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Less stimulating environmental conditions decrease spatial learning ability and reduce GAP-43 and synaptophysin immunostaining in the hippocampal formation in adult male mice

Mitsuru Miyamoto¹, Kozo Sugioka², Takamitsu Arakawa¹, Toshio Kawamata¹, Akinori Miki¹

Environmental conditions affect learning ability and cause neuroanatomical alterations of neurons and synapses in the hippocampal formation. We reared male mice in standard, enriched and wheel-running environments (n = 7 in each group) as well as in an impoverished environment (n = 9) for 102 days and compared their spatial learning ability and histochemical expression of growth-associated phosphoprotein (GAP-43) and synaptophysin. Spatial learning ability, as measured by the Morris water maze, decreased significantly in the impoverished environment group and increased significantly in the enriched environment group. The optical density of GAP-43 immunostaining in the stratum lacunosum-moleculare and mossy fiber layer was significantly lower in the impoverished environment group than in the other 3 groups. GAP-43 immunostaining in the mossy fiber layer was significantly more intense in the enriched environment group. Synaptophysin immunostaining in the mossy fiber layer was significantly less intense in the impoverished environment group than in the enriched and wheel-running groups. These findings suggest that non-social and less stimulating environments can reduce spatial learning ability and impair function of the hippocampal formation.

Key Words
Environmental conditions, spatial learning, synaptic plasticity, hippocampal formation

Introduction

In the past years, many studies have reported that environmental stimulation affects the central nervous system thereby influencing brain plasticity.¹-⁵ The environmental conditions under which animals are reared have been reported to change their performance in several tasks¹,⁵,⁶,⁷ as well as produce a wide range of neuroanatomical alterations in the brain.¹,⁶,⁸,⁹ An enriched environment, consisting of a combination of social or non-social stimulation and physical activity, was reported to enhance the learning/memory and neural consequences.⁶,⁷,¹⁰-¹³ The enriched environment factors lead to an increase in cerebral size¹,⁸,⁹,¹¹, dendritic branching¹³ and density of dendritic spines of the cortex¹³ as well as augmentation of cortical thickness.¹⁰,¹⁴

Studies have demonstrated that long-term potentiation (LTP) activates specific neural activity.¹⁵ In particular, in the hippocampal formation, augmentation of LTP was reported to induce improvement of the spatial learning ability.¹⁶ Compared to mice housed in standard cages, those reared in an enriched environment showed
enhanced LTP in the Schaffer collateral pathway of CA1 hippocampus. Messenger RNA levels of the nerve growth factor were also significantly higher in the visual cortex and hippocampus of rats reared in an enriched environment than those housed individually in single cages without stimulus enrichment. It has also been reported that the cAMP response element binding protein is activated in the brain of mice housed in an enriched environment. These neuroanatomical alterations occur at any age. An elderly rat that was housed in an enriched environment had increased cortical thickness. Middle-aged or elderly female mice reared in an enriched environment had improved spatial learning ability and increased synaptophysin levels in the cortex. Furthermore, an enriched environment stimulates neurogenesis in the dentate gyrus of adult rats.

On the other hand, several studies have reported that physical activity alone enhances brain plasticity in a manner similar to rearing in an enriched environment. In young mice, physical activity alone improved learning ability better than that achieved by rearing in an enriched environment. In addition, running led to a greater increase in heart weight than rearing in an enriched environment, showing that the physiological effect resulting from a higher cardiovascular load was achieved by running. Analyzing the heart weight as an indication of physical activity and investigating whether it is related to spatial learning ability and brain plasticity can help us elucidate whether an enriched environment or physical activity is the most effective promoter of these characteristics.

Although many studies have been conducted to examine the effect of the enriched environment or physical activity, only few reports have focused on the neurological changes in animals reared in an impoverished environment in which the animals are given little stimulation, including few social, material and physical stimulations. It is important to investigate the effect of less stimulating environment, which is considered to lead to negative effects on behaviour such as lower spatial learning, poorer spatial memory and lower brain function, to establish evidence to promote a desirable existence or avoid an undesirable existence for animals.

