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Exercise combined with low-level GABA<sub>A</sub> receptor inhibition up-regulates the expression of neurotrophins in the motor cortex.

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## Abstract

Neurotrophins play a crucial role in neuroplasticity, neurogenesis, and neuroprotection in the central nervous system. Aerobic exercise is known to increase the expression of BDNF in the cerebral cortex. Several animal studies have evaluated the tonic inhibition of GABAergic synapses to enhance hippocampal plasticity as well as learning and memory, whereas the effects of GABAergic inhibition on plasticity in the cerebral cortex related to motor learning are not well characterized. The objective of the present study was to examine the interactive effect of low-level GABA<sub>A</sub> receptor inhibition and exercise on the expression of neurotrophins including BDNF in the murine motor cortex. ICR mice were randomly distributed among 4 groups based on two factors of GABA<sub>A</sub> receptor inhibition and exercise, i.e. control group, an exercise group, a bicuculline group, and an exercise plus bicuculline group. We administered GABA<sub>A</sub> receptor antagonist, bicuculline intraperitoneally to the mice (bicuculline and exercise plus bicuculline group) at a non-epileptic dose of 0.25 mg/kg, whereas the mice (exercise and exercise plus bicuculline group) were exercised on a treadmill for 1 hour every day. After two week intervention, the expression of mRNA and protein abundance of neurotrophins in the motor cortex was assayed using Real time PCR and ELISA. BDNF gene expression was significantly increased by approximately 3-fold in the bicuculline group relative to the control, exercise, and bicuculline plus exercise groups. Protein abundance of BDNF expression was significantly increased by approximately 3-fold in the bicuculline plus exercise group relative to other groups. Therefore, the present study revealed that combined GABA<sub>A</sub> receptor inhibition and moderate aerobic exercise up-regulated BDNF protein expression in the motor cortex without producing side effects on motor or cognitive functions. Alterations in BDNF expression could positively contribute to plasticity by regulating the balance between EPSPs and IPSPs in the motor cortex and thus providing a more appropriate neuronal condition for motor learning and recovery.

## 1. Introduction

Kinesiotherapy is a primary intervention designed to improve and rehabilitate the motor function of patients with central nervous system disorders. Motor recovery in patients with central nervous system disorders such as cerebrovascular accident (CVA) is regulated by cortical neuroplasticity, which is a product of broad spectrum synaptic modification (i.e., synaptic facilitation or inhibition). An important therapeutic goal of motor recovery is therefore to maximize neuronal plasticity and the facilitation of motor learning during the kinesiotherapy in patients.

Neuronal activity involves excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs). Glutamatergic synapses expressing N-methyl-D-aspartate (NMDA) receptors and aminomethylphosphonic acid (AMPA) receptors mainly regulate EPSPs, whereas GABAergic inputs from interneurons produce IPSPs. In a clinical trial designed to control the balance between motor-related EPSPs and IPSPs, transcranial direct current stimulation (tDCS) to the cortex was used to enhance the motor recovery of CVA patients receiving kinesiotherapy [1]. The application of tDCS to the motor-related cortex enhances motor learning [1]. Stagg et al. reported that tDCS inhibited GABAergic neurons and reduced IPSPs to enhance the excitability of the target neuronal network [2, 3]. In addition to the acute neuronal effects of tDCS, several animal studies have evaluated the tonic inhibition of GABAergic synapses using GABA<sub>A</sub> or GABA<sub>B</sub> receptor antagonists to enhance hippocampal plasticity as well as learning and memory [4-9]. While these strategies should take care to avoid excitotoxicity induced by the over-activation of NMDA receptors, the non-epileptic inhibition of GABAergic synapses could positively affect neuronal plasticity in motor learning during kinesiotherapy.

Neurotrophins play a crucial role in neuroplasticity, neurogenesis, and neuroprotection (neuronal maintenance and survival) in the central nervous system. The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4). Of these, BDNF has a high affinity for the tyrosine receptor kinase B (TrkB) receptor, which transduces intracellular signals for neuroplasticity in the brain [10, 11]. Therefore, it is reasonable to hypothesize that the

up-regulation of BDNF could enhance motor learning and neuroplasticity. Aerobic exercise is known to increase the expression of BDNF in the cerebral cortex, hippocampus, and cerebellar cortex [12-16]. Moreover, previous literature indicates that aerobic exercise enhances cognitive function by increasing BDNF expression in the hippocampus [14, 15]. We thus hypothesized that aerobic exercise would enhance motor learning through the up-regulation of BDNF in the motor-related cortex. Furthermore, if we can find a novel therapeutic intervention which efficiently enhances the exercise-induced up-regulation of BDNF expression in the motor cortex, we can expect an effective pre-conditioning for kinesiotherapy to rehabilitate the motor function of patients with central nervous system disorders such as CVA. Thus, we focused on exercise combined with drug-based low-level GABA<sub>A</sub> receptor inhibition in this study. In particular, , we can expect more chronic drug-based inhibition of GABAergic synapses compared with the transient inhibition by tDCS.

