

Expression of *c-fos* in cortical neuron cultures under dynamic magnetic field is not suppressed by calcium channel blockers

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SUMMARY Previously, we developed a dynamic magnetic field (DMF) device using neodymium magnets that induced *c-fos* expression in cortical neurons, while *activity-regulated cytoskeleton-associated protein (Arc)*, and *brain-derived neurotrophic factor (BDNF)* remained unaffected. The precise signal transduction pathway for *c-fos* induction under DMF was unclear. This study aimed to investigate the mechanism of immediate early gene (IEG) induction using calcium channel blockers (CCBs). Six experiments were conducted with cortical neurons, employing an NMDA receptor antagonist and an L-type voltage-dependent calcium channel blocker as CCBs. Neuronal cultures were exposed to DMF, CCBs, or both, and IEG expression (*Arc*, *c-fos*, *BDNF*) was measured through polymerase chain reaction. Results showed a tendency for increased *c-fos* expression with DMF exposure, which was unaffected by CCBs. In contrast, *Arc* and *BDNF* were not induced under DMF exposure but were significantly inhibited by CCBs. These findings suggest that *c-fos* induction under DMF involves a distinct pathway, potentially relevant to stress resistance and drug discovery.

Keywords dynamic magnetic field, immediate early gene, cortical neurons, calcium channel blockers, *c-fos*

1. Introduction

We developed a dynamic magnetic field (DMF) device based on Arago's disc principle (1,2). This DMF device, using a rotating magnet, generates both electromagnetic and Lorentz forces (Figure 1). When rat cortical neurons were cultured under DMF with rotating neodymium magnets, *c-fos* expression was induced, while a transcription factor; *activity-regulated cytoskeleton-associated protein (Arc)*, and *brain-derived neurotrophic factor (BDNF)* were not (2). This selective induction suggests a distinct signaling pathway for *c-fos*, though the physiological mechanism remains unclear.

c-fos functions as a marker for neuronal activation (3) and is essential for neuronal excitability and survival (4). Its molecular evolution might contribute to stress resistance, such as from heat shock and ultraviolet (UV) irradiation (5,6), as well as to the establishment of long-term memory in engram cells (7). *c-fos* acts as a signaling hub, playing a central role in both calcium-dependent and calcium-independent pathways, including the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA)-activated cAMP response element-

binding protein (CREB) and the stress-responsive p38 pathways (8,9). Understanding the unique signaling pathways of *c-fos* not only helps unravel the evolution from ancestral neurons to engram neurons but also holds potential for new drug discovery, enhancing neuronal survival, growth, and stress resistance.

In this study, to investigate *c-fos*'s unique signaling pathway, we examined the induction of immediate early genes (IEGs) in primary cortical neurons using calcium channel blockers (CCBs), N-methyl-D-aspartate (NMDA) receptor antagonist amino-5-phosphonovaleric acid (APV), and the L-type voltage-dependent calcium channel (VDCC) blocker (nicardipine). This research explores the distinct DMF response pathway in cortical neurons that might contribute to *c-fos* expression.

2. Materials and Methods

2.1. DMF exposure system

The device comprises a stand for holding a cell culture container, a rotating component made of acrylic resin with a circular surface embedded with permanent

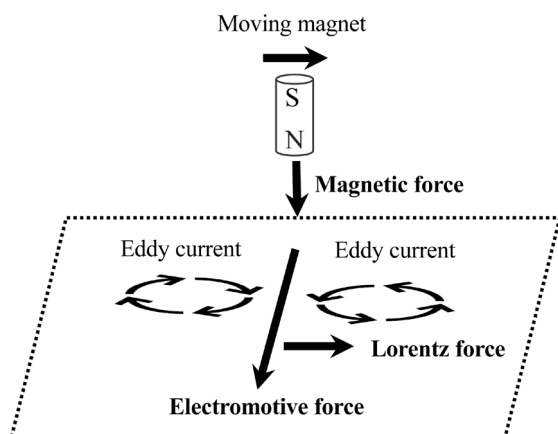


Figure 1. Principle of Arago's Disk through Dynamic Magnetic Field (DMF) exposure. When a magnet approaches an object, such as a neuron, the magnetic force arising from its magnetic flux induces electromotive force, leading to the flow of eddy currents. This process also generates mechanical force within the neuron.

