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Functional Alterations in Immature Cultured Rat Hippocampal Neurons After Sustained Exposure to Static Magnetic Fields

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In cultured rat hippocampal neurons, gradual increases were seen in the expression of microtubule-associated protein-2 (MAP-2), neuronal nuclei (NeuN) and growth-associated protein-43 (GAP-43), in proportion to increased duration, up to 9 days in vitro (DIV). Sustained exposure to static magnetic fields at 100 mT for up to 9 DIV significantly decreased expression of MAP-2 and NeuN in cultured rat hippocampal neurons without markedly affecting GAP-43 expression. Although a significant increase was seen in the expression of glial fibrillary acidic protein (GFAP) in hippocampal neuronal preparations cultured for 6–9 DIV under sustained magnetism, GFAP and proliferating cell nuclear antigen expression were not affected markedly in cultured astrocytes prepared from rat hippocampus and neocortex, irrespective of cellular maturity. No significant alteration was seen in cell survivability of hippocampal neurons or astrocytes cultured under sustained magnetism. In hippocampal neurons cultured for 3 DIV under sustained magnetism, marked mRNA expression was seen for *N*-methyl-D-aspartate (NMDA) receptor subunits, NR1, NR2A-2C, NR2D, and NR3A. In addition, significant potentiation of the ability of NMDA to increase intracellular free Ca^{2+} ions was observed. Differential display analysis revealed a significant decrease in mRNA expression for the transcription factor ALF1 in response to sustained magnetism for 3 DIV. These results suggest that sustained exposure to static magnetic fields may affect cellular functionality and maturity in immature cultured rat hippocampal neurons through modulation of expression of particular NMDA receptor subunits.

Key words: static magnetic fields; MAP-2; GFAP; NMDAR; ALF1

Signal propagation is believed to involve both neurotransmission mediated by neurotransmitters and conduction mediated by electrical currents in neurons. Compared to the many biochemical, pharmacologic, and molecular biological studies dedicated to elucidation of mechanisms underlying neurotransmission at synapses, relatively little attention has been paid to mechanisms associated with modulation of signal conduction by endogenous and exogenous stimuli. In the central nervous system (CNS), both oscillatory and static magnetic fields can modulate a variety of cellular functions in glia and neurons through modulation of electrical currents and/or mechanical mobility in vivo and in vitro. Brief exposure to static magnetic fields at 200 mT leads not only to marked morphologic alterations, for example, but also results in significant reduction of thymidine incorporation and inositol lipid signaling in the human neuronal cell line FNC-B4 (Pacini et al., 1999). Action potentials are blocked by static magnetic fields in cultured sensory neurons (Cavopol et al., 1995), whereas alternating magnetic fields increase expression of glial fibrillary acidic protein (GFAP) in cultured astrocytes (Chan et al., 1999). In clinical studies, repetitive transcranial magnetic stimulation (rTMS) has been shown to be beneficial for treatment and therapy of selected patients with depression, bipolar affective disorder, and schizophrenia, as a possible alternative to electroconvulsive therapy (ECT) often used for the treatment of refractory depression (Hasey, 2001; McNamara et al., 2001; Post and Keck, 2001). The efficacy of weak magnetic fields in the treatment of parkinsonism and motor complications of chronic levodopa therapy has been proposed (Sandyk et al., 1992). In healthy subjects, however, rTMS induces transient mood enhancement (George et al., 1995; Kirkcaldie et al., 1997).

We have shown previously in immature cultured rat hippocampal neurons (Hirai et al., 2002a) that a marked but transient increase is seen in DNA-binding activity of the nuclear transcription factor activator protein (AP1), 30 min after brief (15 min) exposure to static magnetic

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fields at 100 mT. AP1 is a hetero- and homodimer between Jun and Fos family member proteins and modulates gene transcription through specific recognition of the core consensus sequence TGACGTCA in the nucleus. An immunohistochemical study demonstrated that marked expression of c-Fos protein in the visceral and vestibular nuclei of rat brain stem in vivo (Snyder et al., 2000) was induced by a 30-min exposure to magnetic fields at 9.4 T. 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 3–6 hr after stimulation (Hausmann et al., 2001). Similarly marked expression has been shown with c-Fos protein after several sessions of rTMS in parietal cortex and hippocampus (Hausmann et al., 2000) as well as in other telencephalic regions including frontal cortex, striatum, dentate gyrus, Ammon's horn, and amygdala (Doi et al., 2001).

These previous studies raise the possibility that exposure to magnetic fields would lead to long-term consolidation and amplification of different functional alterations induced by transient extracellular signals through modulation of de novo protein synthesis at the level of gene transcription in the brain. We have evaluated cellular maturation and differentiation in rat hippocampal neurons cultured under sustained exposure to static magnetic fields.

MATERIALS AND METHODS

Materials

Antibodies against microtubule-associated protein-2 (MAP-2), glial fibrillary acidic protein (GFAP), and growth-associated protein-43 (GAP-43) were purchased from Sigma (St. Louis, MO). Antibody against proliferating cell nuclear antigen (PCNA) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and antibody against neuronal nuclei (NeuN) was purchased from Chemicon (Temecula, CA). Anti-mouse and anti-rabbit IgG antibodies conjugated with peroxidase as well as ECL detection reagent were obtained from Amersham Life Science (Buckinghamshire, UK). Fluo-3 acetoxymethyl ester was provided by Molecular Probes (Eugene, OR), Versene and Dulbecco's modified Eagle medium (DMEM) were obtained from Gibco BRL (Grand Island, NY), and fluorescein isothiocyanate (FITC) was purchased from ICN Pharmaceuticals (Aurora, OH). Other chemicals used were all of the highest purity available commercially.

Neuronal Cultures

Primary hippocampal neuronal cultures were obtained from 18-day-old embryonic Wistar rats according to the method of Di Porzio et al. (1980), with minor modifications. In brief, hippocampi were dissected and incubated with Versene at room temperature for 12 min. Cells were then dissociated mechanically using a Pasteur pipette with a fire-narrowed tip in the culture medium, and dissociated cells were plated at a density of 1.0 (low) or 2.5 (high) $\times$ 10^5 cells/cm^2 (35-mm plate diameter; Nunc) in a one-well dish after a Trypan Blue dye exclusion test. Culture dishes were coated previously, first with poly-L-lysine (7.5 μg/mL; Sigma) and then with 10% fetal bovine serum (FBS) in DMEM. Culture medium was DMEM without FBS supplemented with 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 for different days in vitro (DIV) at 37°C in a 5% CO_2/95% air humidified incubator.

