Effects of Memantine, an N-Methyl-d-aspartate Receptor Antagonist, on Fatigue and Neuronal Brain Damage in a Rat Model of Combined (Physical and Mental) Fatigue

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Most of the fatigue in everyday life is a combination of physical and mental fatigue. Recently, an animal model of combined fatigue was designed by housing rats in a cage filled with water. We have previously hypothesized that mental fatigue is caused partly by neuronal brain damage through the activation of N-methyl-d-aspartate (NMDA) receptors by quinolinic acid (QUIN), a metabolite of tryptophan (TRP). Therefore, we investigated whether the same mechanism also participates in combined fatigue. Rats were housed for 5 d under water-immersed conditions, and the extent of fatigue was evaluated by a weight-loaded forced swimming test. The swimming time of the water-immersed group was shorter than that of the control group, indicating that rats were fatigued by water-immersion. However, unexpectedly, the blood and brain levels of QUIN in the water-immersed group were lower than those of the control group. QUIN levels in both the blood and brains of a food-restricted nonimmersed group, where body weight was matched with the water-immersed group, were also decreased, suggesting that decreased QUIN in the water-immersed group originated from a reduced intake of TRP-containing food. On the other hand, hippocampal neuronal damage was shown in the water-immersed group, similar to that seen in other fatigue models where QUIN increased. Memantine, an NMDA receptor antagonist, inhibited not only the reduction in swimming times but also neuronal brain damage by an endogenous NMDA receptor agonist other than QUIN participates in combined fatigue by water immersion.

Key words combined fatigue; water-immersion; neuronal brain damage; memantine; N-methyl-d-aspartate receptor

Fatigue is roughly classified into physical and mental fatigue. It is generally accepted that physical fatigue mainly arises from a disorder of energy metabolism in muscle (not limited to lactic acid accumulation) caused by long-duration or intensive exercise.1,2) On the other hand, the detailed mechanisms underlying mental fatigue have not been clarified, although various psychological factors such as stress or insomnia participate in it. Most of the fatigue experienced in everyday life is a combination of physical and mental fatigue. However, no appropriate animal models have been available to study combined fatigue so far. In recent years, Watanabe and co-workers designed an animal model of combined fatigue by housing rats in a cage filled with water for several days.3–7) Under such conditions, rats cannot rest or sleep, and for a long period, under an air-conditioned dark (lights on from 8:00 until 20:00), with unrestricted access to water and food. This study was approved by the Animal Experiment Committee of Kampo Research Laboratories, Kracie Pharma, Ltd. (Takaoka, Japan), in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Animals Male Sprague-Dawley rats 6 weeks of age were purchased from Japan SLC Inc. (Hamamatsu, Japan). The animals were used in experiments after 1 week of acclimation and were housed in an air-conditioned room (23±2°C, 55±10% humidity) under a 12-h dark/12-h light cycle (lights on from 8:00 until 20:00), with unrestricted access to water and food. This study was approved by the Animal Experiment Committee of Kampo Research Laboratories, Kracie Pharma, Ltd. (Takaoka, Japan), in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Fatigue Model Induced by Water-Immersion Fatigue was induced by housing the rats under water-immersed conditions according to the methods of Tanaka et al.3,6) Rats were housed individually for 5 d in a plastic cage (27×44×20 cm; Toyo-riko Co., Ltd., Tokyo, Japan) filled with 23°C water to a height of 1.5 cm. The water was changed every day. For the control group, rats were housed individually for 5 d in a plastic cage of the same size, and the floor was covered with wood shavings and paper (Soft-chip™ and Paper-clean™, Japan SLC Inc.). In both cases, rats were allowed free access to water and food (normal pellet diet; CE-2; Clea Japan, Tokyo, Japan).
Japan), except for a food-restricted group for which food was supplied by 15 g/rat·d.

**Evaluation of Extent of Fatigue** After the rats were housed for 5 d, the extent of fatigue was evaluated by a weight-loaded forced swimming test. Steel wires that weighed 8% of body weight were attached to the tails of the rats, and the rats were forced to swim in a bucket filled with 23°C water. Swimming times, defined as the time from the start of swimming until the rats were exhausted and sank below the surface of the water for 10 s, were measured. Blood was collected from the orbital plexus under ether anesthesia 1 h before the start of swimming, and the serum QUIN levels were measured using GC/MS. Data are represented as mean±S.E. of 9 rats. ***p<0.001, significantly different from the control group (Student’s t-test).