The objective of the present study was to examine the interactive effect of GABA<sub>A</sub> receptor inhibition and aerobic exercise on the expression of neurotrophins including BDNF and NT-4 in the murine motor cortex. We hypothesized that aerobic exercise in the presence of low-level GABAergic synaptic inhibition would enhance the expression of BDNF and NT-4 in the motor cortex in a manner potentially related to the neuroplasticity of cortical neurons.

## 2. Materials and methods

### 2.1. Animals

Twenty-nine 30-week-old female ICR mice were randomly distributed among 4 groups: a control group (Con, n = 7), an exercise group (Ex, n = 7), a bicuculline group (Bic, n = 8), and an exercise plus bicuculline group (Bic&Ex, n = 7). Mice were housed in a temperature- and humidity-controlled room on a 12-hour light/dark cycle with food and water available *ad libitum*. All study procedures were approved by the ethics committee for animal research of Hokkaido University in Japan and conducted according to the guidelines of the committee.

## 2.2. Aerobic exercise

According to previous literature reporting the significant up-regulation of BDNF mRNA after moderate treadmill exercise [13, 15], mice (Ex and Bic&Ex) were familiarized with treadmill exercise at a slow speed using mild electric shocks and subsequently exercised on a treadmill every day for 2 weeks. In each session, the treadmill speed was gradually increased to 15 m/min in order to avoid stress (i.e., 3 min at 6m/min, 3 min at 9 m/min, 5 min at 12 m/min, and 50 min at 15m/min in the absence of electric shocks). Mice were exercised at the same time each day (between the hours of 14:00 and 16:00). Sedentary mice (Con and Bic) were kept in their home cages during exercise sessions.

## 2.3. Drug administration

Castellano et al. [4, 5] reported that intraperitoneal administration of the GABA<sub>A</sub> receptor antagonist bicuculline produced dose-dependent improvements in learning and memory retention, with sufficient effects at a non-epileptic dose of 0.5 mg/kg. Accordingly, we administered bicuculline intraperitoneally to mice (Bic and Bic&Ex) at a subeffective dose of 0.25 mg/kg every day for 2 weeks; this dose was selected to avoid possible neurotoxicity during the 2-week administration period. Bicuculline was administered to Bic&Ex mice 30 min before treadmill exercise and no seizures were noted after drug administration.

## 2.4. Behavioral tests

Motor and cognitive functions were evaluated to examine the possible negative effects of bicuculline administration after exercise intervention. To evaluate motor control, we used the rotarod test, inclined plane test, and wire hang test. In the rotarod test, mice were placed on a rod with a diameter of 30 mm (MK-630B, Muromachi Kikai, Japan) and the rod was rotated at 30 rpm. The latency to falling off of the rod was measured. If a mouse remained on the rod for 180 s, the test was stopped and the latency was recorded as 180s. In the wire hang test, a 50-cm-wide plastic-coated metal wire with a diameter of 2 mm was placed at a height of 1 m above the safe floor. Mice were made to hang from the wire and the latency to falling was measured. In the inclined plane test, a 50-cm square acrylic platform with an angular gauge was fixed to a laboratory bench.

Mice were placed on the platform and the platform was inclined at 10 °s (raised on the cranial side). The incline angle at which mice slipped was measured. The mean value from three trials was recorded for each test.

To evaluate recognition memory, the novel-object recognition test was used according to the methods of Wang et al. [17]. The test included two sessions. After habituation to the test arena (40 cm × 40 cm), 2 identical cylinder blocks were placed at opposite corners of the arena. Each mouse was allowed to explore for 10 min during the first session. In the second session (24 h after the first session), one of the cylinder blocks was exchanged for a novel cube block. Mice were placed into the test arena and allowed to explore for 5 min during the second session. Object investigation was scored as the number of nose touches to each block during the second session. The ratio of nose touches to the novel block versus that of both blocks was calculated to evaluate recognition memory.

In addition, to assess the effects of interventions on the wakefulness (sleeping) during night-time when mice are regularly active, we monitored sleeping mice with eyes closed at 12 time points during night-time.

## 2.5. Tissue collection

Mice were sacrificed and decapitated 30 h after the final exercise or at a corresponding time in order to avoid the acute effects of interventions. The motor cortex of each hemisphere was surgically excised under sterile RNase-free conditions on ice. The right motor cortex was prepared for real-time PCR while the left motor cortex was prepared for analysis by enzyme-linked immunosorbent assay (ELISA). Tissue or tissue suspensions were immediately frozen in liquid nitrogen and stored at –80°C until use.