Analyzing the quantity of synapses is thought to be essential for investigating neuronal plasticity. Growth-associated phosphoprotein (GAP) 43 is a presynaptic membrane protein expressed at high levels in neuronal growth cones during development and axonal regeneration. This protein is considered to play a major role in neurite formation, regeneration and synaptic plasticity. Synaptophysin is also a presynaptic vesicle membrane protein whose increase is indicated by augmentation of synapse density or number, which in turn exhibits sufficient specificity and selectivity for accurate quantitation of synapses. Thus, to examine whether environmental conditions affect synaptic plasticity, we investigated the changes in GAP-43 and synaptophysin in the hippocampal formation caused by several housing environments, including enriched, running, impoverished and control housing environments.

### Materials and Methods

All procedures were approved by the Institutional Animal Care and Use Committee of Kobe University.

#### Housing Conditions

Thirty male C57BL/6 mice (SLC Japan.com; 12 weeks old) were reared for 102 days in 4 different environments. The mice were randomized into 4 groups according to the type of environment: control (CT), enriched (EC), wheel-running (WR) and impoverished (IP) (Fig. 1). CT, EC and WR groups were reared in large cages (width: 740 mm, length: 440 mm, height: 430 mm) with 7 animals per cage. The EC group was housed in a cage containing plastic tunnels of various types and colours. These tunnels covered the entire cage in vertical and horizontal directions, which allowed the mice to search criss-crosswise in the cage. The EC group was allowed to move freely...
Less stimulating environment affects spatial learning and the hippocampal formation.

in the coloured tunnels without limitation, and also use a rotary-type running wheel 180 mm in diameter. In addition, the cage was attached to a huge pipe 2 m in length outside the cage, allowing extra movement. The WR group was also reared in a large cage with free access to 6 connected running wheels (diameter, 160 mm). The CT group was reared in a large cage of the same size but with no equipment. The IP group mice (n = 9) were housed individually in small cages of smaller size (width: 250 mm; length: 180 mm; height: 120 mm) with no equipment, thus depriving them of social and physical stimulation. All groups received food and water freely available in the laboratory room on a 12/12-h light/dark cycle (lights on at 06:00) under their respective experimental conditions. The body weight of each mouse was measured at the start of rearing in each environment and on the 91st day before the Morris water maze task was started.

Morris water maze experiment
The mice were trained on a Morris water maze with 2 trials per day for 10 consecutive days starting from the 91st day of the rearing periods. The diameter of the pool was 60 cm and the depth of the water was 10 cm. Water was conditioned to be maintained at 25°C. The area of the pool was divided into quarters. The maintained escape platform was hidden 1 cm below the surface of the water, which had been made opaque using nontoxic India ink and kept at the centre of the specific quadrant area. The start points to swim were randomly changed among the trials. Each trial was terminated either when the mouse had found the platform or after 60 s had elapsed. The time to reach the platform was recorded as escape latency. Mice that could not find the platform were placed on the platform for 10 s. After completion of the last training trial, the platform was removed for the probe test. In the probe test, each mouse started from the diagonal area of the removed platform area and swam freely for 60 s at one time. In the probe test, the total time for which a mouse stayed in the platform area and the number of crossings of the area of the platform was measured by monitoring using a video camera mounted on the ceiling.

Morphological analysis
After housing in the various environments for 102 days, the mice were anesthetized with sodium pentobarbital (40 mg/kg) and were perfused via the ascending aorta, initially with 0.9% saline

