at a density of 1.0 $\times$ 10^5 cells/cm^2 or 2.5 $\times$ 10^5 cells/cm^2 for 3 DIV,

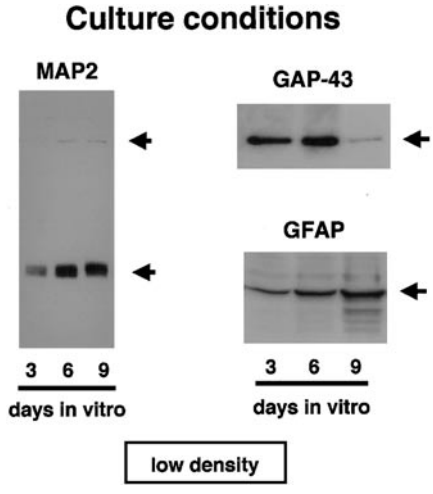

FIG. 2. Expression of marker proteins in hippocampal neurons cultured for different periods. Hippocampal neurons were plated at a density of 1.0×10^5 cells/cm^2 and cultured for 3 to 9 DIV. Cultured neurons were harvested after the culture for different DIV, followed by SDS–PAGE and subsequent immunoblotting using an antibody against the neuronal marker protein MAP2, the neuronal differentiation marker protein GAP-43 or the glial marker protein GFAP. Typical immunoblots are shown with similar results in 3 separate determinations.

followed by brief exposure to static magnetic field for 15 min and subsequent harvest 30 min after exposure for determination of AP1 DNA binding. As shown in Fig. 3, brief exposure almost doubled AP1 binding in

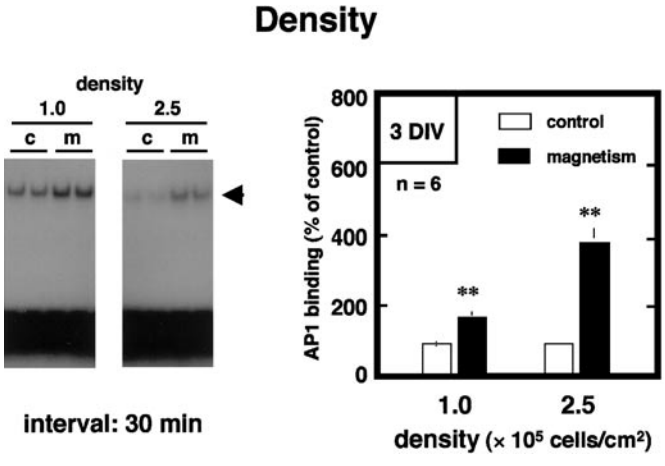

FIG. 3. AP1 DNA binding in hippocampal neurons cultured at 2 different densities. Hippocampal neurons were plated at a density of 1.0 or 2.5×10^5 cells/cm^2 and cultured for 3 DIV. Cultured neurons were exposed for 15 min to static magnetic field, followed by harvest 30 min later and subsequent preparation of nuclear extracts for determination of AP1 DNA binding. Typical autoradiograms are shown in the left panel where the black arrow indicates the position of AP1 binding. Densitometric quantitative data are shown in the right panel where each value represents the mean ± S.E. obtained in 6 separate determinations. $^{**}P < 0.01$, significantly different from each control value obtained in cultured neurons not exposed to static magnetic field.