## 2.6. RNA purification and reverse transcriptase-polymerase chain reaction (RT-PCR)

The right motor cortex was roughly dissociated in 300 µL of a RNA stabilization reagent (RNAlater; QIAGEN, Venlo, Netherlands) in a microfuge tube. Purification of total RNA was performed using the RNeasy Lipid Tissue Mini Kit (QIAGEN, USA). Tissue was transferred to a new microtube for dissociation

with a disposable homogenizer (BioMasherII, Nippi, Japan), 1 mL of kit Qiasol reagent was added to the tube, and finally the suspension was homogenized three times with a Powermasher homogenizer (PowermasherII, Nippi, Japan) set at 450 rpm. The remainder of the procedure was performed according to kit manufacturer specifications. The absorbance of purified total RNA was measured at 260 nm (OD<sub>260</sub>) and 280 nm (OD<sub>280</sub>) with a UV mini-1240 spectrophotometer (NanoDrop ND-2000, ThermoFisher Scientific, USA) to assess the level of purity and RNA concentration of each sample. An aliquot containing 100 ng of RNA was used for reverse transcription polymerase chain reaction (RT-PCR).

RT-PCR was performed using a High Capacity cDNA Reverse Transcription Kit (QIAGEN) according to manufacturer specifications. Briefly, 2.5 ng of RNA per sample was used for each reaction. The thermal profile was a single cycle of 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s followed by incubation at 4°C using a Dice thermal cycler (TP600, TakaraBio, Japan).

## 2.7. Quantitative PCR based on real-time PCR

Real-time PCR was performed using a Step One Plus kit (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes specific for the target genes (TaqMan Gene Expression Assay) were obtained from Applied Biosystems (Foster City, CA, USA). The expression levels of target neurotrophins (BDNF and NT-4/5), the neurotrophin receptor (TrkB), and a marker of neuronal activity (c-fos) were assayed using  $\beta$ -actin as an internal control. Individual codes of TaqMan Gene Expression Assays were as follows: BDNF (Mm334042), NT-4 (Mm01701591), TrkB (Mm00435422), c-fos (Mm01302932), and  $\beta$ -actin (4352341E). Individual reactions contained 0.5  $\mu$ L of target primer, 0.5  $\mu$ L of  $\beta$ -actin primer (TaqMan Gene Expression Assay, Applied Biosystems), 3  $\mu$ L of RNase/DNase-free H<sub>2</sub>O, 5  $\mu$ L of TaqMan Gene Expression Master Mix (Applied Biosystems), and 1  $\mu$ L of sample cDNA in a total volume of 10  $\mu$ L. The thermal profile was a single cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

The relative expression level of each target gene was determined relative to the transcript of  $\beta$ -actin using the comparative ( $\Delta\Delta$ Ct) method. Averages of triplicate Ct values were used for the analysis. Transcript

abundance was normalized to the average of the adult control group for each target gene.

## 2.8. ELISA

Cortical neurotrophins were assayed using the ELISA method. Total protein from the left motor cortex was extracted using a Minute Total Protein Extraction Kit (Invent Biotechnologies, Inc. USA) according to manufacturer specifications. Additionally, we added 1 mL protease inhibitor cocktail PIC-2 (ITSI-Biosciences, PA, USA) including 100 mM AEBSF, 80  $\mu$ M aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, and 1 mM pepstatin to 20 mL of Native Cell Lysis Buffer (SN-002) supplied in the protein extraction kit. Each frozen tissue was placed on a filter cartridge and homogenized by grinding with a plastic rod 50–60 times. A volume of 200  $\mu$ L lysis buffer was added to the tube and the tissue was additionally homogenized with the plastic rod 30–60 times. The filter cartridge was centrifuged at 13,000 rpm to collect supernatants containing native total protein extract. Following total protein extraction, the density of extracted total protein was assayed using a spectrophotometer (NanoDrop ND-2000, Thermoscientific, USA).

Cortical BDNF or NT-4 was assayed using an ELISA BDNF E-maxR kit (Promega, USA) or a biosenseisR Neurorophin 4/5 (NT4/5) Rapid™ ELISA Kit (Biosensis, USA) according to manufacturer specifications. Aliquots of 100  $\mu$ L sample protein including approximately 1.0 mg total protein were transferred to a 96-well ELISA plate. Final absorbances were read at 450 nm using a plate reader (Model 680 Microplate Reader, Bio-Rad Laboratories, Inc., CA, USA).

## 2.9. Statistics

A one-way analysis of variance (ANOVA) was performed using SPSS ver.11.6 (SPSS Inc., Chicago, IL, USA) for each measurement. When a significant effect of group was detected in ANOVA, Boferroni correction method was used for post hoc multiple comparison. Statistical significance was established at  $P < 0.05$ . All data were shown in mean  $\pm$  S.E.M.

## 3. Results

### 3.1 Behavioral tests

There were no significant group effects observed for the rotarod test, wire hang test, or novel-object recognition test (Fig.1A-D), indicating that neither exercise nor bicuculline administration significantly affected motor function or cognitive function. The probability of mice's sleeping (%) was shown Fig.1E. An ANOVA showed significant group effect and post hoc analyses demonstrated that sleeping probability was significantly increased in the Bic&Ex group relative to the Con, Ex, and Bic groups. The sleeping probability was also increased in the Ex group than in the Con group.