**Measurement of Serum and Brain QUIN Levels** The serum QUIN levels were measured according to the methods of Yu et al.13) using GC/MS (GC/MS-QP2010; Shimadzu Corporation, Kyoto, Japan) after the serum was appropriately diluted with pure water. For the measurement of brain QUIN levels, the cerebral cortex was dissected from the brain on an ice-cold dish, and 10 mg was weighed out. Twenty-five volumes (W/V) of pure water were added to it, and the mixture was suspended by sonication. Subsequently, QUIN concentrations in the sample were measured using the GC/MS method described above, and the brain QUIN level was calculated from the resulting value.

**Histological Observation of Hippocampal Neurons** Rats were anesthetized with ether, and then the brain was perfused with 90 mL of 0.1 M phosphate buffer (pH 7.0) followed by 90 mL of 4% paraformaldehyde solution from the left ventricle of the heart. Subsequently, the brain was removed and fixed by immersion in 4% paraformaldehyde solution for over 24 h followed by embedding in paraffin. The paraffin block was sliced into 4-μm-thick sections, and the slices were treated with cresyl violet (Nissl) staining. Using these histological preparations, the number of pyramidal neurons with a clear cell body in a 50×100 μm area of the CA1 and CA3 regions of the hippocampus was measured microscopically, and the cell number per 1 mm³ was calculated. The cell number was measured at 3 areas for each specimen, and the mean value was calculated.

**Statistical Analysis** The results were expressed as the mean±S.E. Statistical differences were assessed using the
Student’s t-test (comparison between two groups) or the Tukey-Kramer test (comparison between each pair of groups). StatView Version 5.0 software (SAS Institute Japan Ltd., Tokyo, Japan) was used for the analysis, and the difference was judged significant when the p value was less than 0.05 ($p<0.05$).

RESULTS

Influence of Water-Immersion on the Extent of Fatigue and the Levels of Serum QUIN

At first, the influence of water-immersion on the extent of fatigue and the levels of serum QUIN were investigated after the rats were housed for 5d. The body weight of the water-immersed group, which did not increase during the 5-d housing period, was significantly lower than that of the control group (Fig. 1A). The swimming time of the water-immersed group, which was about 50% that of the control group, was significantly shorter than that of the control group (Fig. 1B).

Blood was collected from the orbital plexus under ether anesthesia 1h before the start of swimming, and the serum QUIN levels were measured. The serum QUIN level of the water-immersed group, which was about 50% that of the control group, was significantly lower than that of the control group (Fig. 1C).

Influence of Food-Restriction on the Extent of Fatigue and the Levels of Serum and Brain QUIN

As described above, body weight gain in the water-immersed group was suppressed during the 5-d housing period. It was thought that the reason for the suppression of body weight gain was that food intake was reduced during water-immersion, which could lead to decreased energy production and therefore decreased swimming time in the water-immersed group. Therefore, we...
The extent of fatigue was evaluated by a swimming test as described in Fig. 1. Data are represented as mean ± S.E. of 6 rats. *p<0.05, **p<0.01, significantly different from the control group; #p<0.05, significantly different from the MEM-nontreated group (Tukey-Kramer test).

**Fig. 5. Effect of MEM on Body Weight and Fatigue in Water-Immersed Rats**

Rats were housed individually for 5 d in a plastic cage filled with 23°C water to a height of 1.5 cm. Control rats were housed individually for 5 d in a plastic cage in which the floor was covered with wood shavings and paper. MEM was administered intraperitoneally to water-immersed rats at either 5 or 10 mg/kg doses once a day during the 5-d housing period. The rats in the MEM-nontreated immersed group were injected with saline instead of MEM. After the rats were housed for 5 d, the extent of fatigue was evaluated by a swimming test as described in Fig. 1. Data are represented as mean ± S.E. of 6 rats. *p<0.05, **p<0.01, significantly different from the control group; #p<0.05, significantly different from the MEM-nontreated immersed group (Tukey-Kramer test).

**Fig. 6. Effect of MEM on Hippocampal Neurons in Water-Immersed Rats**

Rats were housed individually for 5 d in a plastic cage filled with 23°C water to a height of 1.5 cm. Control rats were housed individually for 5 d in a plastic cage in which the floor was covered with wood shavings and paper. MEM was administered intraperitoneally to water-immersed rats at 10 mg/kg once a day during the 5-d housing period. The rats in the MEM-nontreated immersed group were injected with saline instead of MEM. After the rats were housed for 5 d, the brains were removed without swimming, and the number of pyramidal neurons in the CA1 and CA3 regions of the hippocampus was measured in the same manner as described in Fig. 4. Data are represented as mean ± S.E. of 6 rats. **p<0.01, significantly different from the control group; *p<0.05, **p<0.01, significantly different from the MEM-nontreated immersed group (Tukey-Kramer test).

next evaluated various parameters of a food-restricted nonimmersed group for which body weight was matched with the water-immersed group.