magnets (NeoMag Co., Ltd., Tokyo, Japan) and a control device to manage the motor mounted at the center of the rotating part, as previously described (2). The dish stand, made of aluminum, has a 35.5 mm hole that fits a 35 mm diameter dish. The height of the stand is adjustable to ensure the dish does not touch the magnets. In our experiment, we set the minimum distance between the dish and the magnets to 3 mm. The circular surface includes ten rectangular neodymium magnets evenly spaced from the center, with their N poles facing the dish. The surface exhibits a magnetic flux density of 484 mT (length 40 × width 6 × height 11 mm, magnetization direction along the 11mm dimension). The circular surface is driven by a Direct-current (DC) motor (Pololu Co., Ltd, USA: item #4863, 20.4:1 metal gearmotor 25 Depth x65 Length mm Motor Power 12 Volt with 48 count per revolution encoder), controlled by a microcontroller (Arduino CC, Italy: Arduino Uno R3) and a motor driver (Pololu Co., Ltd, USA : Dual TB9051FTG Motor Driver Shield for Arduino) to regulate the motor speed. Speed is controlled using PID (proportional, integral, differential) control, by supplying voltage to the motor, measuring the speed, and adjusting the target RPM (revolutions per minute) every 0.3 seconds to achieve the desired speed. The ten default magnets on the circular surface correspond to low speed (30.0 rpm), middle speed (60 rpm), and high speed (240 rpm), with the speed adjustment knob set to achieve frequencies of 5 Hz, 10 Hz, and 40 Hz, respectively. These speeds can be modified to expose cells to frequencies in the delta band (0.5-4 Hz), theta band (4-8 Hz), alpha band (8-13 Hz), beta band (14-30 Hz), gamma band (30-80 Hz), and high-frequency band above 80 Hz.

2.2. DMF exposure

Primary cultures of cortical neurons were obtained from Sprague-Dawley rats at embryonic day 17 and cultured

until day 10 (Japan SLC, Shizuoka, Japan), following the method detailed by previous papers (2,10). The preparation and treatment of these cells were conducted under the University of Toyama's Animal Care and Experimentation Committee guidelines, approval number A2022PHA-6. In brief, the cerebral cortex was extracted from embryonic brains, treated with trypsin and DNase I, and the resulting dissociated cells were seeded at a density of 2.0×10^6 cells on poly-L-lysine (PLL) coated dishes (AGC Techno Glass Co., Ltd., Shizuoka, Japan) for Reverse Transcription Polymerase Chain Reaction (RT-PCR). Half of the culture medium was refreshed every 3 days. PLL, a positively charged synthetic polymer, enhances cell adhesion by attracting negatively charged molecules on the cell surface.

This research comprised 6 experiments with a total of 24 dish samples. Each experiment involved 4 conditions: a vehicle control condition, a condition treated with the NMDA receptor antagonist (APV) and the L-type calcium channel blocker nifedipine (Nica), a non-magnetic-exposure control condition, and a condition exposed to a DMF. Gene expression was evaluated immediately after 3 consecutive days of exposure, comparing 4 groups: vehicle condition without DMF, vehicle condition with DMF, APV + Nica without DMF, and APV + Nica with DMF. The APV + Nica exposure (APV: 200 μ M, Nica: 5 μ M) was administered 10 minutes before the first day of DMF exposure.

Preliminary experiments showed that γ -band (40 Hz) exposure for 6 hours per day suppressed the effect (data not shown). Hence, the neurons were exposed to a rotating magnetic field of 484 mT at 40 Hz for 1 hour daily over 3 consecutive days. The exposure setup was inside an incubator (MCO-5AC-PJ. PHC Co., Ltd., Tokyo, Japan) with controlled temperature (37°C), humidity (95%), and CO₂ concentration (10%). A microcomputer-controlled the rotation speed of the magnetic disks outside the incubator.

The study focused on the expression of IEGs such as *c-fos*, *Arc*, and *BDNF* (exon IV-IX and CDS), which are activity-dependent and essential for neuronal morphology and function (11). Gene expressions were normalized to *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, a stable housekeeping gene. RT-PCR procedures and primer sequences for *c-fos*, *Arc*, *BDNF* exon IV-IX, *BDNF* CDS, and *GAPDH* are described as previous paper (2).

