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MODULATION BY STATIC MAGNETISM OF NEURONAL ACTIVITY

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In neurons, the signal propagation involves both the conduction mediated by local electric currents through voltage-sensitive cation channels in axons and the transmission mediated by the exocytotic release of neurotransmitters from nerve endings into synaptic clefts. A great number of desperate efforts have been dedicated to biochemical, pharmacological and molecular biological studies on the elucidation of mechanisms underlying the neurotransmission at synapses, while relatively little attention has been paid to the comprehensive evaluation of the conduction except for local anesthetics. According to a physical theorem, exposure to magnetism should lead to the generation of a certain mechanical force in neurons with concomitant electric currents in a particular situation. In particular, repetitive transcranial magnetic stimulation is beneficial for the treatment of selected patients suffering from depression, bipolar affective disorder and schizophrenia as a possible alternative to the electroconvulsive therapy for refractory depression. In this review, therefore, we will summarize our recent advances made on the neurochemical and molecular biological elucidation in cultured rat hippocampal neurons toward better understanding by the readers of different disciplines of mechanisms associated with the modulation by magnetism of the neuronal activity in the brain.
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INTRODUCTION

The signal propagation is believed to involve both the signal transmission mediated by the exocytotic release of a variety of neurotransmitters from nerve endings into synaptic clefts and the signal conduction mediated by local electric currents through voltage sensitive cation channels at axons in the mammalian central and peripheral nervous systems. Compared to the number of biochemical, pharmacological and molecular biological studies dedicated to the elucidation of mechanisms underlying the neurotransmission at synapses, relatively little attention has been paid to mechanisms associated with the modulation of signal conduction by endogenous and exogenous stimuli. In the central nervous system (CNS), both oscillatory and static magnetic fields could modulate a variety of cellular functions in glia as well as neurons through the electromagnetic induction and/or the mechanical force at biomembranes *in vivo* and *in vitro* (1). Strong static magnetic fields are proposed to modulate synaptic functions through the altered diamagnetic anisotropy in membranes in the CNS and neuromuscular junctions (2). Furthermore, it is noteworthy that the ferromagnetic mineral magnetite is really present in different human tissues including brain (3,4).

In cultured GH3 cells, exposure to a 120 mT static magnetic field results in inhibition of the activity of voltage-activated calcium channels on whole-cell patch clamp analysis (5). Brief exposure to static magnetic fields at 200 mT not only leads to marked morphological alterations, but also results in a significant reduction of both thymidine incorporation and inositol lipid signaling, in the human neuronal cell line FNC-

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B4 (6). Action potentials are blocked by static magnetic fields in cultured sensory neurons (7), while alternating magnetic fields increase the expression of the glial marker protein glial fibrillary acidic protein (GFAP) in cultured astrocytes (8). An immunohistochemical study shows that marked expression of c-Fos protein is induced by exposure for 30 min to magnetic fields at 9.4 T in the visceral and vestibular nuclei of rat brain stem *in vivo* (9). 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 (10). Similarly marked expression is shown with c-Fos protein in parietal cortex and hippocampus (11) as well as other telencephalic regions including the frontal cortex, striatum, dentate gyrus, Ammon's horn and amygdala (12) after several sessions of repetitive transcranial magnetic stimulation (rTMS).

Transcranial magnetic stimulation has been used as a diagnostic tool in neurobiological fields due to its painless and noninvasive properties (13). In clinical studies, moreover, rTMS is shown 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 (14-16). The efficacy of weak magnetic fields in the treatment of Parkinsonism and motor complications of chronic levodopa therapy is also proposed (17). In healthy subjects, however, rTMS is shown to induce transient mood enhancement (18,19).

On the other hand, 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 on inducible target genes (20). 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 (21). Such consolidation mechanism would be operative in certain situations including neuronal plasticity and degeneration.

Accumulating evidence for the expression of AP1 complex in response to neuronal activities *in vitro* and *in vivo* is available in the literature. In primary cultures of rat cerebellar neurons, for example, 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 an increase in AP1 DNA binding (22,23). Intraperitoneal (24) and intracerebroventricular (25) injections of NMDA result in the expression of c-*fos* mRNA and c-Fos protein in the rodent brain *in vivo*. Punching out dissection technique on frozen sections reveals that the systemic NMDA induces a rapid but transient increase in AP1 DNA binding only in the dentate granule layer of murine hippocampus without affecting that in the CA1 and CA3 pyramidal layers (26). Hippocampal dentate layers are shown to contain neural progenitor cells that spontaneously undergo proliferation and subsequent migration in a manner sensitive to the prevention by activation of NMDA receptors even in matured brain (27,28). Expression of AP1 complex would be therefore a guidepost for the transduction of extracellular signals into intracellular and nuclear signals for long-term consolidation and amplification.

These previous studies raise the possibility that exposure to magnetic fields would lead to long-term consolidation as well as amplification of different functional alterations induced by transient extracellular signals through the modulation of *de novo* protein synthesis at the level of gene transcription in the brain. In this article, we will summarize our recent findings on mechanisms associated with functional alterations in rat hippocampal neurons cultured under brief, sustained and repetitive exposure to static magnetic fields.

BRIEF EXPOSURE TO MAGNETISM

Several independent lines of evidence indicate that the cellular maturity is one of crucial determinants for the 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 (29,30) and hippocampal (31) 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 for other Glu receptor subtypes (32). Long-term potentiation (33) as well as sensitivity to Mg^{2+} block (34,35) is declined in proportion to postnatal periods in rat hippocampus.

In primary cultures of rat cortical neurons cultured for 3 to 9 DIV, no significant changes are seen in AP1 DNA binding following the brief exposure for 15 min to static magnetic fields at 100 mT generated by permanent ferrite magnets irrespective of the time after exposure up to 60 min (36). In immature, but not mature, cultured hippocampal neurons, by contrast, magnetic fields rapidly but transiently increase AP1 DNA binding. Moreover, brief exposure almost doubles AP1 binding in immature hippocampal neurons plated at a low density with concomitant quadruplicating binding in those plated at a high density. Prior brief exposure to static magnetic fields leads to a significantly less potent increase in AP1 DNA binding found following the addition of NMDA

compared to neurons not exposed to magnetism at all (36). Neuronal maturity could determine the responsiveness to static magnetic fields, 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 as mentioned afterward in this article. Judging from the findings on LDH release, it is unlikely that magnetic stimulation is neurotoxic to immature hippocampal neurons.