Astroglial Cultures

Astrocytes were prepared as described previously (Murakami et al., 2003). In brief, brain cortices from 19-day-old Wistar rat embryos were cleared of meninges, cut into blocks of about 1 mm^3, and treated with 0.25% trypsin in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS) containing 5.5 mM glucose for 15 min at 37°C with gentle shaking. An equal volume of horse serum supplemented with 0.1 mg/mL of DNase I (DN-25; Sigma) was added to inactivate trypsin, and tissues were centrifuged at 100 $\times$ g for 5 min. Sediments were triturated through a Pasteur pipette with DMEM containing 10% FBS, 100 mg/L streptomycin, and 5 $\times$ 10^4 U/L penicillin. After being filtered through lens-cleaning paper (Fuji Photo Co., Tokyo, Japan), cells were plated on plastic dishes (100 mm diameter; Nunc) coated previously with polyethyleneimine at a density of 0.8–1.3 $\times$ 10^5 cells/cm^2. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 1 week with a medium change every 3 days. After 1 week, astrocytes were replated to remove neurons. At 12–14 DIV, cells were again replated using an ordinary trypsin-treatment technique onto 6-well plates (Nunc) at a density of 4 $\times$ 10^4 cells/well and the culture continued until use. Approximately 90% of these cells were immunoreactive to the astrocyte marker protein GFAP.

Exposure to Magnetism

Neocortical astrocytes as well as hippocampal neurons and astrocytes were cultured in a CO_2 incubator at 37°C for different periods under static 100-mT magnetic fields generated by permanent ferrite magnets placed at both sides of culture dishes. Dishes were invariably put at the center of the magnetic field, 10 cm away from each other. A control group (sham exposure) was also subjected to similar procedures under the same environmental influences as experimental groups. The intensity of magnetic fields was always measured with a Tesla Meter TM-601 (Kanetec), and ranged from 0–0.3 mT in the sham control group.

Conditioned Medium

Hippocampal neurons were cultured for 3 DIV under sustained exposure to static magnetic fields, followed by collection of culture medium as conditioned medium. Astrocytes were cultured for 12–14 DIV, replated using an ordinary trypsin-treatment technique onto 6-well plates at a density of 4 $\times$ 10^4 cells/well, and then cultured for 7 DIV with the aforementioned conditioned medium derived from hippocampal neuronal cultures exposed previously to magnetism for 3 DIV.

Western Blotting

Neurons were cultured from 1–9 DIV at a density of 1.0 or 2.5 × 10^5 cells/cm^2 and astrocytes were cultured for 2 and 7 DIV after replating, respectively. Cells were 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, 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 (Hirai et al., 2002b). 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 dodecylsulfate (SDS), 0.01% bromophenol blue, and 5% 2-mercaptoethanol, and homogenates were then boiled at 100°C for 10 min. A 20-μg aliquot of protein was loaded on 7.5 or 10% polyacrylamide gel containing 0.1% SDS for electrophoresis (constant current of 15 mA/plate for 2 hr at room temperature), followed by blotting to a polyvinylidene fluoride membrane treated previously with 100% methanol. After blocking with 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 the antibodies against MAP-2, NeuN, GAP-43, GFAP, and PCNA, diluted adequately with the latter buffer containing 1% skim milk. After incubation with one of the above antibodies, the membrane was incubated further with anti-mouse IgG antibody conjugated with peroxidase, and subsequently exposed to X-ray films for different periods to obtain appropriate immunoblots for quantitative densitometry. Densitometric determination of X-ray films was carried out with the aid of BIO PROFIL Bio-1D densitograph (VILBER LOURMAT).

Immunofluorescence Staining

For immunofluorescence analysis, cells were plated on glass coverslips (Nunc) coated with poly-L-lysine. Cultures were fixed for 20 min with 4% paraformaldehyde in PBS, washed twice with PBS, and subsequently treated with 10% bovine serum albumin (BSA) in PBS containing 0.1% Triton X-100 for 60 min. Cells were then stained with primary antibodies against MAP-2 and GFAP diluted with 1% BSA in PBS. Staining was visualized with the anti-mouse IgG conjugated with rhodamine and the anti-rabbit IgG conjugated with FITC, respectively, before coverslips were mounted with FluorSave (Calbiochem-Novabiochem). Phase contrast and immunofluorescence images were obtained using a Zeiss Axiovert microscope (Carl Zeiss, Thornwood, NY).

Reverse Transcription-PCR Analysis

For reverse transcription-PCR (RT-PCR) analysis, mRNA was extracted from each sample using a QuickPrep Micro mRNA Purification Kit, followed by synthesis of complementary DNA (cDNA) with 12.5 μM random hexamer primers and Ready-To-Go You-Prime First-Strand Beads (Amersham), as described previously (Hinoi et al., 2001). A reaction with reverse transcriptase was run at 37°C for 1 hr, and an aliquot of synthesized cDNA was used directly for PCR carried out in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP (deoxy nucleotide triphosphate), 20 pmol of each primer, and 0.625 unit of *Taq* polymerase. Gene-specific primer sets were as follows: NR1, 5′-ACGGAATGATGGGCGAGC-3′ and 5′-GGCATCCTTGTGTCGCTTGTAG-3′; NR2A-C, 5′-GGGGTTCTGCATCGACATCC-3′ and 5′-GACAGCAAAGAAGGCCCACAC-3′; NR2D, 5′-CGATGGCGTCTGGAATGG-3′ and 5′-CTGGCAAGAAAGATGACCGC-3′; NR3A, 5′-CCGCGGGATGCCCTACTGTTC-3′ and 5′-CCAGTTGTTCATGGTCAGGAT-3′; GAP-43, 5′-GGAATAAGGATCCGAGGAGGAAAGGAG-3′ and 5′-CTTAAAGTTCAGGCATGTTCTTGGT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GGTGAAGGTCGGTGTCAACGGATT-3′ and 5′-GATGCCAAAGTTGTCATGGATGACC-3′. Amplification from primers specific for NR1, NR2A, NR2B, NR2C, NR2D, NR3A, GAP-43, and GAPDH produced fragments 1,033, 548, 546, 547, 465, 417, 750, and 600 base pairs (bp) in length, respectively. Cycling conditions used for 22 PCR cycles were as follows: NR1, NR2A-C, NR2D, NR3A, and GAP-43: 1 min at 95°C for denaturation, 1 min at 55°C for annealing, 1 min at 72°C for extension. Cycling conditions used for 25 PCR cycles were as follows: NR2A-C and GAPDH: 1 min at 94°C for denaturation, 2 min at 61°C for annealing, and 1 min at 72°C for extension. PCR amplification products were electrophoresed on a 1.5% agarose gel, and DNA was detected with ethidium bromide and subsequent photographing using Gel Doc 1000 (Bio-Rad). The housekeeping gene GAPDH was used as the nonchanging control mRNA. To determine changes in mRNA expression, the density and area of each band of PCR product was analyzed with *NIH Image 1.6* and values were normalized to densitometric values of the corresponding GAPDH PCR products. Under the experimental conditions described above, the number of PCR cycles was selected so that amplified signals were in a dynamic range in any situation (not saturated).