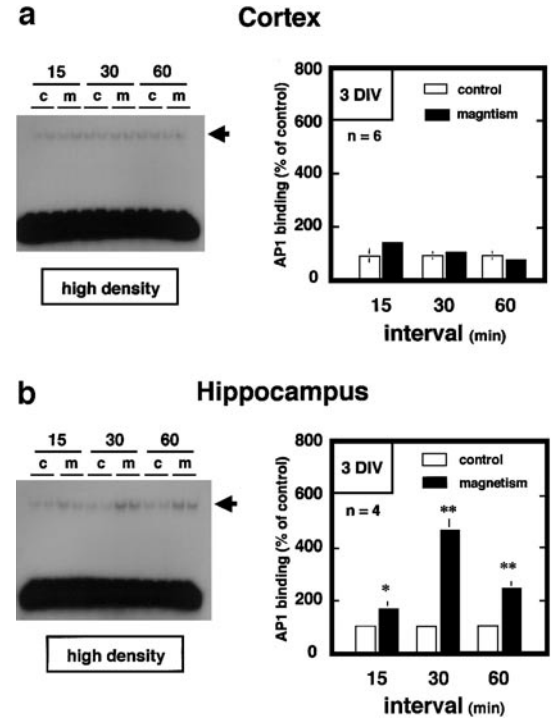

FIG. 4. AP1 DNA binding in (a) cortical and (b) hippocampal neurons cultured at high density. Cortical and hippocampal neurons were plated at a density of 2.5×10^5 cells/cm^2 and cultured for 3 DIV. Cultured neurons were exposed to static magnetic field for 15 min, followed by harvest different times from 15 to 60 min later and subsequent preparation of nuclear extracts for determination of AP1 DNA binding. Typical autoradiograms are shown in the left panels where the black arrow indicates the position of AP1 binding. Densitometric quantitative data are shown in the right panels, where each value represents the mean ± S.E. obtained in 4 to 6 separate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained in cultured neurons not exposed to static magnetic field.

hippocampal neurons cultured at a low density of 1.0×10^5 cells/cm^2 with concomitant quadrupling binding in those cultured at a high density of 2.5×10^5 cells/cm^2.

In cortical neurons cultured at a high density of 2.5×10^5 cells/cm^2 for 3 DIV, brief exposure did not significantly affect AP1 DNA binding determined 15 to 60 min after exposure (Fig. 4a). In hippocampal neurons cultured under the similar conditions, AP1 binding was almost doubled 15 min after brief exposure to static magnetic field for 15 min (Fig. 4b). In these hippocampal neurons, AP1 binding was more than quadrupled 30 min after exposure, with being doubled even 60 min later.

Expression of AP1 Complex

Hippocampal neurons were cultured at a high density of 2.5×10^5 cells/cm^2 for 3 DIV, followed by brief exposure to static magnetic field for 15 min and subsequent harvest 30 min after exposure for preparation

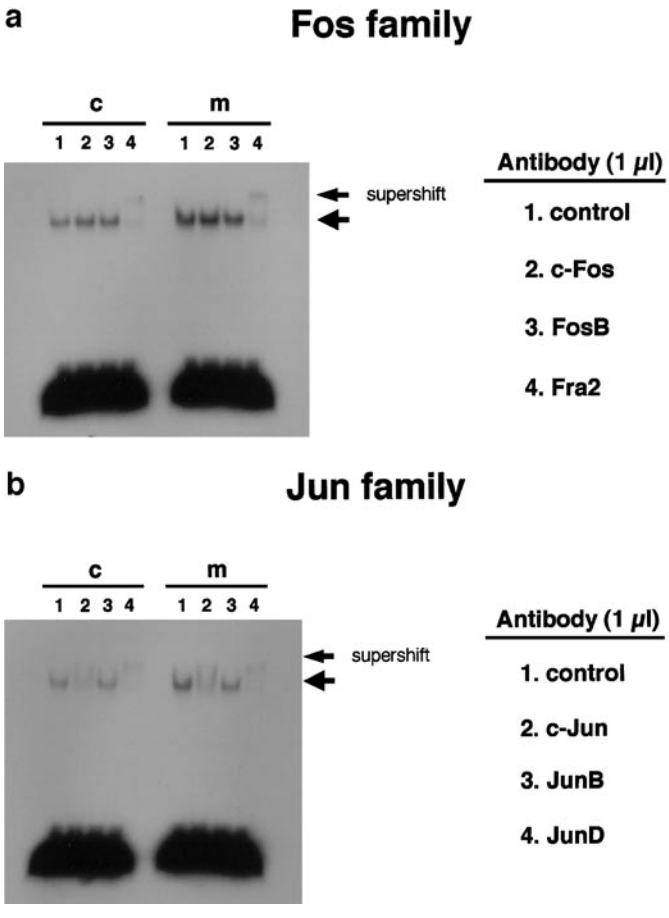

FIG. 5. AP1 DNA binding after reaction with antibodies against (a) Fos family and (b) Jun family member proteins in hippocampal neurons. Hippocampal neurons were cultured for 3 DIV at a density 2.5×10^5 cells/cm^2 and exposed for 15 min to static magnetic field. Cultured neurons were harvested 30 min after exposure, followed by preparation of nuclear extracts and subsequent incubation at 4°C for 18 h with one of antibodies against c-Fos, Fos-B, Fra-2, c-Jun, Jun-B, and Jun-D proteins. These samples were then subjected to determination of AP1 DNA binding on gel retardation electrophoresis. Typical autoradiograms are shown where the small black arrows indicate the position of supershift of probe/protein complex. Similar results were seen in 3 independent experiments.