Of antibodies directed against Fos family member proteins, the anti-Fra-2 antibody induces a marked decrease in AP1 binding in hippocampal neurons exposed and not exposed to magnetic fields with concomitant upward migration of the mobility on the gel (Fig. 1a). Neither the anti-c-Fos nor the anti-Fos-B antibody markedly affects AP1 binding in hippocampal neurons irrespective of the exposure to magnetic force. Of antibodies against Jun family member proteins, both the anti-c-Jun and the anti-Jun-D antibodies are effective in decreasing and migrating AP1 binding in hippocampal neurons previously exposed and not exposed to static magnetic fields. The anti-Jun-B antibody does not markedly affect AP1 binding irrespective of the exposure to magnetic fields. Immunoblotting analysis reveals that the brief exposure to magnetism results in expression of Fra-2, c-Jun and Jun-D proteins with the corresponding molecular weights in hippocampal neurons cultured at a high density when harvested 30 min after exposure (Fig. 1b).

The data cited above are not sufficient to evaluate the functional alterations in neurons exposed to static magnetism. For this purpose, we have determined the levels of intracellular free Ca^{2+} ions permeable to NMDA receptor channels using a Ca^{2+}-sensitive fluorescent dye with the aid of a confocal laser-scanning microscope. The addition of NMDA markedly increases the number of fluorescent cells with an increased intracellular free Ca^{2+} concentration in a concentration-de-

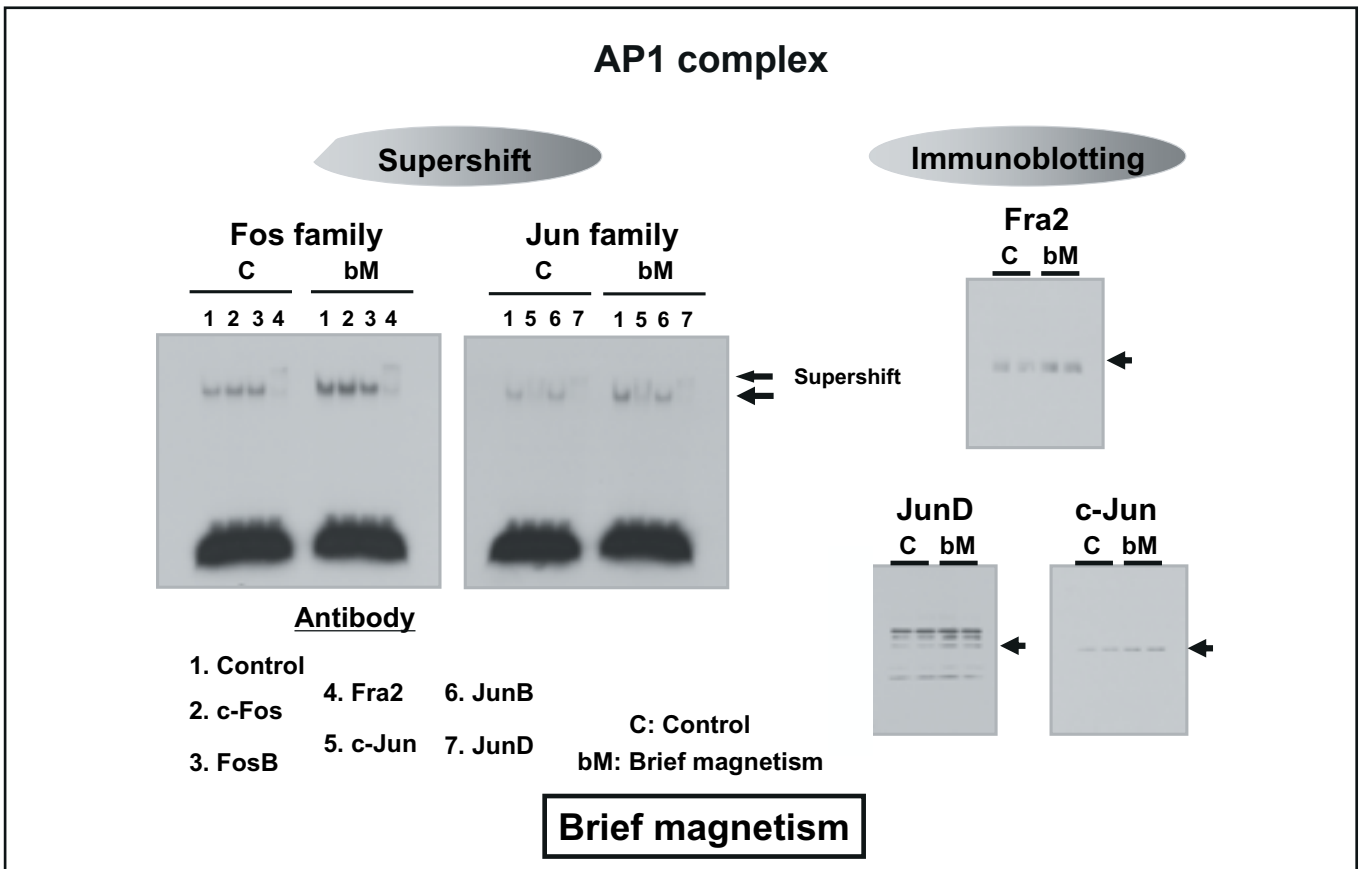


Figure 1. *Expression of AP1 complex by brief exposure to magnetism. Brief exposure to static magnetic fields for 15 min led to marked expression of Fra2, JunD and c-Jun proteins 30 min later in rat hippocampal neurons cultured for 3 DIV. Supershift assays* ***(a)****; Immunoblotting analysis* ***(b)****.*

pendent manner in hippocampal neurons not exposed to static magnetic fields as shown previously (37), while prior exposure to magnetism induces a rightward shift of the concentration-response curve between NMDA and fluorescence 24 h later (Fig. 2). The increase by NMDA is not only inhibited by the antagonist for NMDA receptor channels dizocilpine (MK-801), but also prevented by the blocker of L-type voltage-sensitive Ca^{2+} channels nifedipine, with the inhibitor of Ca^{2+} release across ryanodine-sensitive Ca^{2+} channels from intracellular stores dantrolene being ineffective (38).

Transient magnetic stimulation would therefore induce long-lasting alterations of a variety of cellular functions through the 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 growth-associated protein-43 (GAP-43) give support to the immaturity of hippocampal neurons cultured for 3 DIV. Expression of GAP-43 is associated with the neuronal development, axonal regeneration, synaptogenesis and plasticity in immature neurons but not seen in mature neurons (39). By contrast, static magnetic fields are shown to increase the level of dissolved oxygen in aqueous solutions containing copper (II), iron (II) and heme iron (III) complexes (40), and inhibit the growth of particular bacteria under anaerobic conditions without affecting that under aerobic conditions (41). Nevertheless, it should be emphasized that expression of mRNA and immunoreactive protein is not 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. The modulation could underlie transformation of transient extracellular signals carried by magnetism into long-lasting alterations of cellular functions in immature hippocampal neurons.

