

## Chronic Voluntary Exercise-Induced BDNF and RCAN1 is Associated with Improved Cognition and Depressive Mode in ApoE<sup>-/-</sup> Mice with Obese

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### Abstract

**Background:** Obesity is associated with cognitive dysfunction and dementia, as well as a reduction in brain-derived neurotrophic factor (BDNF). The calcineurin 1 regulator (RCAN1) regulates neuronal plasticity and depressive function by regulating BDNF. Voluntary exercise may improve this condition.

**Methods and findings:** Voluntary exercise was loaded in apolipoprotein E (ApoE)<sup>-/-</sup> mice by a high-fat diet (HFE, n=15) over a period of three months. Non-exercising mice with a high-fat diet (HFD, n=15) became overweight. Mice in voluntary exercise group ran on a wheel 5 days/week on the same period, mice without a high-fat diet as a control (NOR, n=15). A Morris water maze, novel object recognition, and forced swimming were evaluated. Chronic exercise significantly reduced body weight and increased the right psoas muscle weight/body weight ratio in the HFE group compared with HFD. Both of BDNF and RCA1 in HFE were expressed to a significantly higher extent in the hippocampus than with HFD. The Morris water maze test, novel object recognition test and forced swimming test were significantly improved in HFE.

**Conclusion:** Chronic voluntary exercise improved cognitive dysfunction and depressive modes along with increased expressions of BDNF mRNA in the hippocampus and of RCAN1 mRNA in the skeletal muscle.

**Keywords:** Obesity; Brain-derived neurotropic factor; Regulator of calcineurin 1; ApoE<sup>-/-</sup>; Voluntary exercise

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### Introduction

Obesity is associated with cognitive deficits, depression and dementia, with the reduction of brain-derived neurotrophic factor (BDNF) [1]. Calcineurin 1 regulator (RCAN1) controls neuronal plasticity and depressive states by regulating BDNF [2]. Similarly, RCAN1 expression is expressed in both the nervous system and in skeletal muscle [3].

As voluntary exercise has been suggested to improve neural and skeletal functions [1-4], we hypothesized that chronic voluntary exercise could regulate the BDNF and RCAN1 mRNA in the hippocampus and skeletal muscles, thereby affecting cognitive deficit, depressive mode and physiological adaptation.

Synaptosomal dysfunction is induced in ApoE<sup>-/-</sup> mice compared to control animals [5]. Moreover, ApoE gene polymorphism is a

robust genetic factor for Alzheimer's disease (AD). The BDNF have a wide distribution and highly presented in the central nervous system and its constitutive expression is particularly high in the hippocampus [6]. Moreover, brain RCAN1 in patients with Down's syndrome and AD is overexpressed and may be related with the pathogenesis of neurodegeneration [7].

Although BDNF is mainly situated in the brain, recent studies have shown that it is also expressed in non-neurogenic tissues, such as skeletal muscle [8]. Moreover, it is known to have a bearing on memory function and has also been identified as a key component of body mass control and energy homeostasis [9]. Finally, BDNF appears to play a major role not in both central metabolic pathways and energy metabolism in skeletal muscle [8].

Physical exercise is known to improve both cognitive function and depressive mode, with some evidence suggesting that BDNF and

RCAN1 may activate these functions [10]. However, it is not yet known whether the effects of chronic voluntary exercise could regulate BDNF and RCAN1 gene expression in hippocampus and skeletal muscle, thereby inducing improvements in cognitive and depressive states. In recent clinical meta-analyses, physical exercise leads to benefits in older people even without dementia, such as improving mood, reducing mortality, and improving function [11].

To assess whether chronic voluntary exercise improves cognitive dysfunction and depressive states in the murine model of AD by regulating the expression of the BDNF and RCAN1 gene in the hippocampus and skeletal muscles, we monitored voluntary movements on a wheel in obese ApoE<sup>-/-</sup> mice over a three-months period, and then used the test of Morris water maze, novel object recognition test, and forced swimming test to investigate the effectiveness.