2.3. Statistical analysis

All data are presented as the median (interquartile range). Statistical analyses were performed using BellCurve for Excel version 4.05 (Social Survey Research Information Co., Ltd.). The expression levels of IEGs in cultured cortical neurons were compared using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test for *c-fos*, *Arc*, *BDNF* CDS, and *BDNF*

exon IV-IX. This comparison assessed the increase in IEGs due to DMF exposure and the suppression of IEGs due to CCBs exposure. Differences with a P -value < 0.05 were considered statistically significant.

3. Results and Discussion

In this study, primary cultured rat cortical neurons were exposed to a rotating neodymium magnet for three consecutive days, one hour per day, under continuous 40 Hz DMF exposure. The results showed that the group median and interquartile range of *c-fos* mRNA expression without DMF was 6.6 (3.4-9.4) (E-3/GAPDH), compared to 7.6 (5.6-15.7) (E-3/GAPDH) with DMF. For *Arc* mRNA, the group median and interquartile range without DMF were 9.2 (4.9-23) (E-4/GAPDH), and 14.5 (7.9-23) (E-4/GAPDH) with DMF. *BDNF* gene expression analysis focused on *BDNF* exon IV-IX mRNA, regulated in a neuronal activity-dependent manner, and *BDNF* CDS (12,13). The group median and interquartile range of *BDNF* CDS mRNA expression without DMF was 16.3 (9.2-25) (E-3/GAPDH) and 15.8 (11.8-31.6) (E-3/GAPDH) with DMF. For *BDNF* exon IV-IX mRNA, the group median and interquartile range were 4.4 (1.8-6.2) (E-3/GAPDH) without DMF, and 5.2 (2.4-6.9) (E-3/GAPDH) with DMF.

Comparing IEG expression levels with and without DMF exposure revealed a slight increase in *c-fos* mRNA expression upon DMF exposure, consistent with our previous findings (2), though it did not reach statistical significance under stringent Bonferroni multiple comparison testing. *Arc* and *BDNF* mRNA expressions

(both CDS and exons IV-IX) did not show increased expression upon DMF exposure, with no significant differences observed.

Under conditions involving CCBs (APV + Nica), median and interquartile ranges for *c-fos* mRNA expression were 5.3 (3.9-11.9) (E-3/GAPDH) without DMF and 5.4 (4.1-16.4) (E-3/GAPDH) with DMF. For *Arc* mRNA, the group median and interquartile range were 4.1 (2-8.5) (E-4/GAPDH) without DMF and 5.1 (2.8-7.5) (E-4/GAPDH) with DMF. *BDNF* CDS mRNA expression showed median and interquartile ranges of 5.3 (4.7-8.8) (E-3/GAPDH) without DMF and 6 (4.4-9.5) (E-3/GAPDH) with DMF. Lastly, *BDNF* exon IV-IX mRNA expression was 7.1 (5-13.4) (E-4/GAPDH) without DMF and 7.8 (3.8-12.7) (E-4/GAPDH) with DMF. No significant induction of *c-fos* mRNA, *Arc* mRNA, or *BDNF* mRNA (both CDS and exons IV-IX) was detected with DMF exposure (Bonferroni multiple comparison test, $P > 0.05$). However, when exposed to CCBs, *Arc* mRNA and *BDNF* mRNA (both CDS and exons IV-IX) were significantly inhibited (Bonferroni multiple comparison test, $P < 0.05$), while *c-fos* mRNA was not significantly affected (Bonferroni multiple comparison test, $P > 0.05$) (Figure 2).

In this study, two major findings were revealed. 1) Slight induction of *c-fos* mRNA by DMF exposure: Cultured cortical neurons exposed to DMF showed a slight increase in *c-fos* mRNA expression. This pattern was reproducible and consistent with previous our study (2). However, no corresponding increase in the expression of *Arc* or *BDNF* (both CDS and exon IV-IX) was observed under DMF exposure. 2) Differential