The mechanism underlying the differential increase by magnetism in AP1 DNA binding between 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, a 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 (42). The fact that

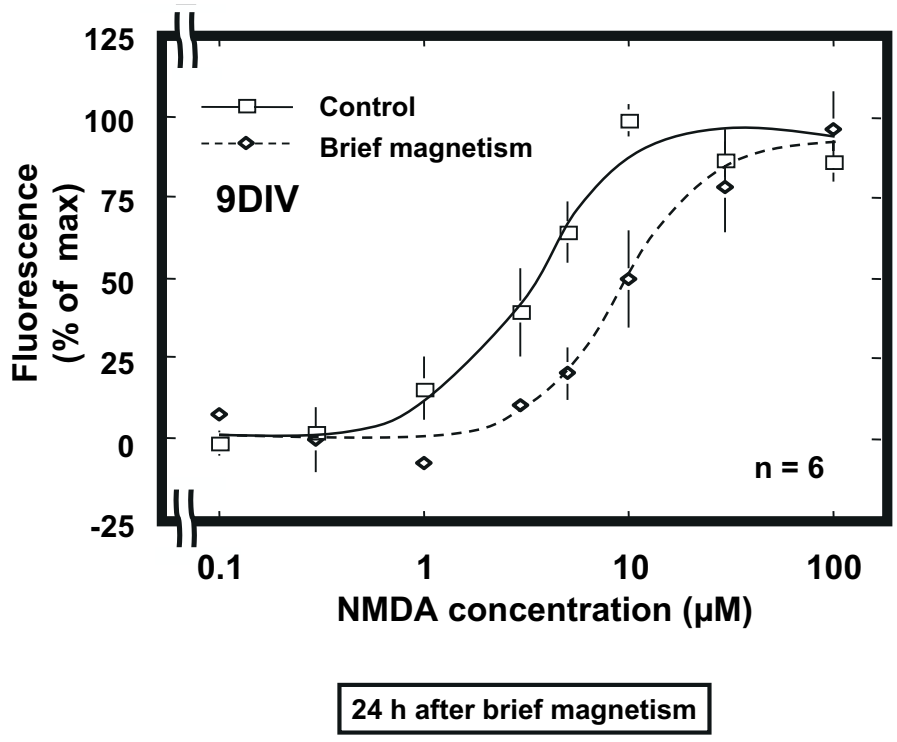


Figure 2. *Desensitization of NMDA receptor channels by brief magnetism. Brief magnetism significantly desensitized the ability of NMDA receptor channels to permeate free Ca^{2+} ions across cell membranes in cultured hippocampal neurons when determined 24 h after the transient exposure for 15 min.*

the exposure to static magnetic fields leads to an increase in AP1 DNA binding in hippocampal neurons in a density-dependent fashion could be accounted for by taking into consideration these previous findings. A number of adhesive molecules would be responsible for the aforementioned density-dependent increase through a mechanism relevant to communications by intimate contacts between cells. An *in situ* hybridization study reveals that high frequency rTMS *in vivo* induces marked expression of $mRNA^{GFAP}$ in the murine hippocampal dentate gyrus with modest expression in cerebral cortex, as seen with electroconvulsive seizures (43). The possibility that the 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.

At any rate, the hippocampus seems to be one of the target architectures highly responsive to magnetism amongst discrete brain structures. In murine hippocampal slices *in vitro*, for example, steady magnetic fields differentially modulate evoked potentials at 8 to 10 mT (44). Direct current-generated magnetic fields induce biphasic effects on the population spike recorded from murine hippocampal slices, which occurs in a manner sensitive to the inhibitor of calcium release from intracellular stores dantrolene but not to the NMDA receptor antagonists (45). In human hippocampus *in vivo*, moreover, the exposure to weak direct current magnetic field stimulation alters the interictal epileptiform spike activity in several patients suffering from mesial temporal lobe epilepsy (46,47). Evocation of epileptiform activity is seen after the exposure to direct current magnetic fields of between 0.9 and 1.8 mT in epileptic patients undergoing pre-surgical evaluation (48), while increased interictal firing rates are confirmed after a series of magnetic field periods in epileptic patients with depth electrode implantation in hippocampus for pre-surgical evaluation (49).

SUSTAINED EXPOSURE TO MAGNETISM

In this section, we will summarize our recent observations on a gene downregulated in response to sustained exposure to static magnetic fields at 100 mT for a period of up to 9 DIV in hippocampal neuronal preparations. The sustained exposure significantly increases the expression of immunoreactive GFAP in hippocampal neuronal cultures, without markedly affecting that of GFAP and proliferating cell nuclear antigen (PCNA) in cultured astrocytes prepared from the rat hippocampus and neocortex (50). No significant cell death is found in hippocampal and neocortical astrocytes cultured under sustained exposure to magnetism. By contrast, the sustained exposure to static magnetic fields significantly decreases the expression of immunoreactive MAP-2 (microtubule-associated protein-2; a neuronal marker protein) to approximately 75% of the control level (Fig. 3). On immunocytochemical analysis,

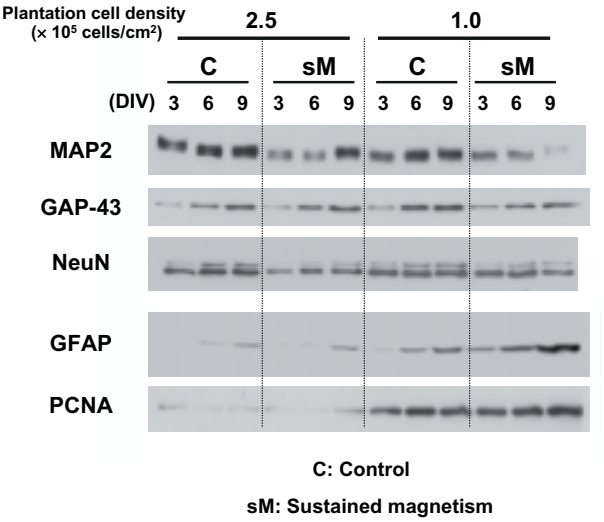


Figure 3. *Expression of different marker proteins by sustained magnetism. Sustained exposure to static magnetism resulted in a decrease in the expression of both MAP-2 and NeuN, but not GAP-43, in cultured hippocampal neurons irrespective of cellular maturity.*

most cells are immunoreactive to MAP-2 at both cell bodies and neurites in cultured immature hippocampal neurons, while sustained exposure to magnetism leads to a marked decrease in MAP-2 expression at neurites rather than cell bodies. Similarly, sustained magnetism significantly decreases the expression of the neuronal marker protein neuronal nucleus (NeuN) without significantly affecting that of GAP-43 in hippocampal neurons. No significant cell death is seen in hippocampal neurons cultured under the sustained exposure to static magnetic fields on morphological analysis using a double fluorescent staining with Hoechst 33342 (bis-benzimide trihydrochloride) and propidium iodide (PI). Moreover, sustained exposure does not significantly affect the cell survivability quantified by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays in cultured hippocampal neurons. However, sustained magnetism induces a significant increase in the maximal response for NMDA to increase intracellular free Ca^{2+} concentrations without significantly altering EC_{50} values.