## Methods

### Animals

Thirty-six female ApoE<sup>-/-</sup> mice (Charles River, Wilmington, USA) were used. All mice were housed in environmentally controlled conditions. The room temperature was kept at 22°C under a 12 h dark: 12 h light cycle. Drinking water and food were available *ad libitum*. All experiments were followed by the guidelines of Kanazawa Medical University. The high-fat diet comprised 56.7% fat and 25.5% protein, as described previously [12]. All mice were raised in accordance with Animal Care Committee Guidelines of Kanazawa Medical University under pathogen-free conditions.

### Methods

Voluntary exercise was loaded in ApoE<sup>-/-</sup> mice on a high-fat diet (HFE, n=15) for a period of three months. Non-exercising mice with the high-fat diet (HFD, n=15) became overweight. All mice were fed from the age of 8 weeks to 20 weeks. Mice in the voluntary exercise ran over a wheel for 5 days/week over the same period. Non-exercising mice not on a high-fat diet (NOR, n=15) were used as a control. The test of Morris water maze was used to evaluate spatial memories, the novel object recognition test to evaluate recognition memory, and the forced swimming test to evaluate depressive mode. All mice were euthanized using pentobarbital followed by cervical dislocation. The procedures were approved by the Kanazawa Medical University Animal Care Committee Guidelines.

**Histological analysis:** At the termination, the brain and skeletal muscle were divided and fixed in 10% neutral-buffered formalin. The brains were gently removed and processed as previously described [13]. Histological analyses were performed in ApoE<sup>-/-</sup> mice of three groups. The morphological fixation process of hippocampus and skeletal muscles were described previously [13]. Hematoxylin and eosin staining was used for histological analyses.

**Gene expression analysis:** The hippocampus and skeletal muscle were immediately harvested for RNA until the gene expression analysis described previously [13]. These RNA samples of hippocampus and skeletal muscle were performed according to

the manufacturer's protocol (RNeasy Kit; Qiagen, Valencia, CA).

The BDNF and RCAN1 RT-PCR were prepared respectively as follows. PCR analysis was performed using Primers specific for RCAN1 isoform 1 and GAPDH (for normalization) are using a CFX96 Real Time PCR machine, all according to the manufacturer (BioRad, Hercules, CA, UAS). Primer sequences were described elsewhere for BDNF, RCAN1 and GAPDH [14,15]. These analyses were examined by the ABI PRISM7700 Sequence Detection System.

**Morris water maze test:** The use of Morris water maze test is assessed in the learning and memory of the animals. The Morris water maze comprised a plastic cylindrical pool surrounded by the wall of 45 cm tall with a lucent plastic platform. Mice were subjected to four different objects situated above the edge of this pool. We examined 4 sessions of this trial on each five days. Mice were released from the randomly chosen points from 4 prefixed positions. The escape latency was measured as the time-duration consumed to reach the platform. Experiment was ended soon after the mouse founded the platform or the time after 60 s passed. The averages of each day were calculated for each mouse. At the end of the final experiment on the last 5th day, the mice were subjected to a trial for 60 s with the removal of platform. The examining trajectory was analyzed using SMART as previously mentioned [16].

**Novel object recognition test:** The use of Novel Object Recognition Test has been assessed to investigate a novel object, spending more time exploring than the familiar one. A cuboid-shaped box of plastic (44 × 26 × 20 cm) was used. Mice were allowed to search for 3 min, and taken away from the area. After 5 minutes, two same objects were situated on the bottom 10 cm apart. Animals were situated again into the same area and allowed to search for 3 min. Murine movement was analyzed by Panlab SMART video tracking system. The consuming time was measured within 2 cm from the border of a box (A1 and A2). On the second day at a 1 day interval, mice sought the box for 3 min in the presence of a familiar and a novel different-shaped object. We measured the time spent within the 2 cm neighbour in these objects apart from the familiar object (A3) and novel one (A4). The ratio of discrimination index was defined as  $A4/(A3+A4)$ . The wider discrimination index shows the better retrieval on familiar object experienced 1 day before. This index of 0.5 means no preference for either object. This test is based on the murine habits of spending more time with a novel object than a familiar one [17].

**Forced swimming test:** For forced swimming, we utilized the test paradigm uniquely designed to test rat behaviour rather than the five-day paradigm previously employed to induce depressive behaviour in mice. The time duration of forced swimming were 10 min daily for two con days in a transparent plastic cylinder (24 cm Ø, 60 cm high) filled with water at 25 C in the depth of 25 cm. Data were analysed by ANY-Maze (Stoelting, Wood Dale, IL, USA) [17].