of nuclear extracts. These nuclear extracts were incubated with one of antibodies raised against c-Fos, Fos-B, Fra-2, c-Jun, Jun-B, and Jun-D proteins prior to gel retardation electrophoresis. Of antibodies directed against Fos family member proteins, the anti-Fra-2 antibody induced a marked decrease in AP1 binding in hippocampal neurons exposed and not exposed to magnetic field with concomitant upward migration of the mobility on the gel (Fig. 5a). Neither the anti-c-Fos nor the anti-Fos-B antibody markedly affected AP1 binding in hippocampal neurons irrespective of exposure to magnetic force. Of antibodies against Jun family member proteins, both the anti-c-Jun and the anti-Jun-D antibodies were effective in decreasing and migrating AP1 binding in hippocampal neurons previously exposed or not exposed to static magnetic field (Fig. 5b). The anti-Jun-B antibody did not markedly affect AP1 binding irrespective of exposure to magnetic field.

Immunoblotting analysis revealed that brief exposure to magnetism resulted in expression of Fra-2, c-Jun, and Jun-D proteins with the corresponding molecular weights in hippocampal neurons cultured at a high density of 2.5×10^5 cells/cm^2 for 3 DIV when harvested 30 min after exposure (Fig. 6).

Delayed Changes

Hippocampal neurons were cultured at a high density of 2.5×10^5 cells/cm^2 for 3 DIV, followed by brief exposure to static magnetic field for 15 min and subsequent further culture for additional 24 h for determination of the number of fluorescent neurons following the cumulative addition of NMDA at 1 to 100 μM in the absence of $MgCl_2$ using laser-scanning microscope. The addition of NMDA markedly increased the number of fluorescent cells in a concentration-dependent manner in hippocampal neurons not exposed to static magnetic field, while prior exposure to magnetism induced a rightward shift of the concentration-response curve between NMDA and fluorescence 24 h later [EC_{50} values (μM); 3.6 ± 0.5 vs. 8.1 ± 1.1**, $^{**}P < 0.01$] (Fig. 7a).

Similarly, hippocampal neurons were at first exposed to static magnetic field for 15 min and additionally cultured for 24 h. Cells were then exposed for 5 min to NMDA at concentrations of 10 to 100 μM in the absence of $MgCl_2$, followed by harvest 2 h later and subsequent preparation of nuclear extracts for determination of AP1 DNA binding. Prior brief exposure to

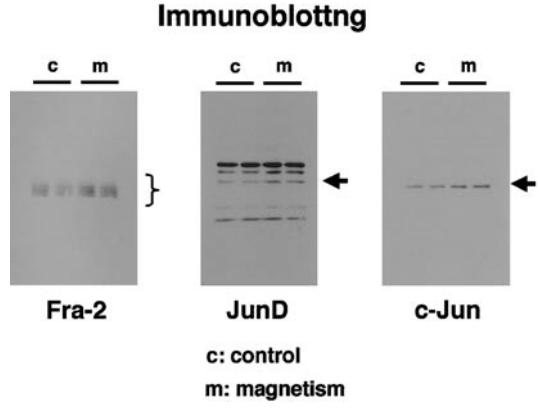

FIG. 6. Immunoblotting of particular Fos and Jun family member proteins in hippocampal neurons. Hippocampal neurons were cultured for 3 DIV at a density 2.5×10^5 cells/cm^2 and exposed for 15 min to static magnetic field. Cultured neurons were harvested 30 min after exposure, followed by SDS–PAGE and subsequent immunoblotting analysis using one of antibodies against Fra-2, c-Jun, and Jun-D proteins. Typical blots are shown in the panel where the black arrows indicate the corresponding molecular weight positions. Similar results were seen in 3 separate determinations.