### 3.2. Expression of mRNA

The relative mRNA expression of BDNF among different groups is shown in Fig. 2A. An ANOVA showed a significant group effect and *post hoc* analyses demonstrated that BDNF gene expression was significantly increased by approximately 3-fold in the Bic group relative to the Con, Ex, and Bic&Ex groups.

The relative mRNA expression of NT-4 among different groups is shown in Fig. 2B. An ANOVA showed a significant group effect and *post hoc* analyses demonstrated that NT-4 gene expression was significantly increased by approximately 10-fold in the Bic and Bic&Ex groups relative to the Con and Ex groups.

The relative mRNA expression of TrkB among different groups is shown in Fig. 3A. An ANOVA showed a significant group effect and *post hoc* analyses demonstrated that the TrkB gene expression was slightly but significantly elevated in the Bic group relative to the Con, Ex, and Bic&Ex groups.

The relative mRNA expression of c-fos among different groups is shown in Fig. 3B. An ANOVA did not indicate a significant group effect.

### 3.3 Protein abundance

The relative protein abundances of BDNF and NT-4 are shown in Fig. 4. An ANOVA showed a significant group effect on BDNF expression and *post hoc* analyses demonstrated that BDNF expression was

significantly increased by approximately 3-fold in the Bic&Ex group relative to the Con, Ex and Bic groups. Alternatively, the ANOVA for NT-4 expression did not show a significant group effect (Fig. 4B).

#### 4. Discussion

The present study revealed that low-level inhibition of the GABA<sub>A</sub> receptor for 2 weeks increased the expression of BDNF mRNA in the motor cortex, indicating enhanced transcription of BDNF. In addition, low-level inhibition of GABA<sub>A</sub> up-regulated the transcriptional expression of the BDNF receptor, TrkB, as well as that of NT-4. However, neither exercise nor GABA<sub>A</sub> receptor inhibition alone modified the protein expression of BDNF, whereas exercise in combination with low-level GABA<sub>A</sub> receptor inhibition significantly increased the protein expression of BDNF in the motor cortex. Since BDNF is a potent neurotrophic factor that enhances neuronal differentiation, survival, and LTP, it is possible that increased BDNF protein expression induced by aerobic exercise in the presence of low-level GABA<sub>A</sub> receptor inhibition could provide a more effective neural condition for motor learning during kinesiotherapy. As described in previous literature, low-level GABA<sub>A</sub> receptor inhibition did not cause the decline of motor or cognitive functions [4, 5], as evidenced by a lack of effect of any intervention in the motor and cognitive behavioral tests evaluated in this study. Thus, this study provides evidence that moderate exercise in the presence of low-level GABA<sub>A</sub> receptor inhibition is likely safe, increases the expression of BDNF in the motor cortex, and could potentially enhance motor learning or motor recovery in patients receiving kinesiotherapy.

Several animal studies have shown that the inhibition of GABAergic signaling improves learning and memory, whereas the enhancement of GABAergic neurotransmission in the hippocampus or prefrontal cortex impairs learning and memory. Jong Hoon Ryu et al. reported that administration of bicuculline or bicuculline methiodide directly into the hippocampus enhanced memory acquisition in a passive avoidance task in a manner related to hippocampal extracellular signal-regulated kinase signaling [7, 8]. Castellano et al. [4, 5] reported that bicuculline or picrotoxin produced dose-dependent enhancements in memory retention, whereas the GABA<sub>A</sub> receptor agonist muscimol produced dose-dependent impairments in memory acquisition and retention. Similar results were reported by Yousefi et al.[9]. Kim et al. reported that bicuculline

methiodide administration into the hippocampus or prefrontal cortex enhanced memory consolidation by increasing hippocampal BDNF expression [6]. In addition, GABA<sub>A</sub> receptor antagonists are reported to induce long term potentiation (LTP) in hippocampal neurons [18] and up-regulate BDNF mRNA expression in hypothalamic neurons [19] *in vitro*. Taken together, these reports suggest that adequate inhibition of GABAergic synapses could positively contribute to plasticity in the central nervous system. Unfortunately, the effects of GABAergic inhibition on plasticity in the cerebral cortex related to motor learning or motor skill acquisition are not well characterized *in vivo* or *in vitro*. Recently, Stagg et al. reported that the inhibition of GABAergic synapses with tDCS prior to motor training enhanced the acquisition of trained motor skills [2, 3]. Lee et al. reported that axonal remodeling for motor recovery after traumatic brain injury required the down-regulation of GABA<sub>A</sub> receptors [20]. Thus, the inhibition of GABAergic synapses appears to provide a more appropriate condition for motor learning. In this study, low-level GABA<sub>A</sub> receptor inhibition enhanced the gene expression of BDNF in the motor cortex, which may indicate an improved capacity for neuroplasticity and thus motor learning. Moreover, when exercise, which is known to potentiate brain BDNF expression, was co-applied with bicuculline, dramatic increases in protein BDNF expression were observed in the motor cortex, indicating that aerobic exercise in the presence of low-level GABA<sub>A</sub> receptor inhibition might facilitate motor cortical plasticity.