**Statistical analysis:** Data were recorded as mean ± SED. For comparative analysis, the significant differences in body weight, peripheral biochemical parameters, and gene expressions were

calculated using student's t-test. Comparisons among groups in Morris Water test, Novel object cognition, test of force swimming were performed using one-way ANOVA followed by Fisher's least significant difference analysis. A value of  $P < 0.05$  was decided to be statistically significant.

## Results

### Organ weight

Body weight (BW) of the HFD was significantly heavier than that in the NOR (**Table 1**). The BW in the HFE group was significantly normalized compared with that in the HFD group. The brain weight in the HFD was statistically lower than that in the NOR. The right psoas muscle weight/body weight ratio was significantly elevated in the HFE compared with the HFD. Liver weight in the HFD group was predominantly heavier than that in the NOR and normalized in the HFE.

### Laboratory data

Blood sugar levels did not differ among the three groups. In contrast, AST and ALT levels in the HFD were significantly elevated than those in the NOR. The LDL cholesterol levels in the HFD and HFE group were higher than in the NOR group (**Table 2**).

### Histological features

The staining by haematoxylin and eosin showed atrophy the pyknotic neurons in the hippocampus of HFD compared with NOR. Mice in HFE showed reduced pyknotic neurons compared with HFD (**Figure 1**). Histological examination of the skeletal muscle sections revealed the development of fatty changes in the HFD group, but fatty changes were reduced in HFE mice (**Figure 1**).

### Gene expression

The enhanced expression of BDNF was revealed on the hippocampus and skeletal muscle in the HFE group compared with the HFD group. Similarly, BDNF expression in skeletal muscle was significantly higher in the HFD than in the NOR (**Figure 2A**). The expression of RCAN1 in the hippocampus did not differ significantly among the three groups, whereas RCAN1 expression

in skeletal muscle of the HFE was statistically elevated than that of the HFD (**Figure 2B**).

### Morris water maze test

The escape latency for the HFD was statistically significantly decreased than that for the NOR group. Similarly, the results of the Morris water maze test were apparently better for the HFE group than for the HFD group (**Figure 3**).

### Novel object recognition test

The exploration time for familiar and novel objects varied but did not differ significantly among the three groups on Days 1 and 2. However, the recognition index on Day 1 for the HFE group was significantly increased than that for the NOR group (**Figure 4**).

### Forced swimming test

The distance travelled and immobility time in the HFD group were significantly impaired compared to those in the NOR group. Chronic voluntary movements induced the distance travelled and shortened the immobility time (**Figure 5**).

## Discussion

Our findings suggest that chronic voluntary exercise improves cognitive dysfunction and depressive state, with induced the hippocampal expression of BDNF and RCAN1 mRNA, and increased RCAN1 mRNA expression in the skeletal muscle of obese ApoE<sup>-/-</sup> mice. They also showed that chronic voluntary exercise reduces body weight, liver weight, liver enzymes, and lipid levels while increasing brain weight and the muscle weight / body weight ratio compared with non-exercising obese mice.

The cells of central nervous system from ApoE<sup>-/-</sup> mice are more naive to excitotoxic and age-related synaptic loss [18], and synaptic dysfunction induced by A $\beta$  in these mice is also enhanced in the contrast to control mice [5]. The gene dose of APOE  $\epsilon$  4 is a dominant risk factor for AD, with the association to age at onset [19], and cognitive decline [20]. Indeed, the APOE polymorphism is one of the strongest and most robust genetic risk factors for the late-onset of AD.

**Table 1** Effects of chronic voluntary movement on body weight (BW) and organ weights in high fat diet ApoE<sup>-/-</sup> mice.