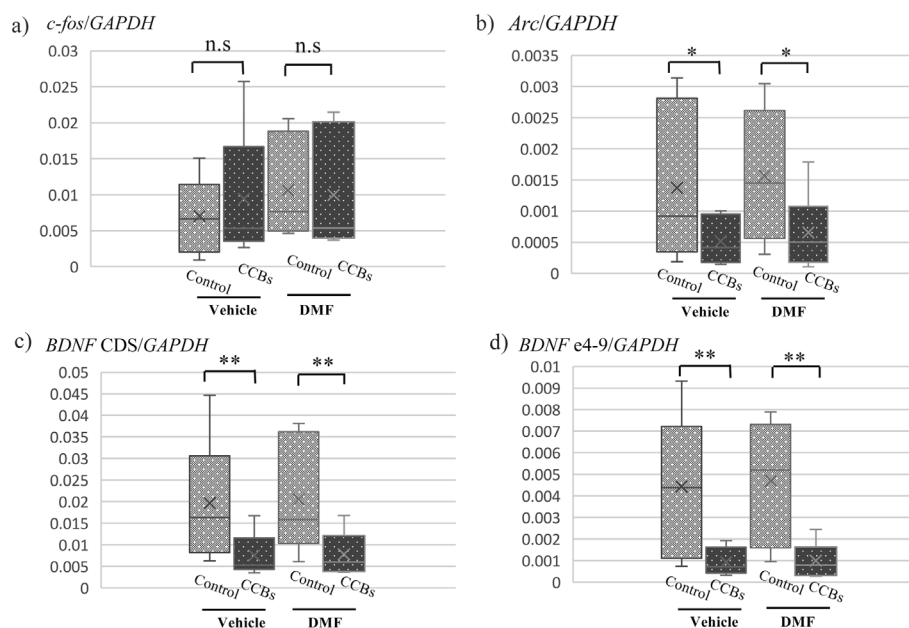


Figure 2. Inhibitory effect of calcium blockers (CCBs: APV + Nica) on the expression of immediate early genes (IEGs) under vehicle conditions or dynamic magnetic fields (DMF) conditions. a) *c-fos* mRNA b) *Arc* mRNA c) *BDNF* CDS mRNA d) *BDNF* e4-9 mRNA. The box plots exhibit the normalized group median and quartile range of IEGs in cultured cortical cells. The Y-axis represents the IEG mRNA normalized to *GAPDH*. Error bars indicate the maximum and minimum values. Statistical significance denoted by asterisks (*) (**) indicates P -values less than 0.05, and P -values less than 0.01, respectively, based on the Bonferroni multiple comparison test.

effects on gene expression by CCBs under DMF: Under DMF exposure with CCBs, *Arc* mRNA and *BDNF* mRNA (both CDS and exon IV-IX) were significantly inhibited, while *c-fos* mRNA was not. These results suggest that the selective induction of *c-fos* under DMF is different from the calcium signaling pathways required for *Arc* and *BDNF* expression and might play a different role, such as in stress resilience, distinct from the roles of *Arc* and *BDNF* in neuronal plasticity and maturation.

The hypothetical pathway of *c-fos* induction in a DMF environment is summarized in Figure 3. Induction of *c-fos* mRNA is usually promoted by calcium influx through NMDA receptors and voltage-dependent calcium channels (14). However, since *c-fos* mRNA expression was not inhibited under NMDA receptor antagonist and L-type calcium channel blocker exposure in this study, alternative pathways of *c-fos* induction need to be investigated. Potential *c-fos* signaling pathways include calcium-dependent pathways through T-type channels or Transient receptor potential (TRP) channels, or calcium-independent pathways through cAMP/PKA/CREB or p38 stress pathways (8,9). T-type VDCC are known for low-threshold pacemaker activity and have been reported to induce mesenchymal stem cell proliferation under a static magnetic field (15). Additionally, TRP channels, which respond to environmental stimuli such as temperature and mechanical forces (16), might also respond to stress from DMF as they detect environmental stress.

The evolutionary significance of *c-fos* is not yet

fully understood. Unlike pseudogenes that evolve rapidly due to neutral mutations (17), genes expressed in the brain evolve slowly under selective pressure (18,19). The stability of the *c-fos* gene might arise from forming a stable activator protein-1 (AP-1) complex with c-Jun, reducing its sensitivity to mutational pressure during adaptive evolution (20). Therefore, *c-fos* has been repeatedly utilized for various functions by serving as a master switch that could convert short-term responses to long-term responses (21), controlling cell proliferation, apoptosis, and deoxyribonucleic acid (DNA) repair (Figure 3). During the evolution from unicellular to multicellular organisms 600-950 million years ago, *c-fos* might have played a role similar to tumor suppressor genes that promote multicellularity (22,23). Under environmental selective pressures such as UV radiation (6), gravity (24), *c-fos* might have evolved to enhance stress resistance in early eukaryotic cells and help neurons adapt to environmental challenges. Increased levels of *c-fos* expression might also confer stress resistance, as demonstrated by increased resilience in mice with high levels of Fos protein (25). Therefore, *c-fos* induction might contribute to stress resistance, especially under extreme environmental conditions.