Since previous reports demonstrated that 60 min of moderate treadmill exercise [13, 15] or voluntary wheel exercise over a period longer than 7 days [12] significantly increased the expression of BDNF in the brain, we used moderate treadmill exercise for a period longer than 7 days in the present study. However, we were unable to detect a positive effect of exercise on BDNF expression in the motor cortex. To this end, we believe that the acute and chronic effects of exercise on BDNF expression should be considered. Huang et al. [12] reported the time-dependent up-regulation of BDNF expression by treadmill exercise, indicating that BDNF up-regulation over a 4-week period of treadmill exercise was confirmed 2-3 h after the last exercise session, but not 2 days later; therefore, the effects of exercise on BDNF expression may be acute. In addition, Ramussen et al. [16] reported that moderate treadmill exercise for 2 h up-regulated BDNF expression in the hippocampus 0–6 h after the last exercise session, while this effect disappeared after 24 h. Therefore, it is

reasonable that the 2-week forced treadmill exercise intervention used in this study had no chronic effect on BDNF expression as assayed 30 h after the final exercise session.

Interestingly, combined exercise and bicuculline administration increased BDNF protein expression. Recent studies show that aerobic exercise reduces short-interval intracortical inhibition (SICI) induced by paired-pulse transcranial magnetic stimulation (TMS) used to assess GABAergic inhibition in human motor cortex [21, 22]. Given that the neuronal expression of BDNF is enhanced in an activity-dependent manner, additional reduction of intracortical inhibition by exercise to GABA<sub>A</sub> receptor inhibition with bicuculline could enhance neural activity and generate an elevation of BDNF.

Of note, combined exercise and bicuculline administration did not have an effect on BDNF gene expression, whereas bicuculline alone increased BDNF gene expression. These data contradicted our protein expression findings, wherein combined exercise and bicuculline administration up-regulated BDNF protein expression. Discrepancies between exercise-induced modifications of BDNF mRNA and protein levels have been previously reported [12, 23]. There are several possible reasons underlying this discrepancy.

First, protein expression reflects mRNA translation after transcription. Exercise can affect intricate intracellular signals that enhance the translation of proteins in the central nervous system, for example by modulating effector proteins that interact with the 5' or 3' untranslated regions of dendritic mRNAs [24, 25]. One possibility is that the translation (rather than transcription) of BDNF was enhanced by combined exercise and bicuculline administration. Therefore, low-level GABA<sub>A</sub> receptor inhibition might have potentiated exercise-induced increases in BDNF translation in the motor cortex. This can also potentially explain our observations regarding NT-4: despite the transcriptional up-regulation of NT-4 by bicuculline administration, there was no effect of bicuculline on NT-4 protein expression.

Second, the discrepancy could be explained by the time lag between transcriptional up-regulation, translation, and subsequent behavioral modification. To assess the chronic effects of exercise and bicuculline administration in this study, mice were sacrificed 30 h after the last intervention. As abovementioned, several studies have indicated that exercise acutely increases the expression of BDNF in a manner that peaks 2–3 h after exercise and gradually decreases to baseline 24 h after exercise [12, 16]. On the contrary, previous

literatures show that protein expression starts to increase after 6 h after exercise and 9 h after GABA<sub>A</sub> receptor inhibition by bicuculline methiodide administration [6, 26]. In addition, mice sleep longer and more deeply after exercise [27]; acute over-excitation induced by bicuculline administration might potentiate this exercise-induced behavioral modification, i.e. mice might sleep longer and more deeply, reducing their regular nighttime activities just prior to sacrifice. In fact, the probability of sleeping during night time was extremely higher in the Bic&Ex group relative to the Con, Ex, Bic groups (Fig.1E). Given that the neuronal expression of BDNF is enhanced in an activity-dependent manner, decreased nighttime activity immediately prior to sacrifice may have limited the extent of BDNF up-regulation in the motor cortex. In agreement with this hypothesis, combined exercise and bicuculline administration tended to decrease the expression of c-fos, a marker for neuronal activity, relative to bicuculline administration alone, although a significant difference was not detected due to the limited sample size of each group.

This study had some limitations. First, we did not directly assay the inhibition of GABAergic synapses using real-time electrophysiological analyses. Second, we did not assay plasticity in the motor cortex nor did we directly evaluate motor learning or skill acquisition. Future studies should address these issues in order to validate our hypotheses and findings. Third, the discrepancy between modifications of BDNF mRNA and protein expression is not clearly explained as in most previous studies [12, 23]. Additional time-course detections of BDNF mRNA and protein are expected to elucidate the mechanism underlying the discrepancy as the next stage following this study.