The essential importance is that sustained exposure to static magnetic fields leads to significant potentiation of the maximal response of NMDA toward an increase in intracellular free Ca^{2+} ions without affecting the concentration for a half-maximal response in cultured rat hippocampal neurons. The data from semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis give support to the idea that

sustained magnetism could facilitate opening processes of NMDA receptor channels through upregulated expression of particular NMDA receptor subunits required for heteromeric assemblies of functional receptor channels. In fact, sustained magnetism results in profound upregulation of expression of mRNA for NR1, NR2A-C, NR2D and NR3A subunits in cultured hippocampal neurons, without markedly affecting that for GAP-43 and the housekeeping gene glycelaldehyde-3-phosophate dehydrogenase (GAPDH) (50). Although the increased maximal response gives rise to the possible upregulation of NMDA receptor channels, Western blotting analysis is needed on expression of all NMDA receptor subunits required for assembled functional channels when antibodies are commercially available against all different NR subunits cloned to date. The inhibition by nifedipine (38) still maintains the possibility that sustained magnetism would modulate properties of voltage-sensitive Ca^{2+} channels.

It should be noted that sustained magnetism increases the expression of the dominant negative NR3A subunit in addition to NR1, NR2A-C and NR2D subunits. NR3A subunit associated with NR1 and NR2 subunits is shown to decrease Ca^{2+} permeation in response to stimulation by NMDA in contrast to NR1/NR2 channels (51). Assembly with NR1 subunit is required for surface expression of functional NMDA channels containing NR2A and NR3A subunits (52). 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 the accumulation of NR3A subunit in the endoplasmic reticulum with less expression at the cell surface (52). The increased expression of mRNA for NR1, NR2A-C, NR2D and NR3A subunits altogether is therefore insufficient to predict the functional alteration of NMDA channels expressed at the cell surface following 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 on the expression of each NR2 subunit is undoubtedly required for further evaluation of heteromeric channel properties including agonist affinity, desensitization, inactivation and developmental expression profiles as shown with mice (53). To our knowledge, our article for the first time deals with the possible correlation between sustained magnetism and altered functionality of NMDA receptor channels in immature cultured rat hippocampal neurons. Sustained magnetism could induce a variety of functional and/or pathological alterations associated with the crisis of neuropsychiatric disorders through upregulated NMDA receptor channels in hippocampal neurons.

Upregulation of NMDA receptors would account for the significant decrease in expression of MAP-2 in hippocampal neurons cultured under sustained magnetism. Microtubules are components of the neuritic cytoskeletons that play an important role in neuronal maturation. MAP-2 is a major constituent of cross-bridges between microtubules in dendrites with the essentiality for dendritic growth through selective stabilization of dendritic microtubules (54), while GAP-43 is a marker of neuronal maturity that is associated with neuronal development, axonal regeneration and synaptogenesis (39). Two high-molecular weight isoforms (MAP-2a and MAP-2b) with an apparent molecular weight of 280 kD and two low-molecular weight isoforms (MAP-2c and MAP-2d) with an apparent molecular weight of 70 kD are known to reside in the brain to date (55). Both MAP-2c and MAP-2d with a low-molecular weight are detected in glia as well as neurons (56), but MAP-2a and MAP-2b with a high-molecular weight are specifically expressed in neurons (57). MAP-2 is also a likely target of transmembrane signal transduction pathways during several stages of neural development. Phosphorylation of MAP-2 occurs in a manner dependent on the subtype of Glu receptors involved and resultant Ca^{2+}-dependent pathways (58). The systemic administration of ammonium acetate is shown to induce proteolysis of MAP-2 through activation of the Ca^{2+}-stimulated protease calpain I after stimulation of NMDA receptors (59). Activation of NMDA receptors leads to rapid but reversible dendritic injury with concomitant proteolysis of MAP-2 (60-62). One possible speculation 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 as well as functional significance of modulation by NMDA receptors, however, remains to be elucidated in future studies. The evaluation on the number of living cells gives rise to an idea that sustained magnetism would modulate expression of MAP-2 or GFAP without affecting cellular viability and proliferation in cultured hippocampal preparations.

There is accumulating evidence that expression of MAP-2 may be a useful marker for diagnosis of schizophrenia and bipolar disorder *in vivo* (63,64) and *in vitro* (65,66). Altered expression of GFAP is also seen in postmortem evaluation of brains from patients with schizophrenia, bipolar disorder and major depressive disorder (67,68). An *in situ* hybridization study reveals that high frequency rTMS *in vivo* induces increased expression of $mRNA^{GFAP}$ in the murine hippocampal dentate gyrus with modest expression in neocortex, as seen with electroconvulsive seizures (43). On both *in situ* hybridization and immunohistochemistry analyses, however, rTMS induces expression of immediately early genes such as c-*fos* with a profile different from that seen after electroconvulsive stimulation in rat brain (69). The possibility that the present alterations of expression of both MAP-2 and GFAP under sustained magnetism may be involved in mechanisms associated with the crisis of a variety of psychiatric disorders sensitive to the treatment with rTMS, thus, is not ruled out. Altered

expression of NMDA receptors would at least in part underlie the clinical usefulness of rTMS, whereas our data suggest that static magnetic stimulation is not toxic to both neurons and astrocytes in terms of cellular survivability.

Moreover, hippocampal neurons cultured under sustained exposure to static magnetic fields are subjected to extraction of total RNA for differential display (DD) analysis using random primers (Fig. 4a) (70). Differentially expressed genes by sustained exposure to static magnetic fields are used as templates for PCR re-amplification (Fig. 4b), while the purified PCR products are subsequently cloned into pT7 blue plasmid and sequenced to search for DNA sequence similarity with the BLASTN algorithm. As a consequence of the study described above, two particular genes are found to be downregulated in response to sustained magnetism in hippocampal neurons. The two cloned differentially expressed fragments are labeled with [α-^{32}P]dCTP for Northern blot analysis. Expression of mRNA for #812 is significantly decreased during *in vitro* cultivation of hippocampal neurons, while a further decrease is seen in expression of mRNA for #812 and #612 in hippocampal neurons cultured under sustained exposure to static magnetic fields at 100 mT (Fig. 4c). Cloning of the genes reveals sequences highly homologous (95% identical) to the 3' non-coding regions of the mouse basic helix-loop-helix (bHLH) transcription factor ALF1 (#812) and that of histone H3.3A (#612), respectively (Fig. 4d).