Measurement of Intracellular Free Calcium

Cultured neurons were washed once 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) and incubated at 37°C for 1 hr in recording medium containing 30 nM Pluronic F-127 and 3 μM fluo-3 acetoxymethyl ester, which is a membrane-permeable form of the Ca^{2+}-sensitive dye. Culture dishes were then washed twice with recording medium and allowed to settle for at least 1 hr in recording medium. The medium was changed once more, followed by the cumulative addition of NMDA at concentrations of 0.1–100 μM in the absence of $MgCl_2$, and subsequent determination of the number of fluorescent neurons every 2 min later. Maximum fluorescence was determined by fluorescence after the addition of the Ca^{2+} ionophore A23187 (10 μM) at the end of each experiment (Nakamichi et al., 2002a). Cells were used invariably within 1–5 hr after these procedures for observation of fluorescence visualized with a confocal laser-scanning microscope equipped with an argon laser. Images were obtained using an objective lens with numeral apertures of 0.5 (Plan-Neofluar) for 20-fold magnification. Fluorescence images labeled with fluo-3 AM were collected using an excitation wavelength of 488 nm. Parameters of illumination and detection were controlled digitally for consistent settings throughout the experiments. Two successive digital images were collected usually at 512 × 512 pixels in the same visual field for evaluation, as described

previously (Nakamichi et al., 2002b). Both maximal response (E_{max}) and half-maximum concentration (EC_{50}) values were obtained by data fitting using Microcal *ORIGIN v. 4.0*. Under the conditions employed, NMDA markedly increased the number of cells with high fluorescence in a concentration-dependent manner. The NMDA-induced increase was not only inhibited by the NMDA receptor channel antagonist dizocilpine (MK-801), but also prevented by nifedipine, a blocker of L-type voltage-sensitive Ca^{2+} channels, with the inhibitor of Ca^{2+} release across ryanodine-sensitive Ca^{2+} channels from intracellular stores dantrolene being ineffective (Nakamichi et al., 2002a).

Cell Viability

Cell viability was quantified by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Culture medium was replaced with HEPES-buffered Kreps-Ringer (HKR) buffer containing 0.5 mg/mL MTT and incubated for 1 hr at 37°C. After incubation, cells were solubilized by addition of a lysis solution containing 99.5% isopropanol and 0.04 M HCl. The amount of MTT formazan product was determined by measuring absorbance at 550 nm on a microplate reader. Relative values were calculated as percentages over values obtained in the control group not exposed to magnetism.

Cell morphology was examined by double fluorescent staining with Hoechst 33342 (bis-benzimide trihydrochloride) and propidium iodide (PI). The DNA-binding dyes Hoechst 33342 and PI were used to measure apoptotic and necrotic cell death, respectively. Hippocampal neurons were plated at a density of 2.5×10^5 cells/cm^2 and cultured for 3 DIV under sustained exposure to static magnetic fields. Cells were washed twice in PBS and then incubated with Hoechst 33342 (10 μg/mL) and PI (5 μg/mL) for 20 min. Nuclei were visualized under epifluorescence microscope (IMT-2; Olympus, Tokyo, Japan) with a V- and G-excitation cubes for Hoechst 33342 and PI, respectively.

Differential Display Analysis

Hippocampal neurons were plated at a density of 2.5×10^5 cells/cm^2 and cultured for 3 DIV under sustained exposure to static magnetic fields as described above. Cells were immediately harvested and total RNA was extracted using Isogen (Nippon Gene) according to the manufacturer's instructions. For differential display (DD) analysis, DD-PCR was carried out using Fluorescence Differential Display Kit (TaKaRa), with 24 upstream and 9 downstream random primers, according to the manufacturer's instruction. Genes expressed differentially by sustained exposure to static magnetic fields were used as templates for PCR re-amplification. Purified PCR products were cloned subsequently into pT7 blue plasmids (Novagen) and sequenced by DYEnamic ET Terminator cycle sequencing kit (Amersham Bioscience). DNA sequence similarity was searched with the BLAST algorithm.

Northern Blotting Analysis

The cloned differentially expressed fragments were labeled with [α-^{32}P]dCTP by the random primer method using Rediprime II random prime labeling system (Amersham) for Northern blot analysis. Total RNA (10 μg per lane) was electrophoresed on 1.0% denatured agarose gel containing 2.2 M formaldehyde and then transferred to a nylon membrane by capillary blotting. Hybridization was carried out in a solution containing 5× SSC, 50% formamide, 5× Denhardt's, 0.5% SDS, and 20 μg/mL salmon DNA at 42°C for 16 hr. The hybridized membrane was washed twice with 2× SSC containing 0.1% SDS at room temperature for 10 min, with 1× SSC containing 0.1% SDS at 42°C for 20 min, and then with 0.5× SSC containing 0.1% SDS at 42°C for 20 min. Hybridization signals were detected by autoradiography, quantified using a densitograph, and normalized with 18S rRNA levels.

Data Analysis

Quantitative densitometric data are expressed as the mean ± SE and statistical significance was determined by two-tailed Student's *t*-test.

RESULTS

Expression of GFAP and PCNA

To evaluate the effect on purity of neurons in cultured neuronal preparations, isolated hippocampal neurons were seeded at a density (low) of 1.0×10^5 cells/cm^2, followed by in vitro culturing for 1–9 DIV under sustained exposure to a static 100-mT magnetic field and subsequent collection to determine expression of immunoreactive GFAP and PCNA. As shown in Figure 1A, GFAP was not markedly detected within 3 DIV but expressed after 3 DIV up to 9 DIV. By 6–9 DIV, sustained exposure to static magnetic fields significantly increased GFAP expression in hippocampal neuronal cultures.

An attempt was next made to determine whether sustained magnetism modulates GFAP and PCNA expression in cultured astrocytes prepared from rat hippocampus and neocortex. No significant changes were seen after replating, however, in GFAP and PCNA expression in hippocampal and neocortical astrocytes cultured for 2–7 DIV under sustained exposure to static magnetic fields (Fig. 1B). No significant cell death was found in hippocampal and neocortical astrocytes cultured for 7 DIV under sustained exposure to magnetism (Fig. 1C).

To evaluate the possible release from hippocampal neurons exposed to magnetism, of certain factors trophic to glial cells, hippocampal neurons were first cultured for 3 DIV under sustained exposure to magnetism, followed by collection of conditioned culture medium and subsequent culturing of hippocampal astrocytes for 7 DIV in the presence of conditioned medium. As shown in Figure 1D, however, no significant changes were found in GFAP and PCNA expression in hippocampal astrocytes cultured for 7 DIV in culture medium conditioned previously for 3 DIV with hippocampal neurons.