There are several limitations to this study: 1) The underlying mechanism of slight *c-fos* induction under DMF exposure remains unknown. In the DMF device, we hypothesized the presence of voltage-dependent, mechanical force, and magnetic-dependent ion channels

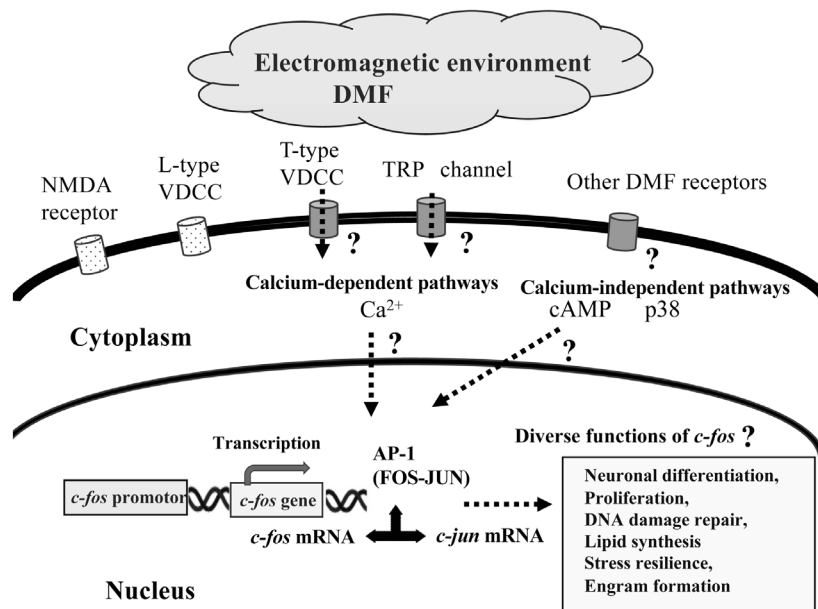


Figure 3. The hypothetical pathway of *c-fos* induction in a DMF environment. The transduction pathway responsible for inducing *c-fos* in the DMF environment might involve mechanisms beyond the calcium-dependent pathway through L-type voltage-dependent calcium channels and NMDA receptors. Instead, hypothetical transduction pathways, such as other calcium-dependent pathways (e.g., T-type VDCC, TRP channels) or calcium-independent pathways (e.g., cAMP, p38), might exist. These pathways could potentially contribute to the acquisition of diverse and significant functions by the *c-fos* gene during neuronal evolution in electromagnetic environments. Abbreviations: DMF: dynamic magnetic field, TRP: transient receptor potential, NMDA: N-methyl-D-aspartate, VDCC: voltage-dependent calcium channel. The question mark (?) indicates an unresolved issue.

as DMF sensors (2), but no ion channels activated by DMF have been discovered, and the physiological mechanisms of DMF effects on neurons are unclear. 2) The magnetic field strength used in this study (484 mT) was much stronger than the geomagnetic field (30-50 μ T). Therefore, it is necessary to investigate the strength, frequency, and exposure duration of the DMF that could induce both *Arc* and *BDNF*, which are more beneficial for neurons. Neurons could mature into engram cells by inducing *Arc* mRNA, which is essential for the formation of neural circuits, synaptic plasticity, and memory retention (26,27), and *BDNF*, which supports neuronal survival and synaptic complexity (28,29).

Many animals, from honeybees and salmon to migratory birds, utilize geomagnetic field for navigation (30). However, little is known about the effects of geomagnetic field on the evolution of neurons. In the future, by using this device to expose neurons to a DMF environment (approximately 30-50 μ T) for an extended period, it might be possible to elucidate how neurons adapt to such conditions. Understanding the *c-fos* induction signaling pathways in neurons under DMF conditions is crucial for comprehending how neurons have evolved in electromagnetic field (*i.e.* brainwave) environments. Furthermore, elucidating the signaling pathways involved in these processes could contribute to the discovery of various drugs that enhance stress resistance and promote synaptic maturation.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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