The gene, mouse ALF1 gene (accession number X64840), is a murine analogue to the class I bHLH protein, HEB/HTF. bHLH proteins are shown to bind as homo- or hetero-dimer to the consensus DNA sequence CANNTG, known as E-box, with categorization into several classes on the basis of distribution profiles, dimerization capabilities and DNA binding

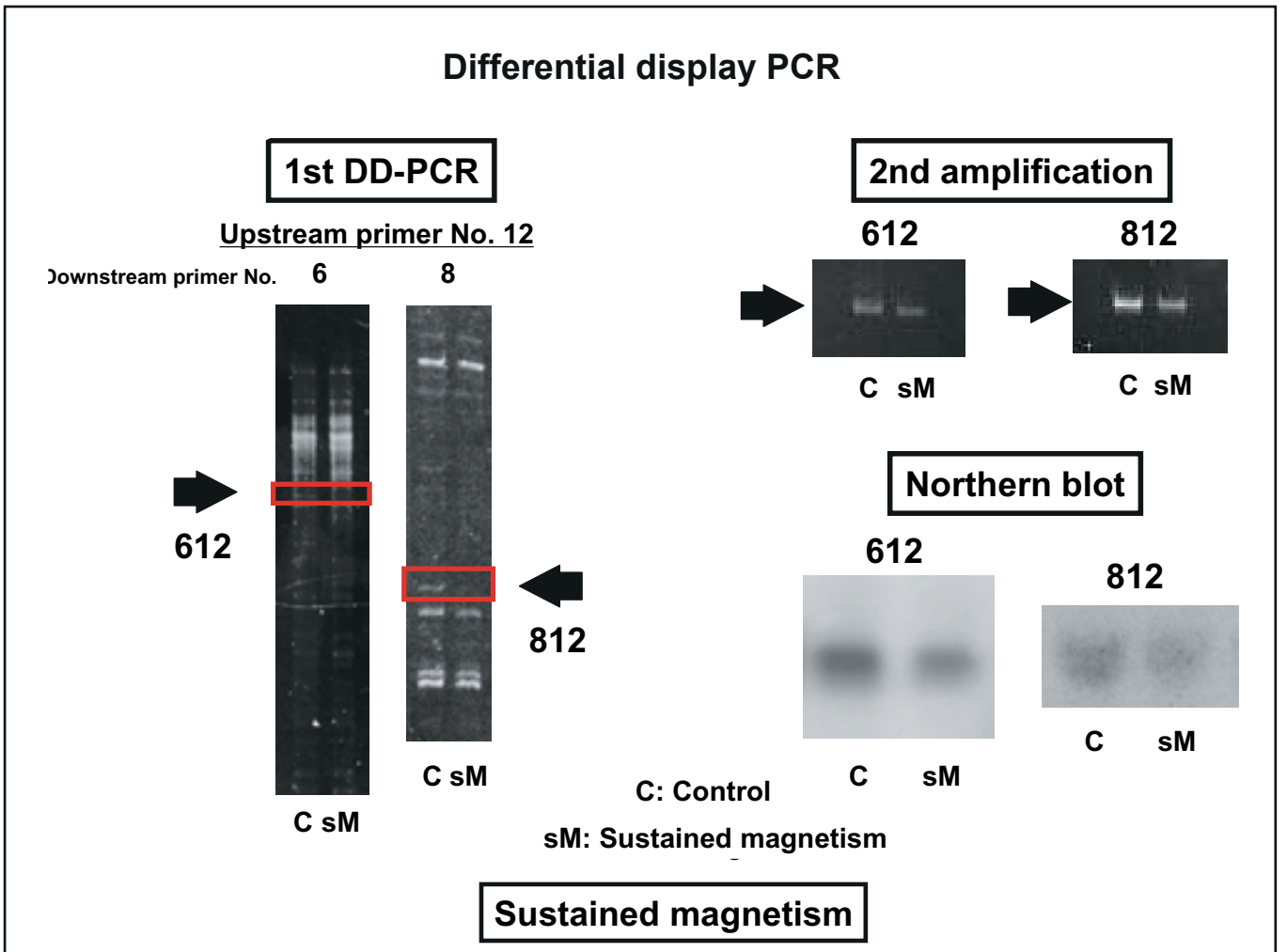


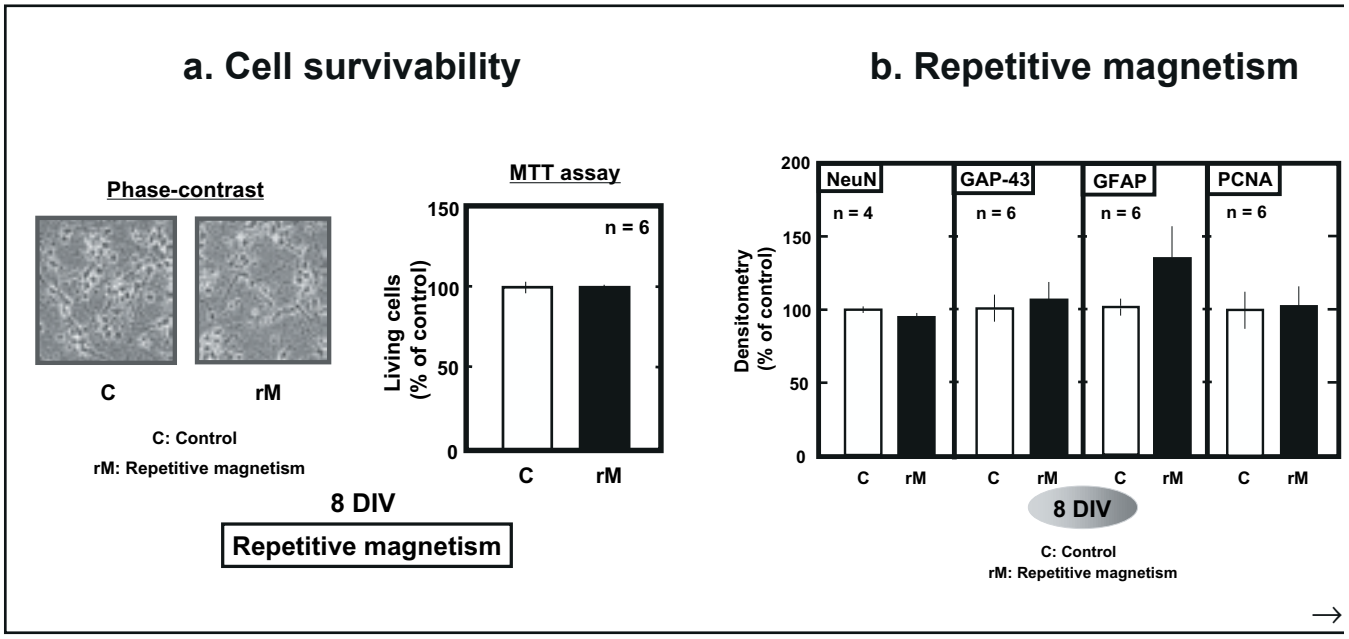
Figure 4. *Downregulation of particular genes by sustained magnetism. Sustained magnetism downregulated two different genes in hippocampal neurons cultured for 3 DIV as revealed by DD-PCR (**a**), 1st DD-PCR (**b**), 2nd amplification (**c**), Northern blotting analysis (**d**) identification.*