Expression of MAP-2

Hippocampal neurons were cultured at a low density for 1–9 DIV under sustained exposure to static magnetic fields, followed by detection of MAP-2 by Western blotting and immunocytochemistry. Expression of MAP-2 of two different molecular weights was increased individually

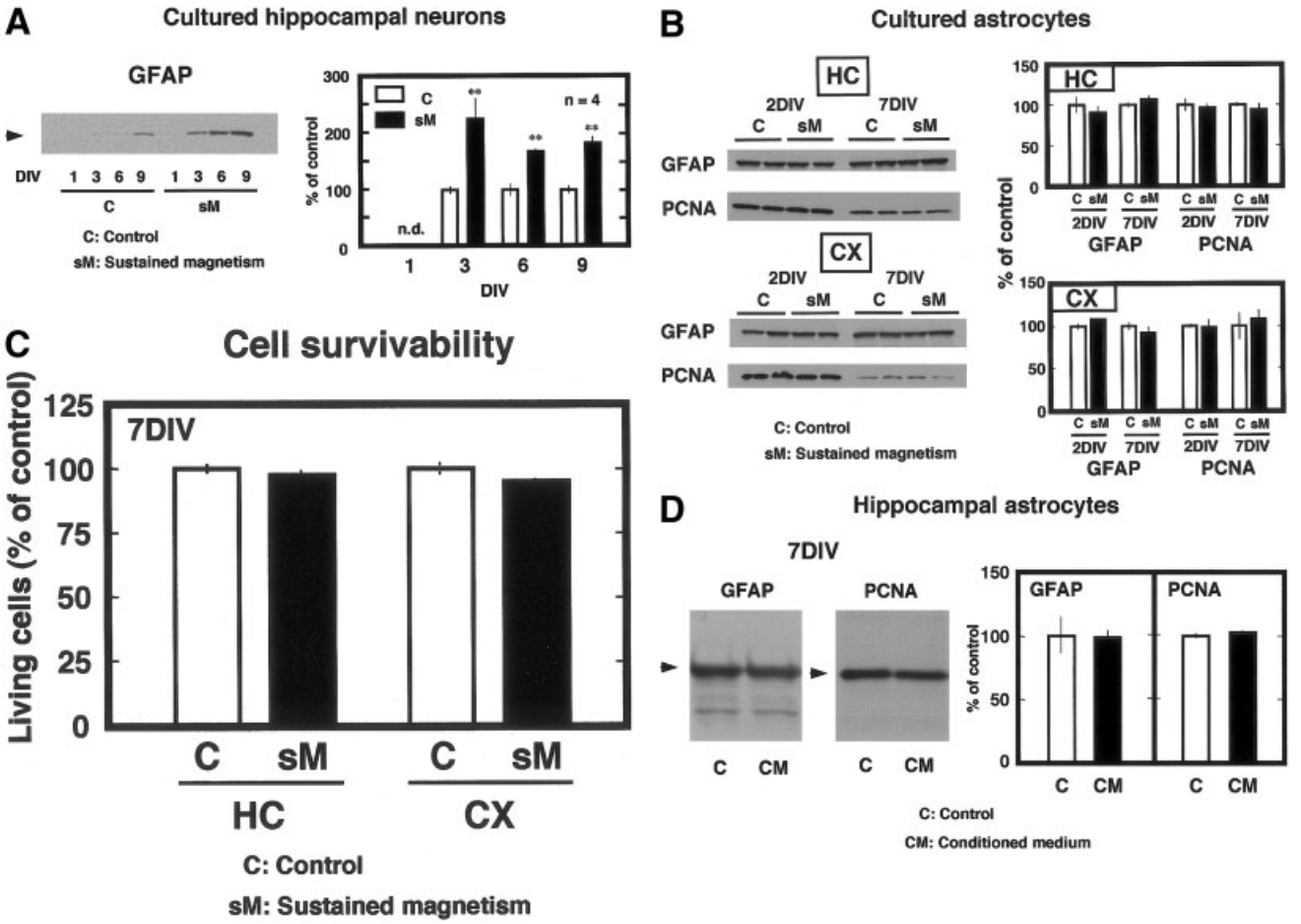

Fig. 1. Effects of sustained magnetism on typical profiles of cultured neurons and astrocytes. **A:** Hippocampal neurons were plated at a density of 1.0×10^5 cells/cm^2 and cultured for 1–9 DIV under static 100-mT magnetic field. Cultured neurons were harvested on days indicated, followed by SDS-PAGE and subsequent immunoblotting using anti-GFAP antibody. Each lane indicates sample from one well in the left panel; each value represents the mean ± SE obtained in four independent experiments in the right panel. $^{**}P < 0.01$, significantly different from each control value obtained in cultured neurons not exposed to static magnetic field (n.d., not detectable). **B:** Astrocytes were prepared from rat hippocampus and cerebral cortex, cultured for 2 and 7 DIV under static 100-mT magnetic field after replating. Cultured astrocytes were harvested on 2 or 7 DIV, followed by SDS-PAGE and subsequent immunoblotting using anti-GFAP and anti-PCNA antibodies. Each lane indicates a sample from one well in the left panel; each value represents the mean ± SE obtained in four independent experiments in the right panel. **C:** Cell survivability was quantified by MTT assay in hippocampal and cortical astrocytes cultured for 7 DIV under sustained exposure to magnetism. **D:** Hippocampal neurons were plated at a density of 1.0×10^5 cells/cm^2 and cultured for 3 DIV under static 100-mT magnetic field for subsequent collection of conditioned medium. Hippocampal astrocytes were cultured for 3 DIV in conditioned medium after replating, followed by SDS-PAGE and subsequent immunoblotting using anti-GFAP and anti-PCNA antibodies. Each lane indicates a sample from one well in the left panel; each value represents the mean ± SE obtained in three independent experiments in the right panel.

in proportion to increased duration of cultivation up to 9 DIV. Sustained exposure to static magnetic fields, however, significantly decreased expression of MAP-2 to approximately 75% of the control level, irrespective of culture durations (Fig. 2A). Upon immunocytochemical analysis of hippocampal neurons cultured for 3 DIV, most cells were immunoreactive to MAP-2 at both cell bodies and neurites, but not to GFAP (Fig. 2B, left panels). Sustained exposure to magnetism, however, led to a marked decrease in MAP-2 expression at neurites rather than at cell bodies (Fig. 2B, upper right), and GFAP-immunoreactive cells were undetectable (Fig. 2B, lower right).