Figure 1. These photographs show the cages that were used in this study to convert the housing environment. A:CT group. B:EC group. C:WR group. D:IP group.
solution and later with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C). Following this, the hearts of the animals in each group were removed. The heart weight was then measured to calculate the heart-to-body weight ratio. The brain of all animals in the 4 groups were removed and postfixed in the same fixative for 48 h, and then embedded in paraffin. The tissue was cut into 7-µm-thick sections throughout the hippocampal formation and dozens of sections were collected onto poly-L-Lysine-coated slides. For immunohistochemistry, adjacent series of sections were incubated with either anti-GAP-43 or anti-synaptophysin. The sections were baked at 60°C for 1 h, and then deparaffinized in xylene and alcohol. To quench endogenous peroxidase activity, the sections were treated with 3% H₂O₂ solution for 10 min and rinsed in Tris-buffered saline (TBS). For GAP-43 immunostaining, the sections were incubated with blocking reagent A (Nichirei Bio Science), and then washed in TBS. The sections were then incubated for 1 h at room temperature with monoclonal anti-GAP-43 (1:1000, Sigma) in diluted buffer and then washed in TBS. Following this, the sections were incubated with blocking reagent B (Nichirei Bio Science) and incubated in simple stain mouse MAX-PO (M) (Nichirei Bio Science) for 10 min. For synaptophysin immunostaining, the sections were incubated for 1 h at room temperature with polyclonal anti-synaptophysin (1:50, Dako) in diluted buffer and then washed in TBS. The sections were then incubated in envision+/HRP for 30 min. Both GAP-43 and synaptophysin sections of the reaction product were visualized by incubating the tissue in a 3, 3-diaminobenzidine tetrahydrochloride liquid system (substrate) containing H₂O₂. After dehydration in alcohol and penetration with xylene, the cover glass was slipped using the mounting medium. These sections were observed using a light microscope.

Semiquantitative analysis of optical density
GAP-43 and synaptophysin immunoreactions were semiquantitatively analyzed as optical density. Ten to twelve left medial sections randomly selected from 4 mice of each group were investigated. Digital images of these sections were taken using the light microscope under the same recording conditions, and converted to gray scale using Adobe Photoshop software (Adobe Systems Incorporated) on a personal computer. Subsequently, optical density measurements were obtained by Image J software (NIH Image), which calculates the average density of immunostaining for each layer of the hippocampal formation divided by white matter region in each same section. The mean value of density measurements was calculated in the stratum oriens, stratum radiatum and stratum lacunosum-moleculare of CA1; the mossy fiber layer of CA3 of the hippocampus and the outer, middle and inner molecular layers of the dentate gyrus.

Statistical analysis
In the Morris water maze experiment, the time to reach the platform (escape latency) and transition of the body weight during the rearing time (before start of the Morris water maze task) were analyzed using 2-way analysis of variance (ANOVA), with session as the within-factor and group as the between factor. In addition, Student’s t-test was used for a probe test within each of 2 groups. Student’s t-test was also used to compare both heart weight or heart-to-body weight ratio and optical density for GAP-43 and synaptophysin in the hippocampal formation within each 2 groups measured.

Results
All mice were housed for 102 days, except for 1 mouse of the EC group that died in a fight with a littermate.

Body weight, heart weight and heart-to-body weight ratio
In the beginning of the experiment (rearing time), there was no difference in the body weight
Less stimulating environment affects spatial learning and the hippocampal formation.

among the groups (Fig. 2A). However, on the
day when the water maze task was started (after
rearing for 91 days), the body weights differed
significantly from that recorded during the rearing
time in all 4 groups \((P < 0.005)\). The mean body
weight of the EC group was significantly lower
than that of CT and IP groups \((P < 0.005\) for both;
Table 1, Fig. 2A). The mean body weight of the
WR group was significantly lower than that of the
IP group \((P < 0.025; \text{Table 1, Fig. 2A})\).

The mean heart weight of the WR group was
higher than that of the EC group \((P < 0.05; \text{Fig.}
2B)\). Furthermore, the heart-to-body weight ratio
of the WR group was higher than that of the EC \((P
< 0.05)\) and IP group \((P < 0.025; \text{Fig. 2C})\).