specificities (71). A class I bHLH factor termed as E-factor, which includes E12, E47, HEB/HTF4 and E2-2, is ubiquitously expressed (72) and able to dimerize with tissue-specific class II factors such as MyoD in skeletal muscle (73) and Neurogenin in neural tissue (74), to regulate cell-specific gene transcription. The activity of these factors is often negatively regulated by another class of HLH protein lacking basic domain including the dominant-negative/inhibitory Id. Expression of the E-factor gene *ALF1* is regulated during trophoblast development, as the expression is extinguished in advance of giant cell differentiation (75). In Schwann cells, discordance is seen for ALF1 expression between protein and transcript levels, with protein levels being sharply down-regulated in terminally differentiated cells which continue to express abundant mRNA (76). In the developing central nervous system, E-factor gene is expressed in proliferating neuroblasts and neurons at the initial stages of differentiation with the absence from matured and differentiated cells (77-79). Reduced expression of the E-factor gene ALF1 mRNA could be at least in part responsible for mechanisms underlying alterations of cellular differentiation and maturation, but not for proliferation and survival, during sustained exposure to static magnetic fields.

REPETITIVE EXPOSURE TO MAGNETISM

As mentioned above, rTMS is shown to be beneficial for the treatment and therapy of selected patients with depression, bipolar affective disorder and schizophrenia as a possible alternative to ECT often used for the treatment of refractory depression (14-16). This fact implies the importance of subsequent evaluation on the functionality in hippocampal neurons cultured under the repetitive exposure to static magnetic fields. Hippocampal neurons are thus cultured under daily brief exposure to static magnetic fields at 100 mT for 15 min each day for the consecutive 8 DIV, followed by cell harvest 24 h after the last exposure and subsequent determination of cell viability and expression of different proteins on Western blot analysis. No marked alterations are found for the morphology (Fig. 5a, left) and survivability (Fig. 5a, right) in hippocampal neurons cultured under daily repetitive exposure to static magnetic fields. However, the repetitive exposure to static magnetic fields significantly decreases by 50% the expression of MAP-2 with different molecular weights that are individually increased in proportion to cellular maturation (Fig. 5c), without significantly affecting that of NeuN and GAP-43 (Fig. 5b). By contrast, no significant changes are seen in the expression of both GFAP and PCNA in hippocampal preparations cultured under daily repetitive exposure to static magnetic fields (Fig. 5b). Daily repetitive magnetism is found to be effective in significantly increasing the expression of NR2B subunit, but not that of NR2A subunit. Repetitive magnetism fails to alter the expression of NR1 subunit essential for the assembly of functional NMDA receptor channels. The cumulative addition of NMDA significantly increases Ca^{2+} fluorescence in a concentration-dependent manner at a concentration range of 1 to 100 μM in hippocampal neurons, while daily repetitive magnetism induces a leftward shift of the concentration-response curve without significantly affecting the maximal response.

Since rTMS elicits beneficial and therapeutic effects on different neuropsychiatric disorders including refractory depression, an analysis is required for the possibility that daily repetitive magnetism is really protective against a variety of



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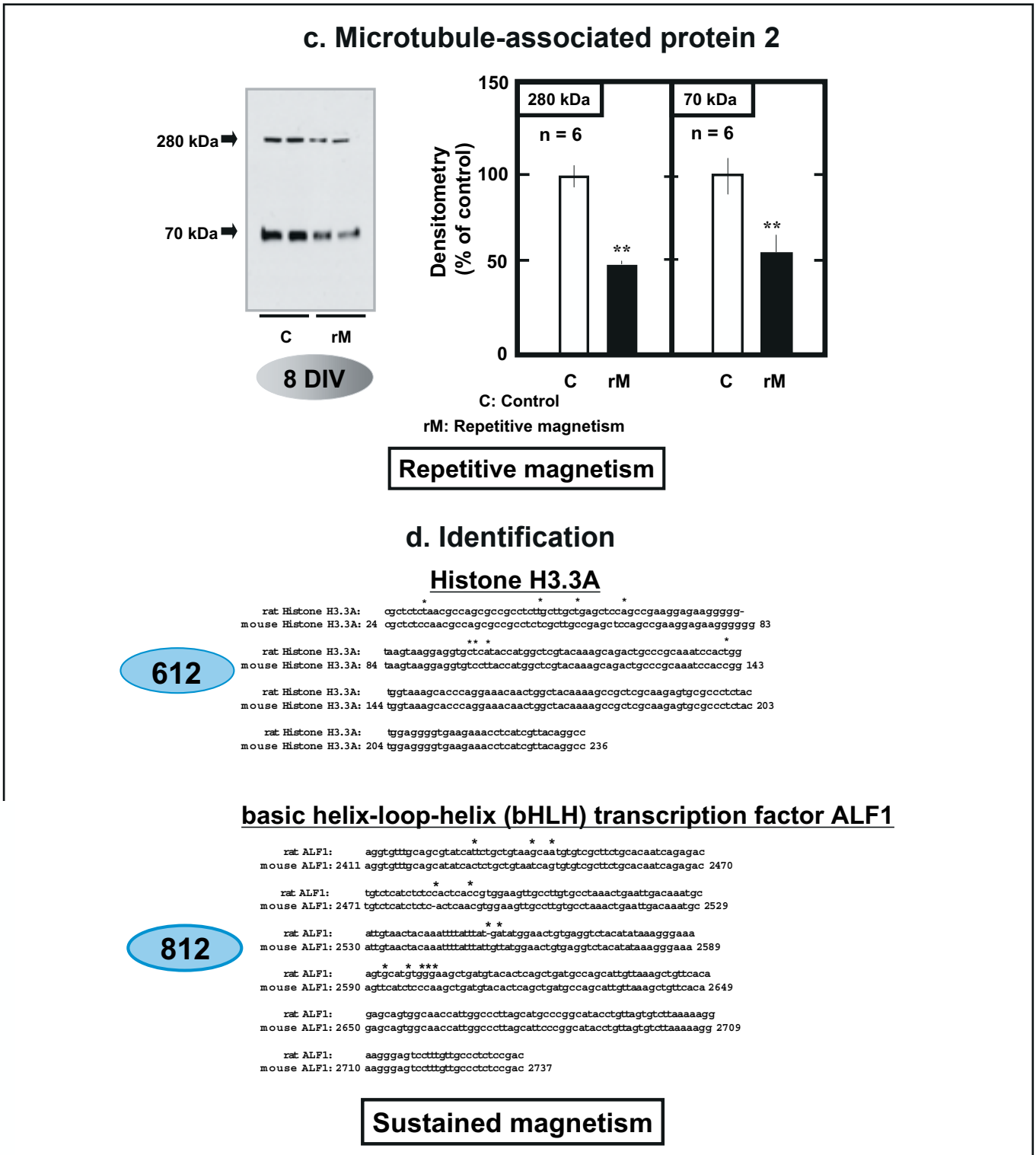