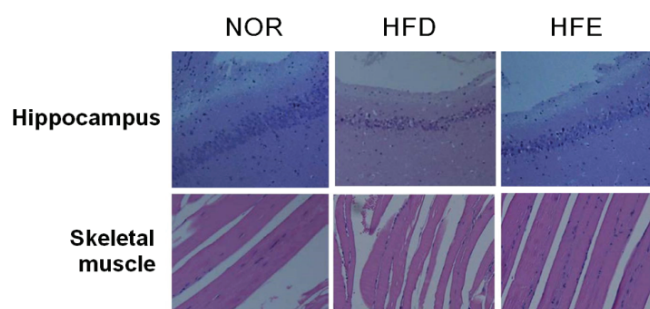
	BW (g)	Brain weight (mg)	Heart weight (mg)	Right iliopsoas muscle weight (mg)	Muscle weight/ BW	Liver weight (mg)	Liver weight /BW ratio (10- 3)
NOR	30.4 $\pm$ 1.6	431 $\pm$ 24	200 $\pm$ 30	264 $\pm$ 24	8.7 $\pm$ 0.6	1460 $\pm$ 330	4.8 $\pm$ 0.95
HFD	49.7 $\pm$ 2.7*	409 $\pm$ 12*	220 $\pm$ 30	244 $\pm$ 24	4.9 $\pm$ 0.8*	3800 $\pm$ 990*	7.65 $\pm$ 1.26*
HFE	36.5 $\pm$ 4.7**	432 $\pm$ 31†	230 $\pm$ 30	308 $\pm$ 30	8.4 $\pm$ 0.7†	2200 $\pm$ 450†	6.03 $\pm$ 1.02

**Abbreviations:** NOR, non-exercised mice without high-fat diet: HFD, ApoE<sup>-/-</sup> mice with high-fat diet: HFE, voluntary exercises were performed in ApoE<sup>-/-</sup> mice with high-fat diet. Statistically significant differences tested by student t. \* $P < 0.05$  vs NOR, † $P < 0.05$  vs HFD.

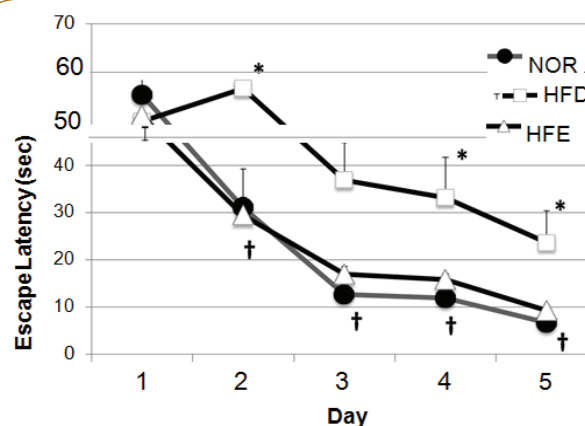
**Table 2** Effects of chronic voluntary movement on Laboratory data in high fat diet ApoE<sup>-/-</sup> mice.

	Blood (mg/dL)	Sugar AST (U/L)	ALT (U/L)	TG (mg/dL)	LDL-C (mg/dL)
NOR	151 $\pm$ 17	557 $\pm$ 103	92 $\pm$ 14	44 $\pm$ 13	78 $\pm$ 22
HFD	145 $\pm$ 20	898 $\pm$ 174*	557 $\pm$ 144*	66 $\pm$ 20	187 $\pm$ 18*
HFE	159 $\pm$ 28	723 $\pm$ 135†	164 $\pm$ 84†	40 $\pm$ 11	150 $\pm$ 24*

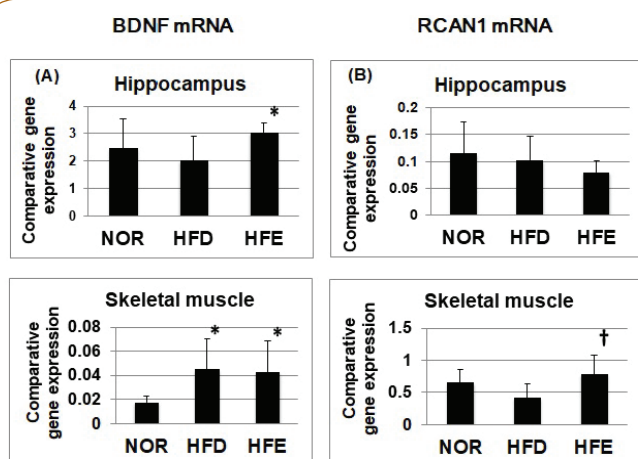
**Abbreviations:** NOR, non-exercised mice without high-fat diet:HFD, ApoE<sup>-/-</sup> mice with high-fat diet: HFE, voluntary exercises were performed in ApoE<sup>-/-</sup> mice with high-fat diet. AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG: triglyceride; LDL-C: low density lipoprotein cholesterol. Statistically significant differences ww test by student t'test. \* $P < 0.05$  vs NOR, † $P < 0.05$  vs HFD.