In conclusion, the present study revealed that combined GABA<sub>A</sub> receptor inhibition and moderate aerobic exercise up-regulated BDNF protein expression in the motor cortex without producing side effects on motor or cognitive functions. Alterations in BDNF expression could positively contribute to plasticity by regulating the balance between EPSPs and IPSPs in the motor cortex. Thus the advantage of the interactive effect of aerobic exercise combined with low-level GABA<sub>A</sub> receptor inhibition could be providing a more appropriate neuronal condition for motor learning and recovery. Therefore, it is expected that this advantage of the interactive effects without neural toxicity could contribute to pre-conditioning for kinesiotherapy to rehabilitate the motor function of patients with central nervous system disorders such as CVA in future.

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## Figure legends

Figure 1. Effects of exercise and bicuculline administration on motor control in the rotarod test (A), inclined plane test (B), and wire hang test (C); on recognition memory as evaluated by the novel object recognition test (D); and on the probability of sleeping during night-time (E). Data are shown as the mean  $\pm$  S.E.M. (n = 7–8).

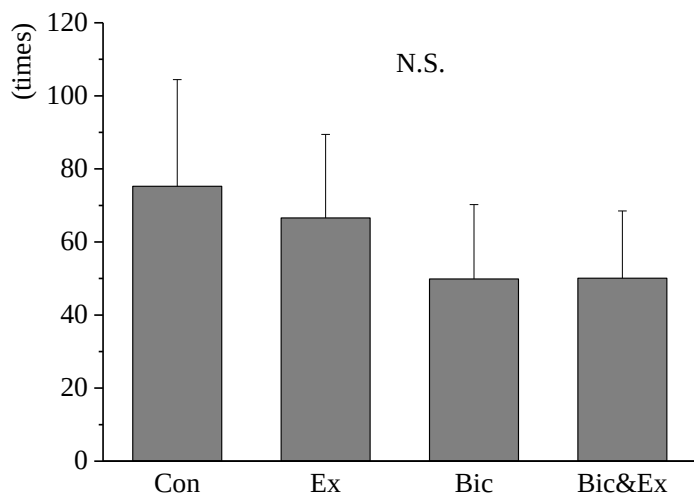
Figure 2. Effects of exercise and bicuculline administration on the mRNA expression of BDNF (A) and NT-4 (B). The expression level of each target gene was determined relative to the transcript of  $\beta$ -actin using the comparative ( $\Delta\Delta C_t$ ) method. Transcript abundance was normalized to the average of the control group for each target gene. Data are shown as the mean  $\pm$  S.E.M. (n = 7–8). \*\*\* indicates  $p < 0.001$  (Boferroni correction).

Figure 3. Effects of exercise and bicuculline administration on the mRNA expression of TrkB (A) and c-fos (B). The expression level of each target was determined relative to the transcript of  $\beta$ -actin using the comparative ( $\Delta\Delta C_t$ ) method. Transcript abundance was normalized to the average of the control group for each target gene. Data are shown as the mean  $\pm$  S.E.M. (n = 7–8). \* indicates  $p < 0.05$  and \*\* indicates  $p < 0.01$  (Boferroni correction ).

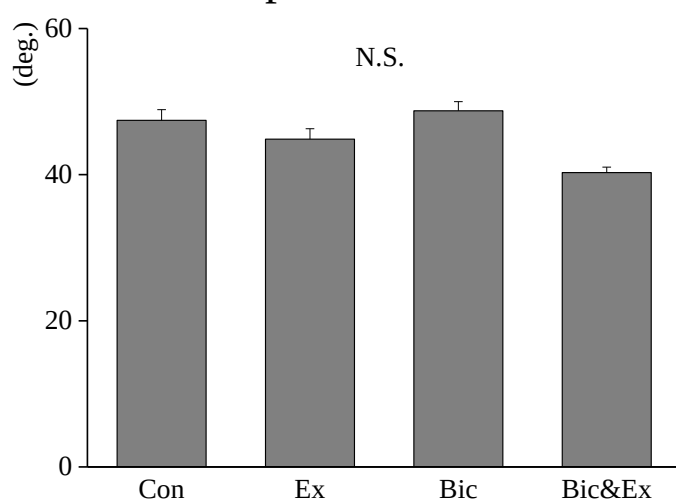
Figure 4. Effects of exercise and bicuculline administration on protein expression of BDNF (A) and NT-4 (B). Protein levels were determined by enzyme-linked immunosorbent assay and standardized to total protein in each well. Data are shown as the mean  $\pm$  S.E.M. (n = 7–8). \*\*\* indicates  $p < 0.01$  ( Boferroni correction).