Figure 5. *Repetitive magnetism. Daily repetitive magnetism markedly affected neither cellular survivability (**a**), nor expression of the neuronal marker protein NeuN, the neurite growth marker protein GAP-43, the astroglial marker protein GFAP and the proliferation marker protein PCNA (**b**), but led to significant downregulation of the expression of MAP-2 with 2 different molecular weights **(c)**, in hippocampal neurons cultured for 8 DIV.*

damages in cultured neurons. For this purpose, hippocampal neurons are cultured in the presence of the NMDA antagonist MK-801 at different concentrations. The antagonist MK-801 not only decreases cell survivability in a concentration-dependent manner at a concentration range of 1 to 100 μM in cultured hippocampal neurons, but also inhibits the expression of MAP-2 with two different molecular weights, 70 kD and 280 kD. Almost complete inhibition is seen for the endogenous level of MAP-2 with both molecular weights in neurons cultured in the presence of 100 μM MK-801. Moreover, semi-quantitative RT-PCR analysis reveals that a significant decrease is seen in the expression of mRNA for both GAP-43 and brain-derived neurotrophic factor in hippocampal neurons cultured in the presence of 10 μM MK-801. Similarly, MK-801 significantly decreases the endogenous level of NR1 subunit by more than 50% with a concomitant increase in that of NR2A subunit when exposed to hippocampal neurons for a consecutive period. These data are suggestive of the neurotoxicity of MK-801 in cultured hippocampal neurons.

Daily repetitive magnetism is ineffective in preventing the loss of NR1 subunit by MK-801, but more than doubles the increased expression of NR2A subunit in neurons cultured in the presence of MK-801 without significantly altering the expression in the absence of MK-801. The expression of NR2B subunit is significantly increased in neurons cultured under daily repetitive magnetism as described above, while in neurons cultured in the presence of MK-801 daily repetitive magnetism fails to increase the expression of NR2B subunit. As a guidepost to evaluate the activity of NMDA receptor channels, the intracellular free Ca^{2+} concentration is determined by fluo-3 fluorescence using confocal laser microscopy. The cumulative addition of NMDA increases the fluorescence intensity in a concentration-dependent manner at a concentration range of 1 to 100 μM in the absence of added Mg^{2+} ions in hippocampal neurons, while in neurons cultured in the presence of 10 μM MK-801 significant decreases are seen in the maximal response and the sensitivity to NMDA. Daily repetitive magnetism is found to significantly protect against the decreased sensitivity to NMDA without affecting the maximal response. Although both daily repetitive magnetism and sustained exposure to MK-801 induce a significant decrease in the endogenous level of MAP-2 with two different molecular weights as mentioned above, daily repetitive magnetism significantly prevents the decrease of MAP-2 with different molecular weights in hippocampal neurons cultured in the presence of MK-801. In neurons cultured under daily repetitive magnetism, sustained exposure to MK-801 fails to further decrease the expression of MAP-2.

The data cited above argue in favor of an idea that repetitive magnetism would be protective against abnormalities and/or malfunctions in hippocampal neurons cultured in the sustained presence of the NMDA receptor antagonist MK-801. Overstimulation of NMDA receptors is well known to induce neurotoxicity through massive influx of extracellular Ca^{2+} ions across the cation channels, whereas blockade of NMDA receptors would also lead to neurotoxicity, such as vacuolization in rat (80) and mouse (81) brains, with behavioral abnormalities related to psychosis. Moreover, widespread apoptotic neurodegeneration would be brought about by the transient blockade of NMDA receptors for only a few hours during the late fetal or early neonatal period in developing rat brain (82). In contrast to fatal NR1 null mice, mice with reduced expression of NR1 subunit survive to adulthood with concomitant exhibition of a variety of psychotomimetic abnormal behaviors that are ameliorated by treatment with the antipsychotic drugs haloperidol and clozapine (83). These previous findings all give support to the toxicity related to psychosis by sustained blockade of NMDA receptor channels in cultured rat hippocampal neurons irrespective of the cell maturity as described in this article. In a quantitative postmortem study on brains of patients with sporadic bipolar disorder, in fact, a significant decrease is seen in expression of both MAP-2 and MAP-1b, in addition to laminar thickness and neuron densities, in layers III, V and VI of the subgenual part of area 24 (66). Immunoreactive MAP-2 is significantly decreased in cortical layers III and V in areas 9 and 32 of the prefrontal cortex, but not in the occipital cortex, in brains of patients suffering from schizophrenia (84). *In situ* hybridization analysis reveals that in rat brain a significant increase is seen in the expression of $mRNA^{MAP\text{-}2}$ in the dentate gyrus within 24 h after the last trial of daily repetitive electroconvulsive shock that is an animal model of ECT in clinical treatment of depression (85). The fact that sustained exposure to MK-801 led to a marked decrease in the endogenous level of MAP-2 in cultured hippocampal neurons, gives rise to the similarity between phenomena found in *in vivo* clinical cases and *in vitro* cultured neurons in terms of the decreased MAP-2 level. The present experimental procedures would give a clue for the elucidation of mechanisms underlying the clinical usefulness of rTMS for the therapy of refractory depression, schizophrenia, Parkinson's disease and Alzheimer's disease in human subjects. The possibility that molecular mechanisms would at least in part involve different phenomena mentioned in this review with respect to the etiology and pathology of crisis of a variety of symptoms relevant to those neuropsychiatric and neurodegenerative disorders is not ruled out.