Morris water maze experiment
(1) Escape latency
During the training period, significant group ef-
facts were observed in all 4 groups \((P < 0.005)\)
and escape latency shortened across training, as
demonstrated by significant session effect in all
groups \((P < 0.05; \text{Table 2; Fig. 3A})\). A significant
session by group interaction for escape latency
appears among all 4 groups \((P < 0.005; \text{Table 2;}
Fig. 3A)\). The EC group showed a significant im-
provement in mean escape latency compared to
the other 3 groups \((P < 0.005 \text{ for WR and IP, } P
< 0.05 \text{ for CT; Table 2, Fig. 3A})\) and the WR and IP
groups exhibited significantly decreased acquisi-
tion of the spatial learning task compared to the
CT group \((P < 0.005 \text{ and } P < 0.05; \text{Table 2, Fig.}
3A)\). However, there was no difference of escape
latency between the WR and IP groups.

(2) Probe test (quadrantal staying times)
The staying time in the target zone area in which
the platform was placed was significantly higher

Table 1. Change in body weight during rearing time before the Morris water maze experiments

<table>
<thead>
<tr>
<th>Day</th>
<th>CT (n=7)</th>
<th>EC (n=6)</th>
<th>WR (n=7)</th>
<th>IP (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25.6 ± 1.13</td>
<td>25.7 ± 0.52</td>
<td>25.9 ± 1.07</td>
<td>25.8 ± 1.46</td>
</tr>
<tr>
<td>91</td>
<td>33.4 ± 2.82</td>
<td>28.8 ± 1.72*</td>
<td>30.6 ± 3.21*</td>
<td>36.4 ± 3.76</td>
</tr>
</tbody>
</table>

The body weight of EC group mice decreased significantly compared to that of CT and IP group mice;
*\(P < 0.005\). The body weight of WR group mice also decreased significantly compared to that of IP group
mice; #\(P < 0.025\).
Table 2. Group means for escape latency during 10 days of Morris water maze experiments

<table>
<thead>
<tr>
<th>Day</th>
<th>CT# ## (n=7)</th>
<th>EC* ** (n=6)</th>
<th>WR (n=7)</th>
<th>IP (n=9)</th>
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<tbody>
<tr>
<td>1</td>
<td>27.6</td>
<td>39.8</td>
<td>33.9</td>
<td>35.7</td>
</tr>
<tr>
<td>2</td>
<td>17.6</td>
<td>18.2</td>
<td>16.6</td>
<td>22.4</td>
</tr>
<tr>
<td>3</td>
<td>12.7</td>
<td>9.6</td>
<td>29.1</td>
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<tr>
<td>4</td>
<td>17.6</td>
<td>13.3</td>
<td>25.4</td>
<td>24.1</td>
</tr>
<tr>
<td>5</td>
<td>19.9</td>
<td>8.1</td>
<td>22.9</td>
<td>25.9</td>
</tr>
<tr>
<td>6</td>
<td>18.4</td>
<td>8.8</td>
<td>23.6</td>
<td>26.1</td>
</tr>
<tr>
<td>7</td>
<td>16.6</td>
<td>10.2</td>
<td>18.1</td>
<td>33.8</td>
</tr>
<tr>
<td>8</td>
<td>14.6</td>
<td>10.4</td>
<td>20.8</td>
<td>23.8</td>
</tr>
<tr>
<td>9</td>
<td>6.0</td>
<td>5.2</td>
<td>23.1</td>
<td>18.1</td>
</tr>
<tr>
<td>10</td>
<td>10.1</td>
<td>7.0</td>
<td>18.1</td>
<td>18.1</td>
</tr>
</tbody>
</table>

*P < 0.05, comparing CT group; ** P < 0.005, comparing WR and IP groups; #P < 0.05, comparing IP group; ##P < 0.005, comparing WR group; 2-way repeated measures analysis of variance (ANOVA).