Properties of Neurons Cultured under Sustained Magnetism

Hippocampal neurons were cultured at a low density for 3–9 DIV, followed by determination of NeuN and GAP-43 on Western blotting. Constitutive expression was seen with both NeuN and GAP-43 throughout in vitro culturing for 3–9 DIV (Fig. 3A, left panels), whereas sustained magnetism significantly decreased expression of

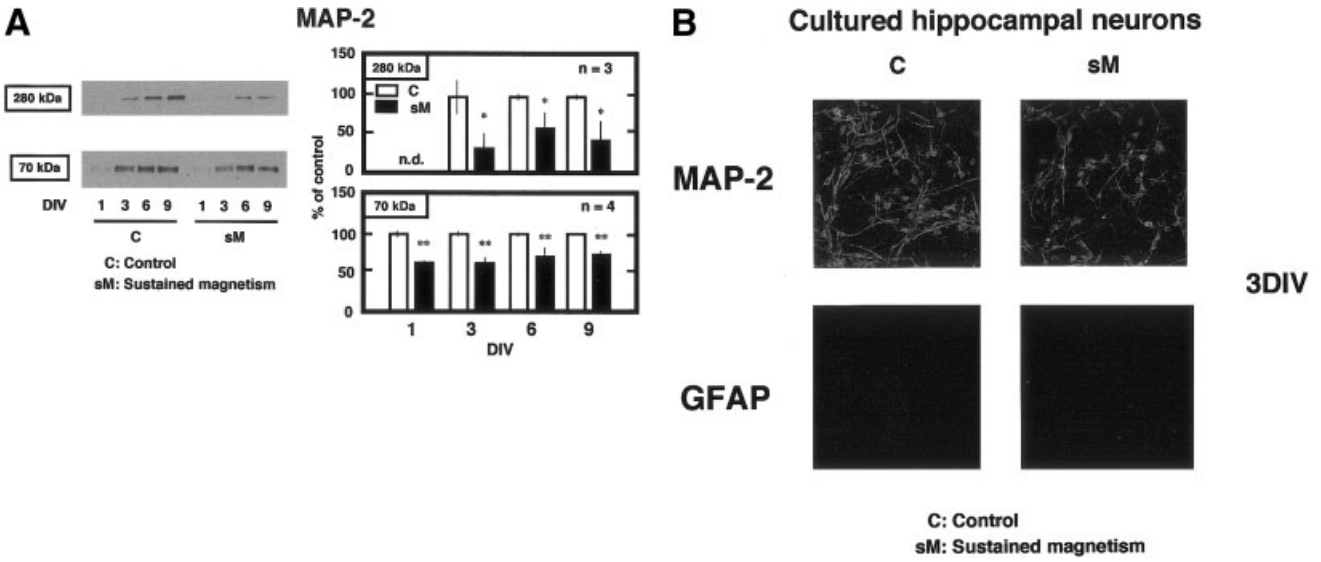

Fig. 2. Effects of sustained magnetism on MAP-2 expression in hippocampal neurons. **A:** Hippocampal neurons were plated at a density of 1.0×10^5 cells/cm^2 and cultured for 1–9 DIV under static 100-mT magnetic field, followed by harvest on indicated day and subsequent immunoblotting using anti-MAP-2 antibody. Each lane indicates a sample from one well in the left panel; each value represents the mean ± SE obtained in 3–4 independent experiments. $\star P < 0.05$, $\star\star P < 0.01$, significantly different from each control value obtained in cells not exposed to static magnetic field. **B:** Hippocampal neurons were cultured for 3 DIV under sustained exposure to magnetic field, followed by fixation with 4% paraformaldehyde and subsequent immunocytochemical detection of MAP-2 and GFAP. Typical images are shown; experiments were repeated at least three times with similar results.

NeuN (Fig. 3A, upper right) for 3–9 DIV without significantly affecting GAP-43 expression (Fig. 3A, lower right).

No marked morphologic alterations were found in cells stained with Hoechst 33342 and PI after 3 DIV under sustained magnetism and the number of cells stained with Hoechst 33342 was unchanged (Fig. 3B). Moreover, no significant alteration was seen in cell survivability when quantified by MTT assay in hippocampal neurons cultured for 3 and 9 DIV under sustained exposure to static magnetic fields (Fig. 3C).

Expression of NMDA Receptors

Hippocampal neurons were plated at high density and cultured for 3 DIV under sustained exposure to static magnetic fields, and semiquantitative RT-PCR analysis was used to determine mRNA expression for NMDA receptor subunits. Sustained magnetism for 3 DIV resulted in profound upregulation of mRNA expression for NR1, NR2A–C, NR2D, and NR3A subunits in cultured hippocampal neurons, without markedly affecting expression for GAP-43 and GAPDH (Fig. 4A). These cultured neurons were loaded with fluo-3 and subjected to determination of fluorescent cell number every 2 min after the cumulative addition of NMDA (concentration range, 0.1–100 μM) in the absence of $MgCl_2$, using a laser-scanning microscope. In hippocampal neurons cultured for 3 DIV, the addition of NMDA markedly increased fluorescent cell number in a concentration-dependent manner, whereas sustained magnetism induced a significant increase in maximal response (E_{max}) without significantly altering the half-maximum concentration (EC_{50}) (E_{max} values, 26.9 ± 1.0% vs. 33.7 ± 1.2%, $P < 0.01$ and EC_{50} values, 3.6 ± 0.6 μM vs. 2.7 ± 0.7 μM, $P < 0.01$) (Fig. 4B).

Differentially Expressed Genes

To identify genes regulated differentially in rat hippocampal neurons exposed to sustained magnetism, DD screening was carried out using 9 upstream and 24 downstream random primers. As shown in Figure 5A, a particular gene was found to be downregulated in response to sustained magnetism in hippocampal neurons cultured for 3 DIV. Cloning of the gene revealed a sequence highly homologous (95% identical) to the 3′ noncoding region of the mouse basic helix-loop-helix (bHLH) transcription factor ALF1 (Fig. 5B). The cloned fragment was radiolabeled with [α-^{32}P]dCTP as a probe for Northern blot analysis to evaluate magnetism-induced differential expression of rat ALF1. Expression of mRNA for ALF1 was decreased significantly during 3-day in vitro cultivation of hippocampal neurons, whereas a further decrease was seen in ALF1 mRNA expression in hippocampal neurons cultured for 3 DIV under sustained exposure to static 100-mT magnetic fields.

DISCUSSION

The primary importance of the present findings is that in cultured rat hippocampal neurons, sustained exposure to static magnetic fields led to significant potentiation of the maximal NMDA response toward increased intra-

cellular free Ca^{2+}, without affecting the concentration of a half-maximal response. Data from semiquantitative RT-PCR analysis support the idea that sustained magnetism could facilitate NMDA receptor channel opening through upregulated expression of particular subunits required for heteromeric assemblies of functional NMDA receptor channels. Although the increased maximal response gives strong support to upregulation of NMDA receptor channels, when antibodies are available commercially against all subunits cloned to date, Western blotting analysis should be carried out for expression of all NMDA receptor subunits required for assembled functional channels. Nifedipine-induced inhibition (Nakamichi et al., 2002a) supports the possibility that sustained magnetism could modulate properties of voltage-sensitive Ca^{2+} channels. From this standpoint, it should be noted that sustained magnetism increased expression of the dominant negative NR3A subunit, as well as the NR1, NR2A–C, and NR2D subunits.