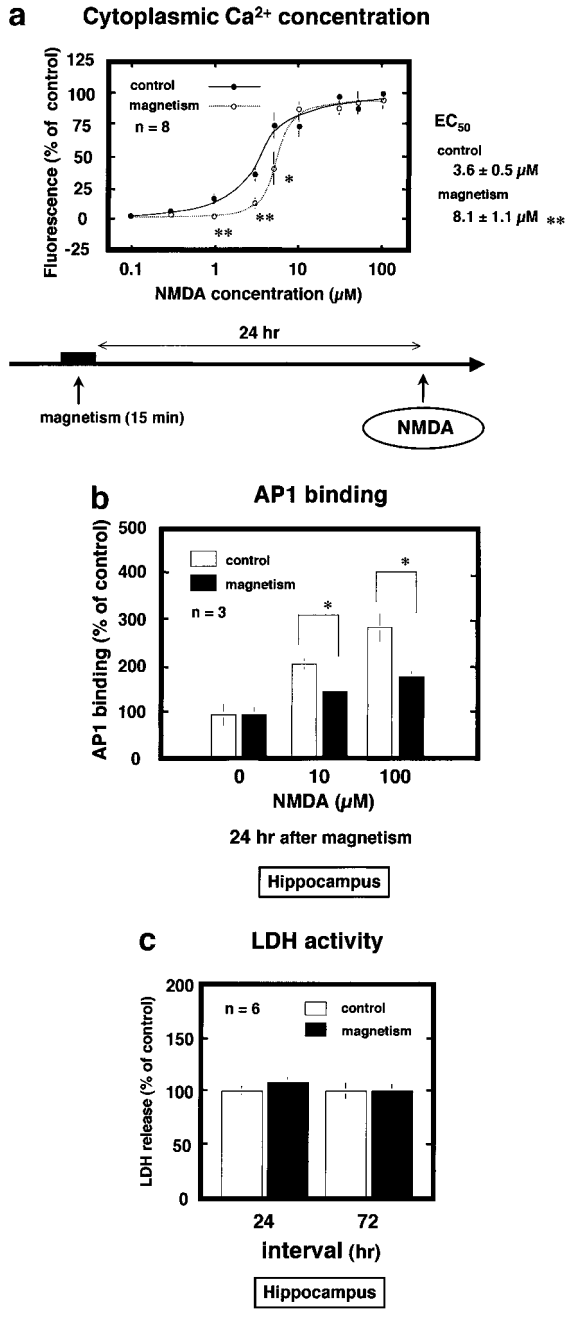

FIG. 7. Effects of prior exposure to static magnetic field on hippocampal neurons. Hippocampal neurons were cultured for 3 DIV at a density of 2.5×10^5 cells/cm^2, followed by brief exposure to static magnetic field for 15 min and subsequent further culture for 24 h. (a) Cells cultured for 3 DIV were then loaded with Fluo-3 and exposed to NMDA in the absence of $MgCl_2$ for determination of the number of fluorescent neurons by laser-scanning microscope. Values are the mean ± S.E. of percentages over the corresponding maximal values in 8 independent experiments. *$P < 0.05$, **$P < 0.01$, significantly different from each control value obtained in neurons not exposed to static magnetic field. (b) Cells cultured for 3 DIV were exposed to NMDA for 5 min in the absence of $MgCl_2$ and collected 2 h later for determination of AP1 DNA binding. Each value represents the mean ± S.E. in 3 separate measurements. *$P < 0.05$, significantly different from each control value obtained in cultured neurons not exposed to static magnetic field. (c) Hippocampal neurons were cultured for 3 DIV, followed by replacement of culture medium and subsequent exposure for 15 min to static magnetic field. The activity of LDH was determined in culture medium collected 24 or 72 h after the exposure to static magnetic field. Each value represents the mean ± S.E. obtained in 6 separate determinations.

static magnetic field led to a significantly less potent increase in AP1 DNA binding following the addition of NMDA compared to neurons not exposed to magnetism when determined in hippocampal neurons 24 h after the exposure (Fig. 7b).

Hippocampal neurons were cultured for 3 DIV, followed by replacement of culture medium and subsequent brief exposure to static magnetic field for 15 min. Cells were further cultured for 24 to 72 h to collect culture medium for determination of LDH activity. However, brief exposure to magnetic force did not significantly affect the release of LDH under these experimental conditions (Fig. 7c).

DISCUSSION

The essential importance of the present findings is that brief exposure to static magnetic field led to rapid but transient expression of AP1 complex with DNA binding activity in immature cultured rat hippocampal neurons. Transient magnetic stimulation would induce long-lasting alterations of a variety of cellular functions through modulation of *de novo* synthesis of particular inducible target proteins at the level of gene transcription by AP1 complex in the nucleus. For instance, prior brief exposure to magnetism could lead to desensitization of NMDA receptor channels with respect to increases in intracellular free Ca^{2+} concentration and nuclear AP1 DNA binding in immature cultured hippocampal neurons. Expression profiles of GAP-43 give support to the immaturity of hippocampal neurons cultured for 3 DIV. Expression of GAP-43 is associated with neuronal development, axonal regeneration, synaptogenesis, and plasticity in immature neurons but not seen in mature neurons (25). Our data on Western blotting analysis are suggestive of an idea that brief exposure to static magnetic field may be effective in inducing AP1 complex with DNA binding activity only in immature hippocampal neurons with high expression of GAP-43 but not in mature hippocampal neurons with low expression of GAP-43. The data cited above led us to employ rat hippocampal neurons cultured for 3 DIV in place of those for 9 DIV as an *in vitro* model of immature neurons to assess signaling processes mediated by static magnetic field to nuclear AP1 complex in the present study.