Table 3. Optical density values of GAP-43 immunostaining

<table>
<thead>
<tr>
<th>Region</th>
<th>CT (n=4)</th>
<th>EC (n=4)</th>
<th>WR (n=4)</th>
<th>IP (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>101.4 ± 6.4</td>
<td>103.5 ± 6.4</td>
<td>101.6 ± 4.9</td>
<td>101.3 ± 4.1</td>
</tr>
<tr>
<td>R</td>
<td>103.2 ± 4.0</td>
<td>104.1 ± 3.7</td>
<td>102.7 ± 5.2</td>
<td>102.9 ± 4.7</td>
</tr>
<tr>
<td>LM</td>
<td>118.1 ± 2.0</td>
<td>120.4 ± 8.0</td>
<td>119.8 ± 6.1</td>
<td>108.6 ± 4.7*</td>
</tr>
<tr>
<td>mf</td>
<td>103.0 ± 2.6</td>
<td>112.9 ± 7.8#</td>
<td>106.2 ± 3.8</td>
<td>98.9 ± 2.2*</td>
</tr>
<tr>
<td>iml</td>
<td>127.3 ± 4.7</td>
<td>125.5 ± 5.7</td>
<td>124.4 ± 4.4</td>
<td>123.3 ± 5.4</td>
</tr>
<tr>
<td>mml</td>
<td>101.7 ± 4.4</td>
<td>102.8 ± 3.5</td>
<td>101.5 ± 4.6</td>
<td>100.0 ± 3.9</td>
</tr>
<tr>
<td>oml</td>
<td>100.0 ± 2.1</td>
<td>102.2 ± 3.4</td>
<td>100.0 ± 3.7</td>
<td>99.4 ± 4.8</td>
</tr>
</tbody>
</table>

Optical density values represent (×100).

The IP group shows a significant decrease in immunostaining compared to the other 3 groups in LM and mf; *P < 0.05. The EC group also shows a significant increase in immunostaining compared to the CT group in mf; #P < 0.05.

Table 4. Optical density values of the synaptophysin immunostaining

<table>
<thead>
<tr>
<th>Region</th>
<th>CT (n=4)</th>
<th>EC (n=4)</th>
<th>WR (n=4)</th>
<th>IP (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>154.7 ± 13.5</td>
<td>161.3 ± 11.3</td>
<td>156.8 ± 14.8</td>
<td>159.8 ± 10.9</td>
</tr>
<tr>
<td>R</td>
<td>153.2 ± 12.7</td>
<td>152.0 ± 13.4</td>
<td>153.7 ± 12.4</td>
<td>151.3 ± 9.3</td>
</tr>
<tr>
<td>LM</td>
<td>113.6 ± 7.5</td>
<td>117.3 ± 8.9</td>
<td>110.0 ± 9.3</td>
<td>115.7 ± 7.7</td>
</tr>
<tr>
<td>mf</td>
<td>167.2 ± 11.1</td>
<td>180.6 ± 6.7#</td>
<td>175.8 ± 14.8</td>
<td>157.3 ± 9.6* **</td>
</tr>
<tr>
<td>iml</td>
<td>141.4 ± 12.4</td>
<td>133.2 ± 12.6</td>
<td>143.2 ± 12.9</td>
<td>142.5 ± 13.9</td>
</tr>
<tr>
<td>mml</td>
<td>123.0 ± 3.0</td>
<td>118.8 ± 8.6</td>
<td>123.5 ± 7.2</td>
<td>126.1 ± 10.2</td>
</tr>
<tr>
<td>oml</td>
<td>137.3 ± 6.2</td>
<td>135.7 ± 10.4</td>
<td>138.3 ± 10.9</td>
<td>136.9 ± 10.0</td>
</tr>
</tbody>
</table>

Optical density values represent (×100).

The densities of mf of the IP group decreased significantly compared to that of EC and WR groups; *P < 0.01, **P < 0.05. In addition, the densities of mf of the EC group show a significant increase in this immunostaining compared with that of the CT group; #P < 0.05.

for the WR group than that for the IP group (P < 0.05; Fig. 3B).

(3) Probe test (crossing times)

No difference was found among the 4 groups (Fig. 3C).