Indeed, the NR3A subunit associated with NR1 and NR2 subunits has been shown to decrease Ca^{2+} permeation in response NMDA stimulation, in contrast to NR1/NR2 channels (Sasaki et al., 2002). Assembly with the NR1 subunit is required for surface expression of functional NMDA channels containing NR2A and NR3A subunits (Perez-Otano et al., 2001). Coexpression of NR1-1a, NR2A, and NR3A subunits, which would result in a mixed population of functional NMDA channels such as NR1-1a/NR2A and NR1-1a/NR2A/NR3A, facilitates accumulation of NR3A subunit in endoplasmic reticulum with less cell surface expression (Perez-Otano et al., 2001). Increased expression of mRNA for NR1, NR2A–C, NR2D, and NR3A subunits is insufficient to predict functional alteration of NMDA channels expressed at the cell surface after sustained magnetism. Other NR2 subunits could compete for NR1 subunit required for intracellular trafficking of NR3A subunit upregulated by sustained magnetism from the endoplasmic reticulum to the cell surface. Analysis of the expression of each NR2 subunit is undoubtedly required for further evaluation of heteromeric channel properties. This includes agonist affinity, desensitization, inactivation, and developmental expression profiles, as described previously for mice (Behar et al., 1999). To our knowledge, the present study deals for the first time with the possible correlation between sustained magnetism and NMDA receptor channel functionality in immature cultured rat hippocampal neurons. Through upregulation of NMDA receptor channels in hippocampal neurons, sus-

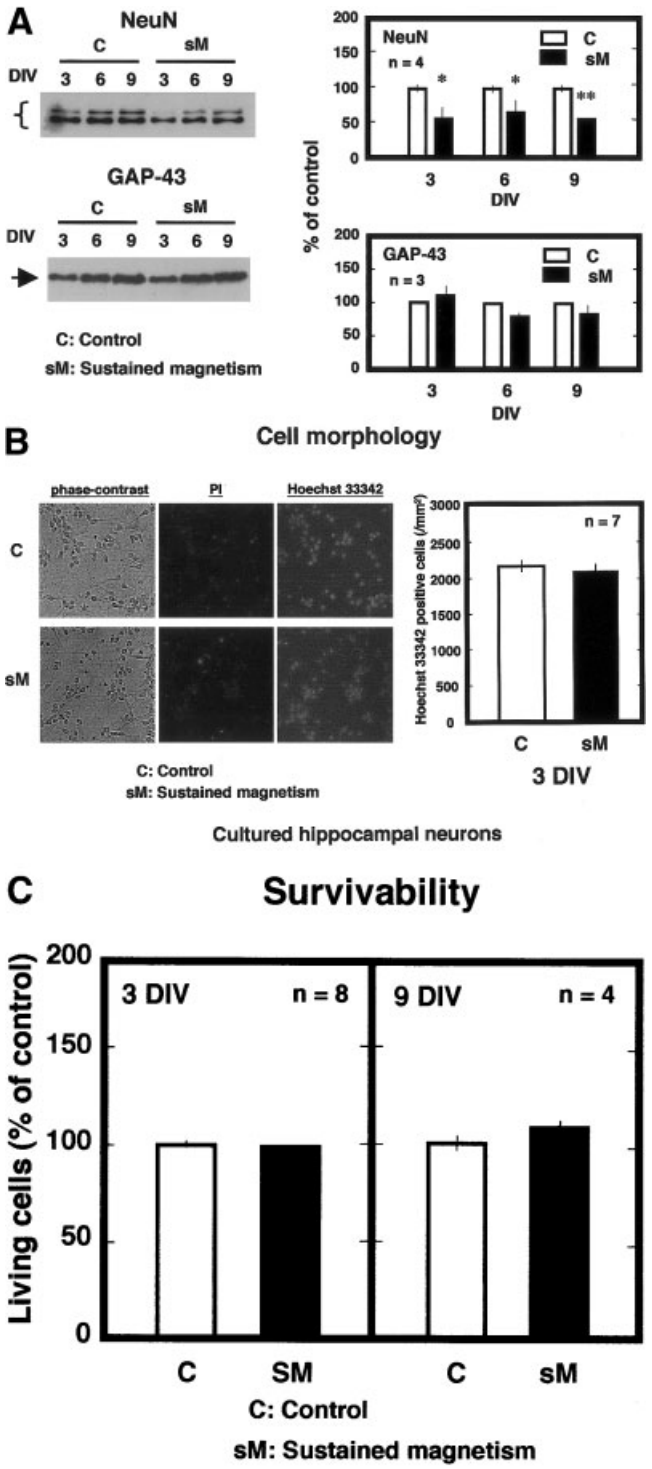

Fig. 3. Effects of sustained magnetism on expression of NeuN and GAP-43 in hippocampal neurons. Hippocampal neurons were plated at a density of 1.0×10^5 cells/cm^2 and cultured for 3, 6, and 9 DIV under sustained static magnetic fields. **A:** Cells were harvested on indicated day, followed by immunoblotting using anti-NeuN or anti-GAP-43 antibody. Each lane indicates a sample from one well in the left panel; each value represents the mean ± SE obtained in 3–4 independent experiments in the right panel. $\star P < 0.05$, $\star\star P < 0.01$, significantly different from each control value obtained in cultured neurons not exposed to static magnetic field. **B:** Cells were harvested after 3 DIV, followed by double fluorescent staining with Hoechst 33342 and PI. Hoechst 33342-stained cells were counted as shown at right. **C:** Cell survivability was quantified by MTT assay in hippocampal neurons cultured for 3 and 9 DIV under sustained magnetism.