Fig.1

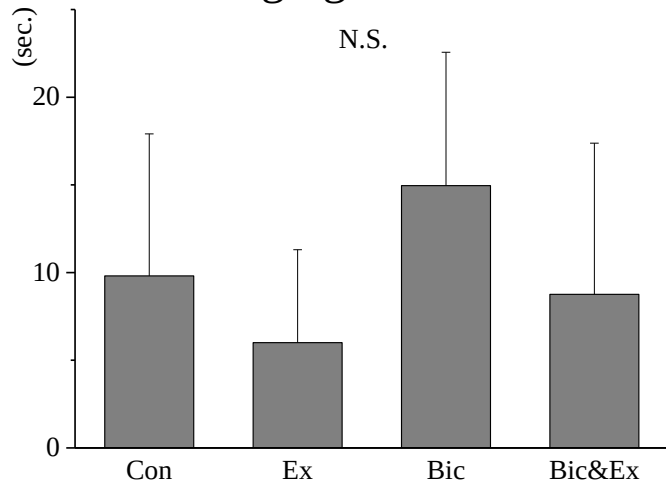
## A. Rotarod test



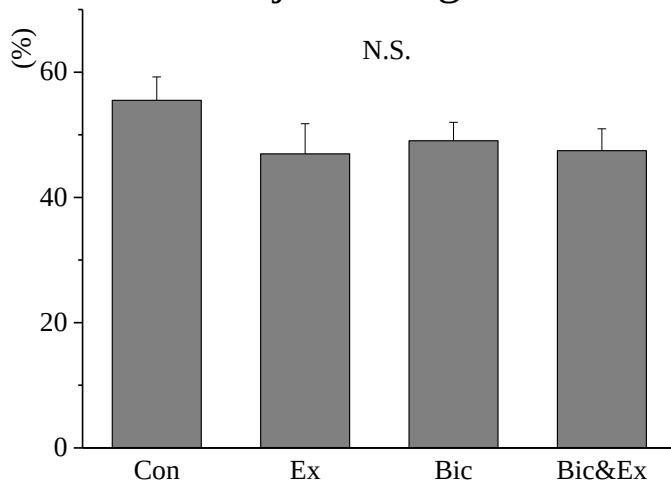
## B. Inclined plane test



## C. Wire hanging test



## D. Nobel-object recognition test



## E. Sleeping probability

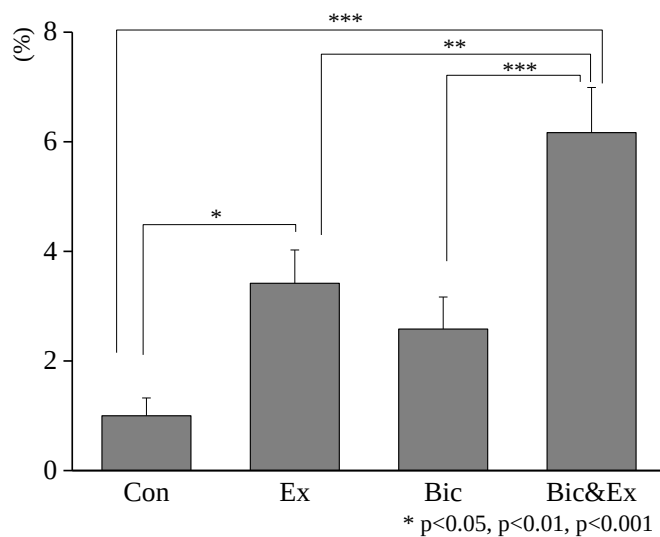
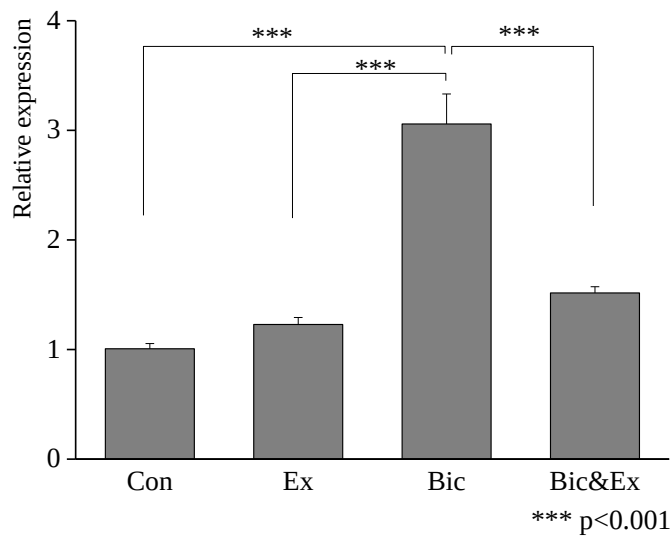
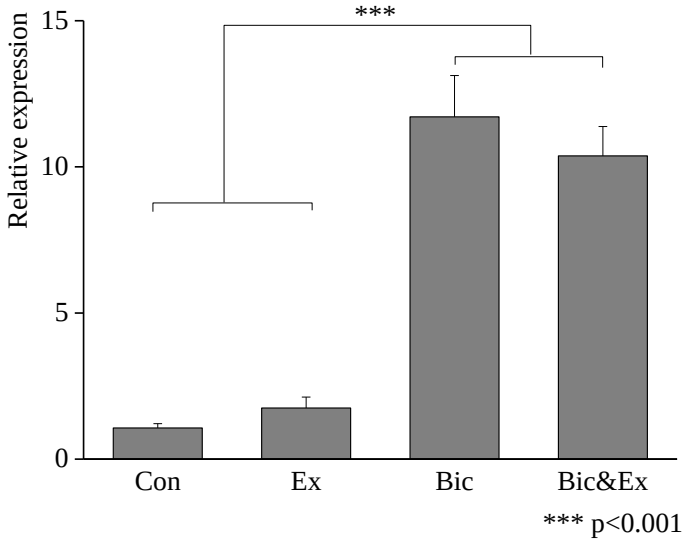