**Figure 1** Pathological changes of hippocampus and skeletal muscle. Mice of HFD showed neuronal atrophy and psychosis in hippocampus and fatty changes of skeletal muscle. Mice of HFE showed reduced neuronal atrophy and reduced fatty changes of skeletal muscle.



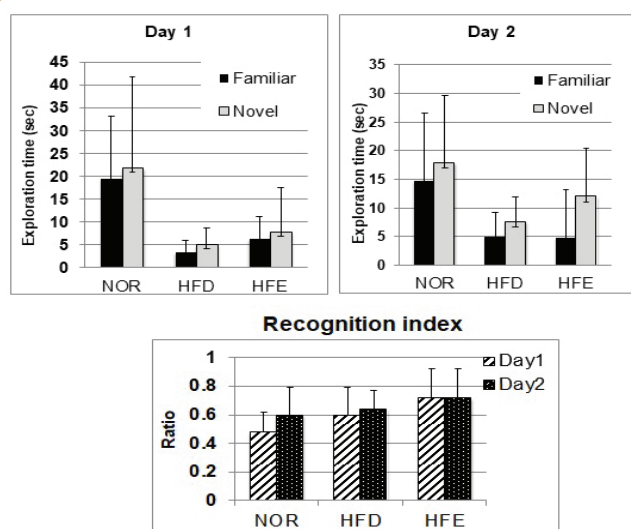
**Figure 3** Chronic voluntary movement significantly elongated the exploration time and the discrimination index on Day2 by Novel object recognition task compared to NOR and HFD. \* $P < 0.05$  vs NOR, † $P < 0.05$  vs HFD.



**Figure 2** Effects of chronic voluntary movement on BDNF and RCAN1 expression in hippocampus and skeletal muscle, (A) Both of BDNF mRNA and RCAN1 mRNA in Hippocampus of HFE were significantly elevated compared to NOR and HFD; (B) BDNF mRNA in skeletal muscle of HFE was significantly higher than NOR, but that was not changed from HFD. RCAN1 mRNA of HFE was significantly elevated from HFD. \* $P < 0.05$  vs NOR, † $P < 0.05$  vs HFD.

Obesity reduces cognitive and motor function and suppresses brain plasticity [21]. The high-fat diet increases oxidative stress in both the brain and in other organs such as skeletal muscle and reduces BDNF, which is known to mediate the effect of obesity on cognition and behavior in the hippocampus [22]. We have shown that chronic voluntary movement reduces obesity and induces BDNF gene expression in the hippocampus and skeletal muscle. The BDNF gene expression induced in skeletal muscle of obese mice could not be explained clearly, although increased oxidative stress in muscle may induce the BDNF gene as an adaptive mechanism.

BDNF has been implied to neural growth and function, including neurogenesis, dendritic degeneration and long-term duration of neuronal function, since animal models provides the constant evidence for exercise-induced expression of BDNF mRNA. Therefore, physical exercise may be a successful strategy for