Morphological analysis

GAP-43 immunoreactivity that appeared in neuropil areas showed different intensity of staining in each layer of the hippocampal formation (Fig. 4A). In general, the stratum lacunosum-moleculare of CA1 of the hippocampus and the inner molecular layer of the dentate gyrus
Less stimulating environment affects spatial learning and the hippocampal formation.

showed higher intensity of immunostaining (Fig. 4A). However, the stratum oriens and stratum radiatum of CA1, the middle as well as the outer molecular layers of the dentate gyrus, including the mossy fiber layer of CA3, showed less intense immunoreactive staining patterns (Fig. 4A). The immunoreactive staining pattern of the stratum lacunosum-moleculare and mossy fiber layer in

Figure 3. These graphs show the escape latency (A), staying time in the target zone (B) and crossing time of the borderlines of target quarters (C) in the probe test of the Morris water maze task.

Figure 4. These photographs show the GAP-43 immunoreactivity in the coronal sections of the hippocampal formation of each of the 4 groups.

the IP group was less intense compared to that of the other 3 groups \((P < 0.05; \text{Table 3, Fig. 4B–I})\). The mossy fiber layer of the EC group showed an increase in immunostaining compared to that of the CT group \((P < 0.05; \text{Table 3, Fig. 4F, G})\).

Synaptophysin immunoreactivity was generally intensely stained compared to the GAP-43 which appeared in neuropil areas, but immunoreactivity was not appeared in the white matter region, pyramidal and granular layers similar with GAP-43 (Fig. 5A). However, the immunostaining pattern of synaptophysin was highly intense and different from that of GAP-43 in the stratum oriens and stratum radiatum of CA1 and outer molecular
layers of the dentate gyrus (Fig. 5A). In addition, the mossy fiber layer showed a moderately dense staining pattern, while the stratum lacunosum-moleculare of CA1–3 and the middle molecular layers of the dentate gyrus showed a light staining pattern, indicating less immunoreactivity in these regions (Fig. 5A). The mossy fiber layer showed a significant decrease in synaptophysin immunostaining in the IP group compared to that in EC ($P < 0.01$) and WR ($P < 0.05$) groups (Table 4, Fig. 5G–I). Moreover, the mossy fiber layer showed increased synaptophysin immunostaining in the EC group compared to that in the CT group ($P < 0.05$; Table 4, Fig. 5F, G).

The results of this study highlight that impoverished environment has a negative effect on the spatial learning ability and brain plasticity of mice.

**Discussion**

Many studies have evaluated the influence of the rearing environment on memory function in various learning tasks. An enriched environment that gives complicated stimulations has been reported as one of the most effective approaches for improving memory function. In the present study, the EC group showed shortening of escape latency compared to the other 3 groups (Table 2, Fig. 3A). In addition, this group showed a significantly low heart weight and heart-to-body weight ratio than the WR group (Figs. 2B, C). This means that the EC group could not engage in highly physical activities unlike the WR group. Thus, in spite of the low impact of physical activity, the animals of the EC group could reach the platform faster in the Morris water maze. In addition, the optical density of GAP-43 and synaptophysin in the EC group showed increased immunoreactivity in the mossy fiber layer of CA3 in the hippocampus (Figs. 4, 5). The mossy fibers originate from the granular cells in the dentate gyrus and terminate in the stratum lucidum in CA3. The stratum lucidum in CA3 is the proximal part of the apical dendrite of the pyramidal cell, and the mossy fiber terminates in this proximal part of the apical dendrite. Further, CA3 pyramidal cell projects Schaffer collaterals to CA1 to project CA1 pyramidal cells, which then terminates in the subiculum and entorhinal area. These synaptic connections play the role of parallel excitatory pathways in the hippocampal formation as the trisynaptic circuit. The mossy fiber’s activation has also been associated with spatial learning, and mossy fiber synaptogenesis can be related to spatial long-term memory formation. Thus, the granule cells of the dentate gyrus play an important role in spatial learning, i.e., in sending the impulse to the pyramidal cells of the CA3 region. Therefore, in the present study, the spatial
memory of animals of the EC group improved by augmentation of the neural network of the hippocampal formation.