CONCLUSION

The findings cited above argue in favor of an idea that static magnetic fields would at first induce particular immediately early genes, such as c-*fos* and c-*jun*, for modulation of the expression of target genes responsible for the homeostatic maintenance of the functionality and integrity in neurons. For example, brief exposure to static magnetism could decrease the endogenous level of MAP-2 required for matured

neuritic cytoskeletons through rapid expression of the nuclear transcription factor AP1 complex consisting of c-Jun, JunD and/or Fra2 proteins and subsequent attenuation of opening processes of NMDA receptor channels permeable to calcium ions in hippocampal neurons as shown in Fig. 6. Similarly, the bHLH transcription factor ALF1 identified on DD analysis could at least in part play a role in mechanisms related to the decreased expression of MAP-2 in hippocampal neurons cultured under sustained exposure to static magnetic fields. By contrast, repetitive magnetism seems to be useful for the prevention of neurotoxicity of an NMDA receptor channel antagonist in terms of the endogenous level of MAP-2 in cultured hippocampal neurons. In addition to the aforementioned *in vitro* studies using cultured neurons, further *in vivo* studies are undoubtedly required for the demonstration of modulating properties of static magnetic fields on a variety of neuronal activities.

It thus appears that static magnetic fields really modulate the neuronal activity through regulation of the gene expression in cultured rat hippocampal neurons. The altered MAP-2 expression is suggestive of rather potent functional alterations in response to static magnetic fields under different exposure conditions. The magnetic modulation would be at least in part responsible for the high incidence of different neuropsychiatric disorders in regions with a higher magnetic density, in addition to less sunshine, than other areas on this globe. Much attention may need to be paid to the clinical use of powerful magnetic generators such as magnetic resonance image and nuclear magnetic resonance for the diagnosis of a variety of disorders associated with brain malfunctions. Both electromagnetic induction and physical force generation could affect the signal conduction mediated by axonal local electric circuitry to lead to altered transmission mediated by exocytotic release of neurotransmitters at synapses in central neurons. Elucidation of the exact underlying molecular mechanisms would give us a clue for the discovery and development of novel strategies useful for the therapy and treatment in patients suffering from a variety of neuropsychiatric and neurodegenerative disorders.

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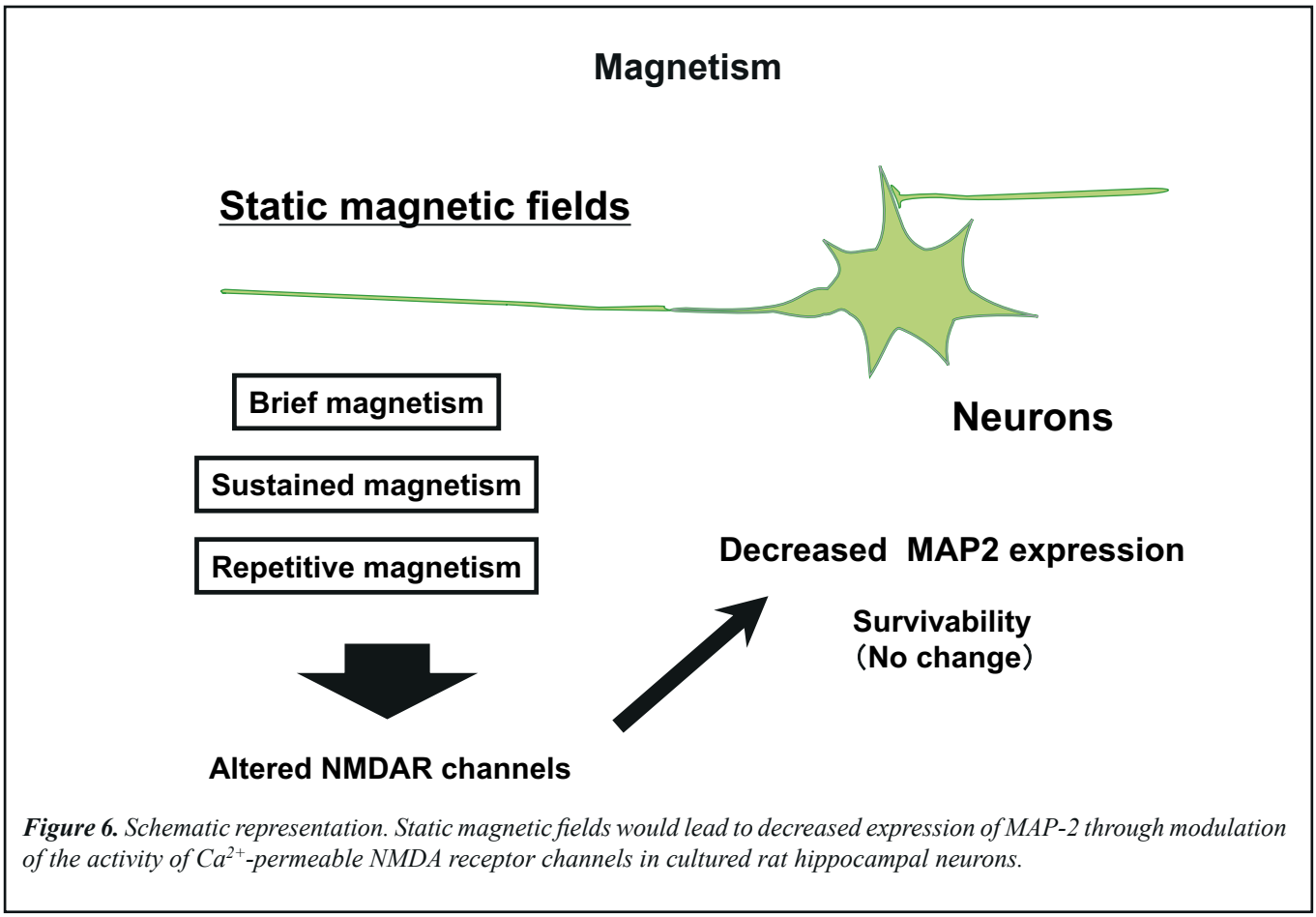

Figure 6. *Schematic representation. Static magnetic fields would lead to decreased expression of MAP-2 through modulation of the activity of Ca^{2+}-permeable NMDA receptor channels in cultured rat hippocampal neurons.*

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