The food intake of the control group was about 24 g/rat-d. When the food supply was restricted to 15 g/rat-d, the body weight of the food-restricted group was well matched with that of the water-immersed group (Fig. 2A). The swimming time of the food-restricted group was almost equal to that of the control group. However, the swimming time of the water-immersed group, which was about 59% that of the control group, was significantly shorter than that of both the control and food-restricted groups (Fig. 2B).

Another experiment was performed to determine the serum and brain QUIN levels of the control, food-restricted and water-immersed groups without swimming after the 5-d housing. The serum QUIN levels of the food-restricted and water-immersed groups, which were about 58% and 56% that of the control group, respectively, were significantly lower than that of the control group. However, no differences were observed between the serum QUIN level of the food-restricted group and that of the water-immersed group (Fig. 3A). Similarly, the brain QUIN levels of the food-restricted and water-immersed groups, which were about 89% and 86% that of the control group, respectively, tended to be lower than that of the control group. However, no differences were observed between the brain QUIN level of the food-restricted group and that of the water-immersed group (Fig. 3B).

**Influence of Food-Restriction and Water-Immersion on Hippocampal Neurons**

Pyramidal neurons in the CA1 and CA3 regions of the hippocampus of control, food-restricted and water-immersed groups were observed without swimming after the 5-d housing. The numbers of pyramidal neurons in the CA1 and CA3 regions of the food-restricted group were almost equal to those of the control group. However, the numbers of pyramidal neurons in the CA1 and CA3 regions of the water-immersed group, which were about 65% and 71% of the values of the control group, respectively, were significantly lower than those of the control and food-restricted groups (Fig. 4; upper). The typical appearance of the CA1 and CA3 regions of the hippocampus is shown (Fig. 4; lower). A reduction in the width of the pyramidal layer, accompanied by the decrease in the number of neuronal cells, is clearly seen in the water-immersed group.

**Effect of MEM on Fatigue and Hippocampal Neurons in Water-Immersed Rats**

The weight of the water-immersed group given MEM at 5 mg/kg was increased to about 123% of that of the MEM-nontreated immersed group; however, this effect was not significant. The swimming time of the water-immersed group given MEM at 10 mg/kg was about 153% of and significantly longer than that of the MEM-nontreated immersed group (Fig. 5; upper).

The swimming time of the water-immersed group given MEM at 5 mg/kg was increased to about 123% of that of the MEM-nontreated immersed group; however, this effect was not significant. The swimming time of the water-immersed group given MEM at 10 mg/kg was about 153% of and significantly longer than that of the MEM-nontreated immersed group (Fig. 5; lower).

Pyramidal neurons in the CA1 and CA3 regions of the hippocampus of the water-immersed group given MEM at 10 mg/kg were observed without swimming after the 5-d housing. The numbers of pyramidal neurons in the CA1 and CA3 regions of the water-immersed group given MEM at 10 mg/kg were about 116% and 117% of those of the MEM-nontreated immersed group, respectively; they were significantly higher than those of the MEM-nontreated immersed group (Fig. 6).

**DISCUSSION**

We had previously suggested that QUIN, an endogenous NMDA receptor agonist, which participates not only in the causes of mental fatigue but also in those of the sensation of fatigue after exercise. Therefore, in the present study, we investigated whether QUIN also participates in causing combined (physical and mental) fatigue using an animal model in which rats were housed under water-immersed conditions. The swimming time of the water-immersed group was shorter than
that of the control group after the 5-d housing, indicating that rats were fatigued by water-immersion. However, unexpectedly, the serum QUIN level of the water-immersed group was lower than that of the control group.

The body weight of the water-immersed group did not increase during the 5-d housing period. It is thought that the reason for this suppression of body weight gain is that food intake was decreased by water-immersion. The decrease in food intake leads to the reduction in energy production, and, consequently, the swimming time may have been reduced in the water-immersed group by the energy shortage because swimming time almost entirely depends on physical endurance. Therefore, we evaluated various parameters of a food-restricted nonimmersed group for which body weight was matched with the water-immersed group. As a result, no differences in the swimming time were observed between the control group and the food-restricted group, although the body weight of the food-restricted group was significantly lower than that of the control group. On the other hand, the swimming time of the water-immersed group, the body weight of which was equal to that of the food-restricted group, was significantly shorter than that of the food-restricted group. These results are consistent with those of Tanaka et al. and Jin et al. who compared the swimming times of these three groups by the same method. Therefore, it is thought that the energy shortage caused by decreased food intake does not contribute to the reduction in swimming time in the water-immersed group.