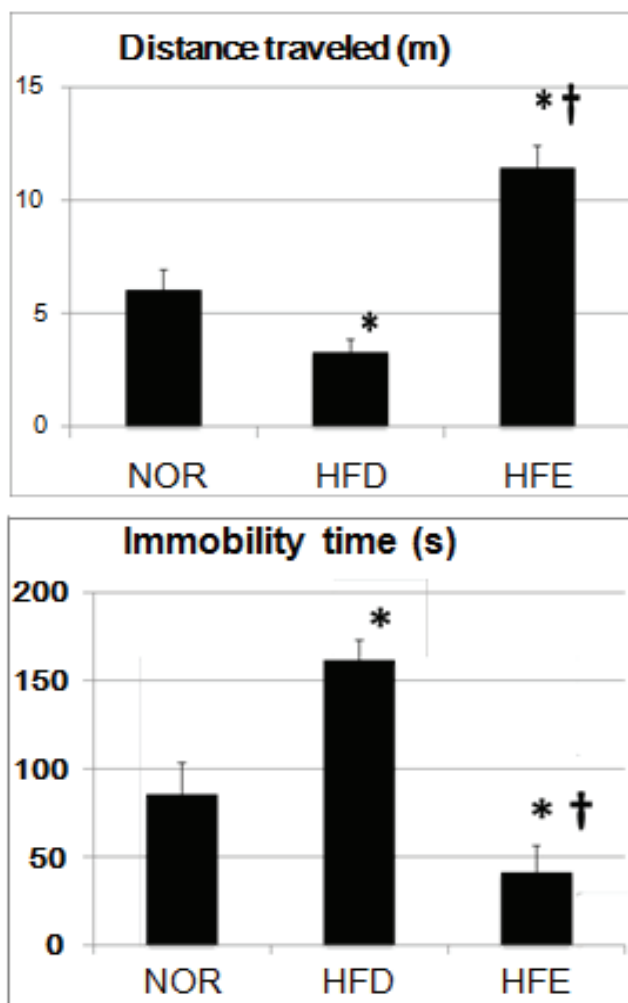


**Figure 4** Chronic voluntary movement significantly elongated the distance travelled, and significantly shortened the immobility time compared to NOR and HFD. \* $P < 0.05$  vs NOR, † $P < 0.05$  vs HFD.

promoting mood or cognition due to its ability to enhance BDNF activity.

Chronic exercise by high intensity alters the induction of BDNF mRNA via the muscle phenotype, reducing the protein levels of BDNF in fast muscles while increasing genetic expressions of BDNF in slow muscles [23]. BDNF is reported to act in an autocrine and paracrine fashion in skeletal muscle for fat oxidation. In the sedentary state, BDNF expression is affected by muscle phenotype, with high-intensity chronic exercise reducing BDNF protein levels in the fast muscles and increasing BDNF mRNA levels in the slow muscles [23]. Muscle-derived BDNF plays an important role in muscle repair, regeneration, and differentiation and our data show that exercise induces BDNF mRNA expression in both the hippocampus and in skeletal muscle, which could have a beneficial effect on cognitive function and anti-depression [24].





**Figure 5** Morris Water Maze Test showed the significant longer escape latency by chronic high fat diet in comparison with mice without high-fat diet and chronic voluntary movement normalized this test. \* $P < 0.05$  vs NOR, † $P < 0.05$  vs HFD.

Elevated RCAN1 levels lasting just several hours can be neuroprotective under acute stress conditions, such as acute oxidative stress. Long-term overexpression of RCAN1 gene may help to understand the mechanism of neuro degeneration in diseases such as AD and Down's syndrome [25]. RCAN1, which is reported to be a physiological modulator of oxidative stress, is regulated in the skeletal muscles after exhaustive physical exercise, which increases RCAN1 protein levels in the gastrocnemius. Indeed, protein oxidation, an index of oxidative stress, has been shown to be increased in the gastrocnemius. Induced expression of RCAN1 may regulate represent important components of the physiological adaptation to exercise-induced oxidative stress [4]. We showed increased expression of RCAN1 in the gastrocnemius skeletal muscle after chronic exercise but no change of RCAN1 gene expression in the hippocampus. Although the role of RCAN1 in the hippocampus after chronic exercise remains unclear, chronic exercise could play an important role in skeletal muscle adaptation in obese animals [26].

Both of Morris water maze test and novel object recognition test were improved by chronic exercise in obese ApoE<sup>-/-</sup> mice [27]. Similarly, chronic exercise was also beneficial in the forced swimming test, as an indicator of depressive condition. The improvement of cognition and mood is associated with BDNF activity. Moreover, chronic consumption of high-fat food and obesity induces plasticity-related changes in reward circuitry that are related with a depressive phenotype, with an alternation in BDNF activity beings implicated in depressive behaviour [5-7]. Chronic exercise in obesity may influence cognition and mood by inducing the expression of BDNF and RCAN1 gene in the hippocampus, and RCAN1 expression in skeletal muscle.

The limitations of this study were the three months period of chronic exercise and the examination of only one skeletal muscle [28]. A longer duration of exercise may alter the gene expression and recognition test results. Similarly, other skeletal muscles may need to be examined to clarify the different effects in slow and fast muscle. As such, further studies are required in this field.

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