Fig.2

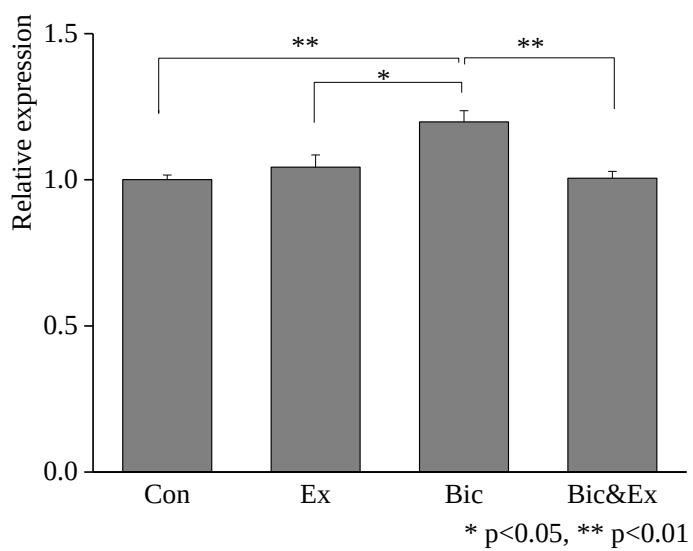
A. BDNF mRNA



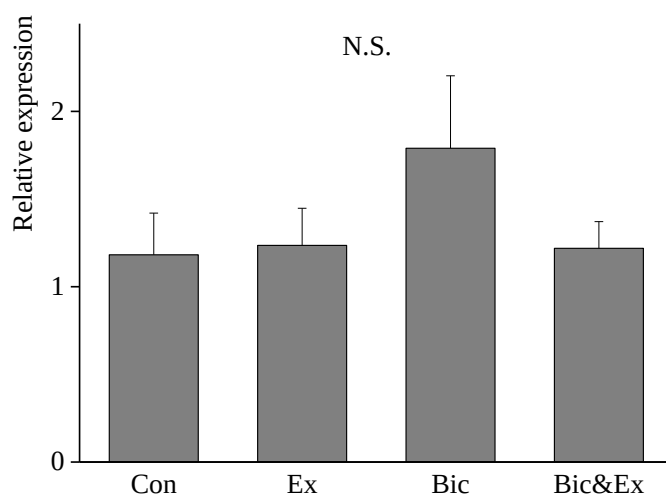
B. NT-4 mRNA



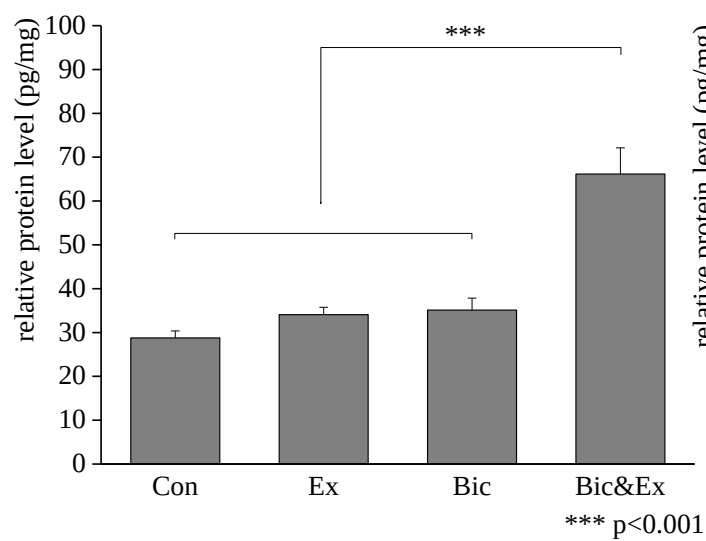
## A. TrkB mRNA



## B. c-fos mRNA



## A. protein BDNF



## B. protein NT-4