Several independent lines of evidence indicate that cellular maturity is one of crucial determinants for responsiveness to a variety of extracellular signals in neurons. In contrast to studies using mature cultured

neurons, NMDA is often neurotrophic in a manner dependent on the cellular maturity in cerebellar (27, 28) and hippocampal (29) neurons in primary culture. In cerebellar slices, NMDA is more potent in depolarizing Purkinje and granule cells obtained from immature rats than those from adults rats, with similarly potent depolarization by agonists at other Glu receptor subtypes (30). Long-term potentiation (31) as well as sensitivity to Mg^{2+} block (32, 33) is declined in proportion to postnatal periods in rat hippocampus. Neuronal maturity could determine responsiveness to static magnetic field, which leads to long-lasting but unidentified functional alterations through modulation of *de novo* synthesis of particular target proteins at the level of gene transcription by AP1 complex in the nucleus. Differential display technique could be useful for the search and identification of target genes transcribed by AP1 complex that is expressed in response to transient magnetic stimulation. Judging from the findings on LDH release, it is unlikely that magnetic stimulation is neurotoxic to immature hippocampal neurons.

The mechanism underlying differential potentiation by magnetism of AP1 DNA binding between immature neurons from hippocampus and cortex is not clear so far. Proliferation, differentiation and/or maturation could undergo with neurons in these 2 separate telencephalic structures in a manner different from each other. In cortical stem cells, cell-cell contact would be critical for the fate of differentiation. Cortical stem cells give rise to neurons, astrocytes and oligodendrocytes in cultures at a high density, with almost exclusive differentiation into smooth muscle at a low density (34). The fact that exposure to static magnetic field led to potentiation of AP1 DNA binding in immature hippocampal neurons in a density-dependent fashion could be accounted for by taking into consideration these previous findings. An *in situ* hybridization study reveals that high frequency rTMS *in vivo* induces marked expression of mRNA for GFAP in the murine hippocampal dentate gyrus with modest expression in cerebral cortex, as seen with electroconvulsive seizures (35). To our knowledge, this paper deals with the first direct demonstration of preferential expression of AP1 complex with DNA binding activity in response to transient exposure to weak static magnetic field in immature cultured rat hippocampal neurons *in vitro.* The possibility that the present expression of AP1 complex by magnetism may be involved in mechanisms associated with clinical usefulness of rTMS for several psychiatric disorders, however, remains to be elucidated in future studies.

An immunohistochemical study shows that marked expression of c-Fos protein is induced by exposure for 30 min to static magnetic field at 9.4 T in the visceral and vestibular nuclei of rat brain stem *in vivo* (36). In organotypic brain slices of rat parietal cortex, magnetic stimulation results in transient expression of c-Fos protein in neurons but not in astroglia via tetrodotoxin-sensitive sodium channels 3 to 6 h after stimulation (37). Similarly marked expression is shown with c-Fos protein in parietal cortex and hippocampus (38) as well as other telencephalic regions including frontal cortex, striatum, dentate gyrus, Ammon's horn and amygdala (39) after several sessions of rTMS. In contrast to these previous findings, c-Fos protein did not participate in the potentiation of AP1 DNA binding following brief exposure to static magnetic field in the present study. Evaluation using repetitive stimulation by magnetic force more potent than that used here would give us a clue to account for the paradoxical expression of Fos family member proteins. Nevertheless, it should be emphasized that expression of neither mRNA nor immunoreactive protein is always followed by alterations of different functions including recognition of particular core nucleotide sequence. From this point of view, determination of DNA binding is crucial for the direct demonstration of functional alterations induced by magnetism.

It thus appears that transient exposure to weak static magnetic field may modulate *de novo* synthesis of particular inducible target proteins at the level of gene transcription by nuclear AP1 complex in immature cultured rat hippocampal neurons. The modulation could underlie transformation of transient extracellular signals carried by magnetism into long-lasting alterations of cellular functions in immature hippocampal neurons.

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