**Physical activity such as running has also been** reported to promote improvement of spatial learning\(^7\,16\,22\,23\) as well as cell proliferation\(^22\), neurogenesis\(^16\,22\,23\) and augmentation of LTP\(^16\), GAP-43 and synaptophysin expression\(^34\) in the hippocampal formation. However, when the animals were tested on a T-maze spatial memory task, rats housed in the cage containing only a running wheel had lower spatial learning ability compared to rats housed in an enriched environment.\(^35\) In this study, the animals of the WR group did not show any improvement in spatial learning (Fig. 3A). Furthermore, the WR group did not have enhanced optical density in any layer of the hippocampal formation as measured by immunostaining for GAP-43 and synaptophysin (Tables 3, 4, Figs. 4, 5). The heart-to-body weight ratio of the WR group was also significantly higher than that of EC and IP groups (Fig. 2C). These findings suggest that exercise load may cause cardiac hypertrophy in the WR group, but it is not effective for improving spatial learning ability and or causing neurochemical alternation in the hippocampal formation. Thus, our results suggest that physical activity alone cannot produce improvement of spatial learning and function of the hippocampal formation. The reasons for these discrepancies between previous studies and our results are unclear, although similar to these previous studies, our study also allowed voluntary running. Not all exercise studies suggest that running activity improves spatial learning ability.\(^35\,36\) Therefore, the degree of learning improvement might be higher following rearing in an enriched environment than following exercise alone, although it is not clear which is more effective for spatial learning ability and brain plasticity among them. However, direct comparisons of the spatial learning task and neurochemical changes between running and enriched groups are needed to obtain definite conclusions.

Here we examined the influence of a less stimulating environment by creating the IP group. In the present study, IP group mice took a significantly longer time to reach the hidden platform than EC and CT group mice (Table 2, Fig. 3A), suggesting that IP group mice have decreased ability for spatial learning. The IP group mice also had a lower staying time in the target quarter zone in the probe test than the WR group mice (Fig. 3B), which also suggested that IP group mice had reduced spatial memory ability. These findings indicate that lack of not only physical activity and environmental stimulation but also lack of social interaction significantly interferes with learning abilities. Previous studies have revealed that rearing in an impoverished environment, wherein the animals were housed in a small cage without littermates or any instruments, led to emotionality in male rat pups\(^37\) and impaired spatial working memory and metabotropic glutamate receptor 5 expression in the prefrontal cortex in young male rats.\(^38\) In the present study, the optical densities of GAP-43 immunostaining in the stratum lacunosum-moleculare and mossy fiber layer and of synaptophysin immunostaining in the mossy fiber layer showed a decrease in the IP group compared to that in the other 3 groups (Tables 3, 4 and Figs. 4, 5). The stratum lacunosum-moleculare is the distal part of the apical dendrite of the pyramidal cells of the CA1 region.\(^39\) The basal dendrite of this cell was terminated from the entorhinal area, as the perforant path.\(^39\) These fibers were reported to be related to LTP.\(^40\,41\) As mentioned previously, the pyramidal cells of the CA3 region and mossy fiber have an important role in the spatial learning\(^32\), and furthermore, applying a psychological stressor by restraint is reported to induce atrophy of the apical dendrites of hippocampal CA3 pyramidal neurons.\(^42\) Thus, the animals of the IP group have impaired function of the essential area for spatial learning. Our results indicate that less social, material and physical stimulation environment markedly inhibited learning abilities. Therefore, an impoverished environment that includes less social, material and physical stimulations causes an undesirable organic effect on the
hippocampal formation, and this effect in turn impairs spatial learning ability and spatial memory. These data might provide evidence that avoiding non-social, immaterial and less physical activities and providing complicated circumstances are important not only for the maintenance of the levels of social activities such as learning and memory but also for the preservation of organic levels of the brain.