The serum QUIN levels of the food-restricted and the water-immersed groups were significantly lower than those of the control group. Similarly, the brain QUIN levels of the food-restricted and the water-immersed groups tended to be lower than those of the control group. However, neither the serum QUIN levels nor the brain QUIN levels were different between the food-restricted group and the water-immersed group. These results suggest that the decrease in serum and brain QUIN levels in the water-immersed group resulted from the decreased intake of TRP-containing food. This is supported by the results of Jin et al. who confirmed that the blood TRP levels were significantly decreased in food-restricted and water-immersed groups compared with a control group after a 5-d housing. On the other hand, the swimming time of the food-restricted group was not different from that of the control group. Taken together, it is thought that some factors other than energy shortage or QUIN cause fatigue in water-immersed rats.

In our previous study, hippocampal neuronal damage as well as the increase in blood and brain QUIN levels was shown concomitantly with fatigue-like symptoms in LPS-treated mice. On the other hand, MEM, which is a low affinity and uncompetitive but selective NMDA receptor antagonist, counteracted not only the development of fatigue-like symptoms but also the neuronal damage induced by LPS without affecting the increase in blood and brain QUIN levels. These findings suggest that increased QUIN and subsequent neuronal damage through the activation of NMDA receptors causes fatigue in LPS-treated mice. Therefore, in the present study, it was worth investigating the relationship between fatigue and neuronal brain damage in water-immersed rats, although QUIN did not increase in these rats. We found that the number of hippocampal neurons in the water-immersed group was significantly decreased compared with that of the control and food-restricted groups after the 5-d housing. However, MEM at 10 mg/kg significantly inhibited hippocampal neuronal damage and counteracted the reduction in swimming time in water-immersed rats. These results suggest that neuronal brain damage participates in the development of fatigue in water-immersed rats.

As described above, the serum and brain QUIN levels of the water-immersed group were decreased compared with those of the control group. These facts indicate that the neuronal damage in the water-immersed rats is not caused through the activation of NMDA receptors by QUIN. However, the neuronal damage in the water-immersed rats was inhibited by MEM. Particularly, MEM significantly inhibited such neuronal damage at 10 mg/kg, that is the dose being effective to block NMDA receptors. This implicates an endogenous NMDA receptor agonist other than QUIN, such as D-serine, in the neuronal damage. It has been reported that the blood levels of L-serine, a biological precursor of D-serine, are significantly higher in food-restricted rats but lower in water-immersed rats than in control rats, implying that L-serine is transformed into NMDA-receptor-active D-serine by water-immersion in the rat. Moreover, the glucocorticoid stress hormones may have caused neuronal brain damage because water-immersion strongly induces stress. Glucocorticoids are known to cause hippocampal atrophy or neuronal damage through an NMDA receptor-mediated mechanism. Glucocorticoids also reportedly increase the number or function of NMDA receptors. In the present study, the serum corticosterone level of the water-immersed group (without swimming) was significantly elevated compared to that of the control group after the 5-d housing (control: 55.9±10.0 ng/mL; water-immersed: 102.4±9.3 ng/mL, n=6, p<0.05). Therefore, it is possible that the hippocampal neurons of water-immersed rats are vulnerable to an endogenous NMDA receptor agonist because of increased corticosterone. Additionally, MEM at 10 mg/kg did not affect the increase in serum corticosterone level by water-immersion (saline/water-immersed: 102.4±9.3 ng/mL; MEM/water-immersed: 113.1±14.6 ng/mL, n=6, not significant). This result is consistent with the results of Zhou et al. who reported that MEM at 10 mg/kg did not affect blood corticosterone levels in normal rats. Therefore, it is unlikely that MEM protected the hippocampal neurons either by attenuating stress or by suppressing corticosterone production.

In conclusion, QUIN is not implicated in the development of combined fatigue in water-immersed rats, although it seems to be causative in some fatigue models. On the other hand, hippocampal neuronal damage was shown in this combined fatigue model, similar to the results of other fatigue models where QUIN increased. MEM inhibited not only fatigue but also the neuronal damage induced by water-immersion. Therefore, it is thought that neuronal brain damage by an endogenous NMDA receptor agonist other than QUIN participates in the development of this combined fatigue. However, the QUIN levels in cerebrospinal fluid, which are closely related to the central action of QUIN, were not measured in the present study. Further studies are required in the future to clarify the detailed mechanisms underlying the development of combined fatigue.
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