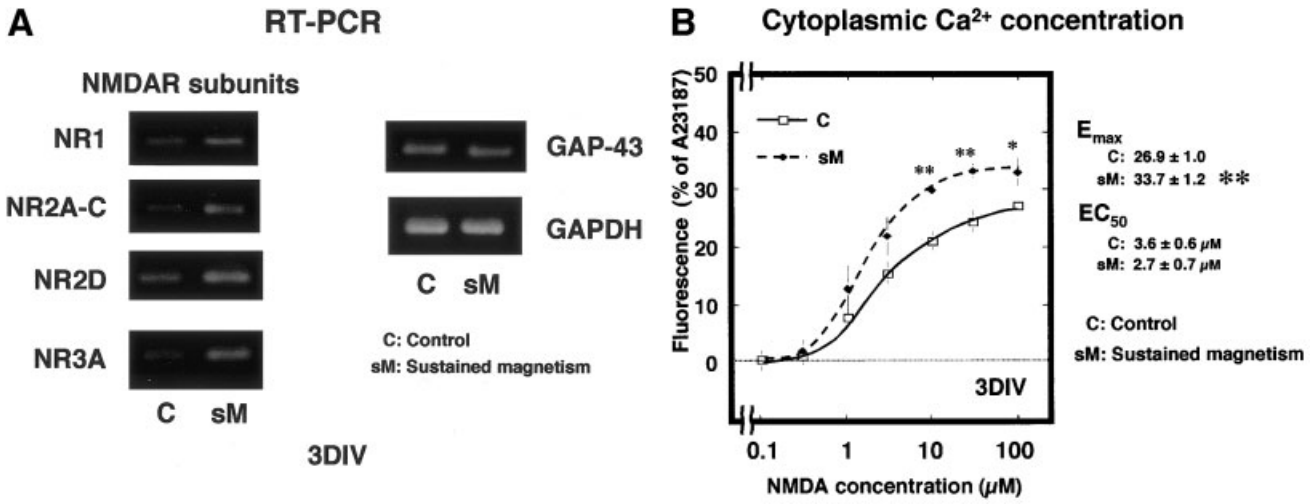

Fig. 4. Effects of sustained magnetism on NMDA receptors in hippocampal neurons. Hippocampal neurons were plated at 2.5×10^5 cells/cm^2 and cultured for 3 DIV under sustained magnetism. **A:** mRNA was extracted from cultured neurons, followed by RT-PCR using specific primers for each NMDA receptor subunit. Experiments were repeated at least three times with similar results. **B:** Cells were loaded with fluo-3, followed by incubation in buffer and subsequent cumulative addition of NMDA (concentration range, 0.1–100 μM) in the absence of $MgCl_2$ for determination of number of fluorescent cells every 2 min by laser-scanning microscope. At the end of each experiment, Ca^{2+} ionophore A23187 (10 μM) was added to incubation medium to obtain maximal values. Values are mean ± SE of percentages over corresponding maximal values in 10 independent experiments. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, significantly different from each control value obtained in cells not exposed to static magnetic field.

tained magnetism could induce a variety of functional or pathological alterations associated with neuropsychiatric disorders.

NMDA receptor upregulation would account for the significant decrease in MAP-2 expression in hippocampal neurons cultured for 1–9 DIV under sustained magnetism. Microtubules are components of neuritic cytoskeletons that play an important role in neuronal maturation. MAP-2 is a major constituent of crossbridges between microtubules in dendrites and is essential for growth through selective stabilization of dendritic microtubules (Harada et al., 2002), whereas GAP-43 is a marker of neuronal maturity associated with neuronal development, axonal regeneration, and synaptogenesis (Benowitz and Routtenberg, 1997). Two high molecular weight isoforms (MAP-2a and MAP-2b) with an apparent molecular weight of 280 kDa and two low molecular weight isoforms (MAP-2c and MAP-2d) with an apparent molecular weight of 70 kDa are known to reside in the brain to date (Kalcheva et al., 1995). Both MAP-2c and MAP-2d (low molecular weight) are detected in glia and neurons (Charriere-Bertrand et al., 1991), but MAP-2a and MAP-2b (high molecular weight) are expressed specifically in neurons (Matus, 1994). MAP-2 is also a likely target of transmembrane signal transduction pathways during several stages of neural development. MAP-2 phosphorylation occurs in a manner dependent on the Glu receptor subtype involved and the resultant Ca^{2+}-dependent pathways (Quinlan and Halpain, 1996).

Systemic administration of ammonium acetate has been shown to induce MAP-2 proteolysis through activation of the Ca^{2+}-stimulated protease calpain I after stimulation of NMDA receptors (Felipo et al., 1993). NMDA receptor activation leads to rapid but reversible dendritic injury with concomitant proteolysis of MAP-2 (Faddis et al., 1997; Minana et al., 1998; Laferriere et al., 1999). One possible explanation is that upregulated NMDA receptors may play a pivotal role in mechanisms underlying the significant decrease in MAP-2 expression in hippocampal neurons cultured under sustained magnetism. The exact mechanism and the functional significance of modulation by NMDA receptors, however, remain to be elucidated. The lack of toxicity gives rise to an idea that sustained magnetism would modulate MAP-2 or GFAP expression without affecting cellular viability and proliferation in cultured hippocampal preparations.

There is accumulating evidence that MAP-2 expression may be a useful marker for diagnosis of schizophrenia and bipolar disorder in vivo (Whitaker-Azmitia et al., 1995; Mazer et al., 1997) and in vitro (Bouras et al., 2001; Marx et al., 2001). Altered GFAP expression is also seen in postmortem evaluation of brains from patients with schizophrenia, bipolar disorder, and major depressive disorder (Johnston-Wilson et al., 2000; Cotter et al., 2001). An in situ hybridization study reveals that high frequency rTMS in vivo induces increased expression of GFAP mRNA in the murine hippocampal dentate gyrus with modest expression in neocortex, as seen with electroconvulsive seizures (Fujiki and Steward, 1997). In both in situ hybridization and immunohistochemistry analyses, however, rTMS induces expression of immediate early genes, such as c-fos, with a