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Abbreviations list for the Tables and Figures
CT, control; EC, enriched environment; Gr, granular layer; iml, inner molecular layer; IP, impoverished environment; LM, stratum lacunosum-moleculare; mf, mossy fiber layer; mml, middle molecular layer; O, stratum orions; oml, outer molecular layer; P, stratum pyramidale; Po, polymorphic layer; R, stratum radiatum; W, white matter; WR, wheel running.

Figure legends:
Figure 1: These photographs show the cages that were used in this study to convert the housing environment. A: This large cage with no equipment was used to rear the animals of the CT group. B: This large cage containing different types of variously coloured plastic tunnels was used to house the EC group. C: This large cage with 6 running wheels was used to rear the WR group. D: These single small cages with no equipment were used to rear the animals of the IP group.

Figure 2: Change in body weight (A), heart weight (B) of mice of each of the 4 groups after rearing for 102 days, and the heart- to-body weight ratio of each group (C). A: In the beginning of rearing and on the 91st day after the Morris water maze experiment was performed. On the 91st day, the mice of the EC group had significantly low body weight than that of the CT and IP groups; *P < 0.005. On the 91st day, the WR group also had significantly lower values than the IP group; #P < 0.025. B: The mean values of the heart weight from the mice of each of the 4 groups after rearing for 102 days. The heart weight of the WR group is significantly higher than that of the EC group; #P < 0.05. C: The heart- to-body weight ratio of each group. The heart-to-body weight ratio of the WR group has a higher value than that of the EC group; *P < 0.05 and that of the IP group; **P < 0.025.

Figure 3: The escape latency (A), staying time in the target zone (B) and crossing time of the borderlines of target quarters (C) in the probe test of the Morris water maze task. A: After starting this test, escape latency to reach the hidden platform decreased steeply in the EC group compared to the other groups at the 4-day point. After 10 consecutive days, the EC group showed significantly improved spatial learning ability compared to the other 3 groups. No difference was found between the WR and IP groups. *P < 0.05, comparing CT group; **P < 0.005, comparing WR and IP groups; #P < 0.05, comparing IP group; ##P < 0.005, comparing WR group; 2-way repeated measures analysis of variance (ANOVA). After training in the Morris water maze, the animals’ staying time in the target zone (B) and crossing time of the borderlines of target quarters (C) in the probe test were measured. B: In the probe test, the WR group had significantly longer staying time in the target zone area than that of the IP group (P < 0.05). No difference was found among the other 3 groups. C: For crossing times of the borderlines of quarters, there was no difference among each of the 4 groups.

Figure 4: These photographs show the GAP-43 immunoreactivity in the coronal sections of the hippocampal formation. A: The whole hippocampal formation of the CT group. LM of CA1 and iml show the most intense immunostaining, although O, R, oml, mml, Po and mf show lighter staining or less immunoreaction. The region of W, P and Gr do not show any immunoreactions. B–E: The immunostaining in the CA1 area and the dentate gyrus in each 4 groups. Immunostaining levels of LM in the IP group (E) appear to be lower than other 3 groups. F–I: The immunostaining in the CA3 area in each of the 4 groups. The mf of the IP group (I) shows significantly lower levels in GAP-43 immunostaining than that
of the other 3 groups. Additionally, mf of the EC group (G) shows increased immunostaining compared with that of the CT group (F).

Figure 5: This photograph shows the synaptophysin immunoreactivity pattern in the coronal sections of the hippocampal formation. A: The CT group. The intensive immunostaining appear in the O, R, oml, iml and Po. Moderately dense staining in visible mf and light staining in LM and mml. The W, P and Gr regions show immunoreactions similar to that of GAP-43. The immunostaining in the CA1 area and dentate gyrus (B–E) and CA3 (F–I) in each of the 4 groups. The mf of the IP group (I) show significantly lower level of synaptophysin immunostaining than the other 3 groups. In addition, mf of the EC group mice (G) shows increased synaptophysin immunostaining compared to that of the CT group mice (F).