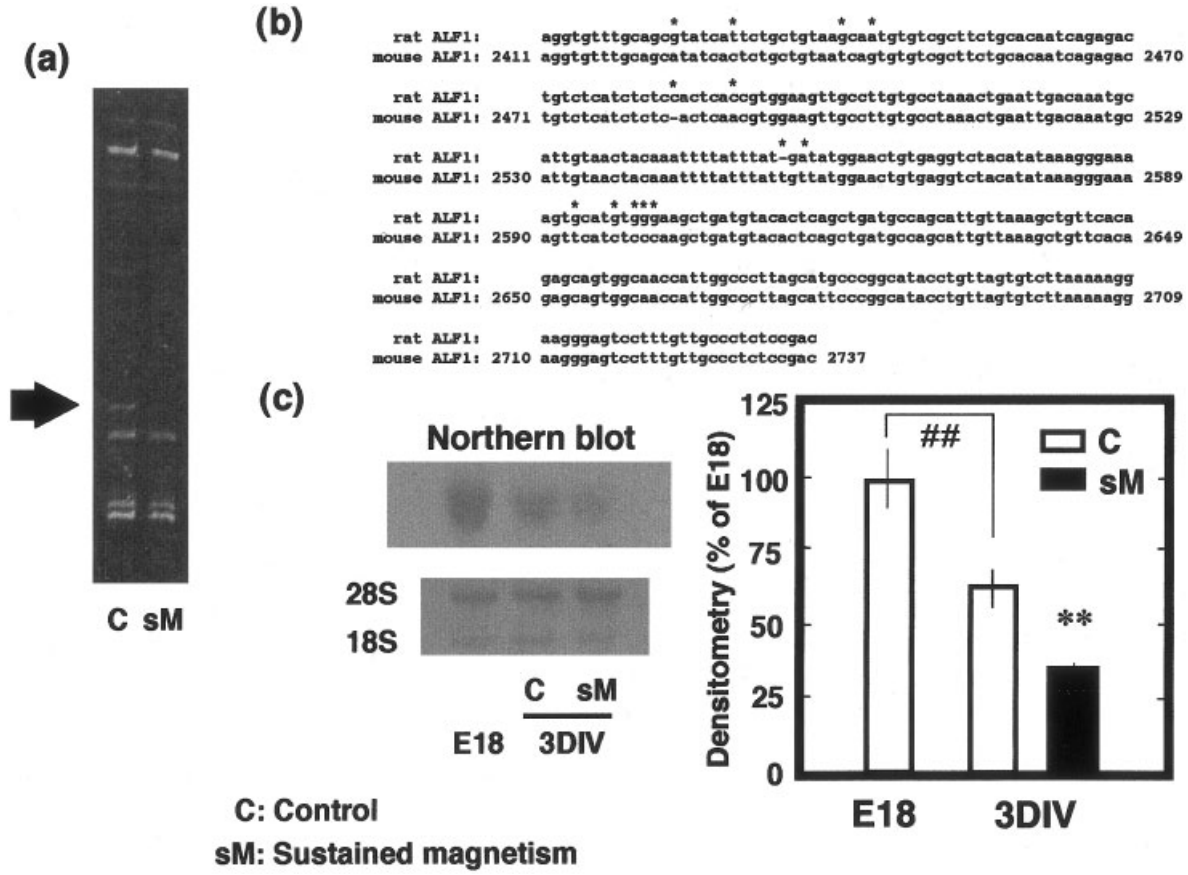

Fig. 5. Analysis of differential expression mRNA by sustained magnetism in hippocampal neurons. Hippocampal neurons were plated at a density of 2.5×10^5 cells/cm^2 and cultured for 3 DIV under sustained static magnetic fields. **A:** Cells were subjected to DD analysis using 24 upstream and 9 downstream random primers. Black arrow, a band expressed differentially by sustained magnetism. **B:** Sequence (obtained by DD analysis) highly homologous (95% identical) to the 2411–2737 of the 3′ noncoding region of mouse ALF1. **C:** Northern blot analysis using cloned differentially expressed fragments labeled with [α-^{32}P]dCTP as probe. Hippocampi were dissected from 18-day-old embryonic rats (E18), followed by extraction of total RNA and Northern blot analysis (DIV 0). Each value represents the mean ± SE obtained in three independent experiments. $\#\#P < 0.01$, significantly different from the values obtained before preparations of hippocampal neurons. $**P < 0.01$, significantly different from each control value obtained in cultured neurons not exposed to static magnetic fields.

profile different from that seen after electroconvulsive stimulation in rat brain (Ji et al., 1998). The possibility has not been ruled out that the present alterations of MAP-2 and GFAP expression under sustained magnetism may also be involved in mechanisms associated with psychiatric disorders sensitive to rTMS treatment. Altered NMDA receptor expression would underlie at least in part the clinical usefulness of rTMS, whereas our data suggest that static magnetic stimulation, in terms of cellular survivability, is not toxic to neurons and astrocytes.

It is not yet clear why static magnetic fields increased GFAP expression in cultured hippocampal neuronal preparations without affecting expression in cultured hippocampal astrocytes. GFAP expression seems to be derived from astrocytes contaminated in hippocampal neuronal preparations cultured even in the absence of FBS. Static magnetic fields increase the level of dissolved oxygen in aqueous solutions containing copper (II), iron (II), and heme iron (III) complexes (Sakurai et al., 2000), and inhibit the growth of particular bacteria under anaerobic conditions without affecting that under aerobic conditions (Kohno et al., 2000). The finding that GFAP and PCNA expression was not changed in hippocampal astrocytes cultured in medium conditioned previously with hippocampal neurons that had been cultured under sustained magnetism do not support the idea that sustained magnetism facilitates release of an endogenous substance from cultured neurons that is trophic to cultured astrocytes. The presence of neurons could be required for acceleration of proliferation or maturation of astrocytes by sustained magnetism. The fact that increased GFAP and PCNA expression was seen in neurons plated at a low but not at a high density argues in favor of the proposition that close intercellular communication between neurons and astrocytes could affect expression of particular functional proteins in

hippocampal neurons cultured under sustained magnetism.

Nucleotide sequence analysis followed by BLASTN homology searching revealed that one of the DD-PCR product had high homology to the mouse ALF1 gene (accession number X64840), a murine analogue to the Class I bHLH protein, HEB/HTF. bHLH proteins bind as homo- or heterodimers to the DNA consensus sequence CANNTG, known as E-box, and are categorized into several classes based distribution profiles, dimerization capabilities, and DNA-binding specificities (Massari and Murre, 2000). A Class I bHLH factor termed E-factor, which includes E12, E47, HEB/HTF4, and E2-2, is expressed ubiquitously (Roberts et al., 1993) and is able to dimerize with tissue-specific Class II factors such as MyoD in skeletal muscle (Lassar et al., 1991) and Neurogenin in neural tissue (Sun et al., 2001), to regulate cell-specific gene transcription. Activity of these factors is often regulated negatively by another class of HLH protein lacking the basic domain including the dominant-negative/inhibitory Id. E-factor gene ALF1 expression is regulated during trophoblast development; expression is extinguished before giant cell differentiation (Scott et al., 2000). In Schwann cells, there is a discrepancy in ALF1 expression between protein and transcript levels, with sharply downregulated protein levels in terminally differentiated cells that continue to express abundant levels of the mRNA (Stewart et al., 1997). In the developing CNS, the E-factor gene is expressed in proliferating neuroblasts and neurons at initial differentiation stages but is absent from matured and differentiated cells (Neuman et al., 1993; Soosaar et al., 1994; Chiaramello et al., 1995). During sustained exposure to static magnetic fields, reduced ALF1 mRNA expression could be at least partly responsible for mechanisms underlying alterations in cellular differentiation and maturation, but not for those underlying proliferation and survival.

It thus seems that sustained exposure to static magnetic fields may modulate cellular maturation and development but not survivability at least partly through heteromeric NMDA receptor channel upregulation in immature cultured rat hippocampal neurons. Evaluation of the underlying mechanisms could greatly benefit therapy and treatment of patients suffering from neuropsychiatric disorders associated with NMDA receptor channel malfunction.

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