Orally administered L-ornithine elevates brain L-ornithine levels and has an anxiolytic-like effect in mice

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Orally administered L-ornithine elevates brain L-ornithine levels and has an anxiolytic-like effect in mice

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The number of figures is four.

CONFLICT-OF-INTEREST NOTIFICATION FIELD

There are none.

Abstract

Intracerebroventricular injection of L-ornithine has demonstrated sedative and hypnotic effects in neonatal chicks exposed to acute stressful conditions. However, whether orally administered L-ornithine can reduce acute mental stress remains to be defined. To clarify the nutritional importance of L-ornithine in controlling the stress response, in Experiment 1 we first investigated whether orally administered L-ornithine can be transported into the brain of mice. Mice were orally administered L-ornithine (3 mmol/water 10 ml/kg, per os). L-Ornithine levels were significantly elevated in the cerebral cortex and hippocampus at 30 and 60 min post administration. In Experiment 2, the effect of orally administered L-ornithine (0, 0.1875, 0.75 and 3 mmol/water 10 ml/kg, per os) on anxiety-like behavior in mice exposed to the elevated plus-maze test was examined at 30 min post administration. There was a significant increase in the percentage of time spent and entries in the open arms in the group receiving 0.75 mmol of L-ornithine compared to control group. Furthermore, locomotion activity in a novel environment was not significantly changed between the control group and 0.75 mmol of L-ornithine group in Experiment 3. Therefore, it appears that orally administrated L-ornithine is bioavailable to the rodent brain and reduces anxiety-like behavior as demonstrated by the elevated plus-maze test.

Key words:

L-Ornithine

Oral administration

Stress

Anxiety

Amino acids

Introduction

Mental and/or physical stresses in both acute and chronic conditions are an unavoidable part of human existence. Extreme forms of stress and chronic stress can cause an abnormal mental state and behavior, and are risk factors of some psychiatric disorders including depression and schizophrenia.[1,2] Thus, controlling stress can contribute to the prevention of some psychiatric disorders.

L-Ornithine, a metabolite of L-arginine, is found in animals as a free amino acid, in various foods such as Corbicula (an Asian clam), and is common in the natural world. L-Ornithine is found in the liver where it acts as an intermediate in the urea cycle.[3,4] L-Ornithine is converted to pro-proliferative polyamine via ornithine decarboxylase. Polyamines such as putrescine, spermidine and spermine are small ubiquitous cationic molecules required for cell growth and homeostasis.[5,6] Furthermore, intracerebroventricular injection of L-ornithine has been demonstrated to induce sedative and hypnotic effects in neonatal chicks exposed to acute stressful conditions.[7]

Hamasu et al.[8] investigated changes in the content of amino acids in the telencephalon and diencephalon of chicks exposed to stressors. L-Arginine was reduced in both the telencephalon and diencephalon under stressful conditions, which suggested that this amino acid may be rapidly metabolized in the brain. Suenaga et al.[9] reported that central L-arginine attenuated the stress response. Furthermore, the sedation and hypnosis induced by L-arginine was shown to be mediated by L-ornithine and by cooperation with other free amino acids having sedative and hypnotic effects in the CNS.[7] Suenaga et al.[7] confirmed that

L-arginine administered by intracerebroventricular injection was rapidly metabolized to L-ornithine in the brain. On the other hand, polyamines, unlike L-ornithine, do not induce a hypnotic effect, whereas only putrescine caused a sedative effect among the three polyamines.[10] Several putative metabolites from L-ornithine, L-citrulline and D-ornithine were also investigated.[11] D-Ornithine weakly attenuated the stress responses while L-citrulline had no effect. Therefore, it appeared that the sedative and hypnotic effects of L-ornithine were mainly induced by L-ornithine itself.[10]

Therefore, while it appears that stress can be attenuated by increasing the brain L-ornithine content during stressful conditions, it remains to be determined whether orally administrated L-ornithine can cross the blood-brain barrier. In Experiment 1, we investigated whether orally administered L-ornithine enhances brain L-ornithine levels in mice. In Experiment 2, we examined whether orally administered L-ornithine can reduce stress in mice exposed to a stressful condition such as the elevated plus-maze test which is commonly used to study anxiety.[13-15] In Experiment 3, to ascertain whether the locomotor activity was affected by orally administrated L-ornithine, open field test was performed in a novel environment.

Material and Methods

Animals

Six-week-old male ICR mice, purchased from Japan SLC, Inc. (Hamamatsu, Japan), were used. Mice were housed 3 per cage under a light/dark

cycle (lights on at 08:00, lights off at 20:00) at a room temperature of 23 ± 1°C. Mice had ad libitum access to food (MF; Oriental Yeast, Tokyo, Japan) and water. This study was performed according to the guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Low (No.105) and Notification (No.6) of the Government of Japan.

Experimental procedure

In Experiment 1, after 1 week of acclimation, mice were divided into six groups; one control group and five L-ornithine groups. The control group was sacrificed by cervical dislocation and the cerebral cortex and hippocampus were immediately dissected and weighed to clarify the values before treatments. The samples were frozen in liquid nitrogen, and stored at -80°C until analysis. The remaining mice were administered 3 mmol/10 ml/kg, p.o. of L-ornithine monohydrochloride (provided by Kyowa Hakko Bio Co., Ltd, Tokyo, Japan) dissolved in water. These mice were then sacrificed by cervical dislocation at 30, 60, 120, 240 and 480 min post administration, and the cerebral cortex and hippocampus were immediately dissected and weighed. The samples were frozen in liquid nitrogen, and stored at -80°C until analysis.

In Experiment 2, after 1 week of acclimation, mice were divided into four groups; one control group and three L-ornithine groups. The control group was administered water and the L-ornithine groups were administered L-ornithine monohydrochloride (0.1875, 0.75 or 3 mmol/10 ml/kg, per os) dissolved in water. The mice were then given the elevated plus-maze test at 30 min post

administration as described below.

In Experiment 3, after 1 week of acclimation, mice were divided into two groups; a control group and a 0.75 mmol of L-ornithine group. The control group was administered water and the 0.75 mmol of L-ornithine group was administered L-ornithine monohydrochloride (0.75 mmol/10 ml/kg, per os) dissolved in water. The mice were then given the open field test at 30 min post administration as described below.

In both Experiments 2 and 3, behavior was recorded in the digital versatile disc using video camera.

Analysis of free amino acids

The concentrations of free amino acids were analyzed by high-performance liquid chromatography (HPLC). This system quantified major amino acids such as L-aspartic acid, L-serine, L-proline, L-glutamic acid, L-citrulline, glycine, L-alanine, L-valine, L-cystine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, β-alanine, L-threonine, γ-aminobutyric acid, L-ornithine, L-lysine, L-histidine, and L-arginine, L-1-methylhistidine and so on. The cerebral cortex and hippocampus were homogenized in an ice-cold 0.2 M perchloric acid solution containing 0.01 mM ethylenediaminetetraacetic acid disodium salt dihydrate and left for deproteinization in ice. After 30 min, the mixtures were centrifuged at 20,000 × g for 15 min at 0°C. After centrifugation, the pH of the supernatant was adjusted to approximately 3.0 by adding 1 M sodium acetate and was filtered through a 0.20 μm filter. A 20 μL sample of the filtrate was loaded into an amino

acid analyzer (JLC-500/V; JEOL, Tokyo, Japan). A standard L-amino acid solution was prepared by diluting a commercially available L-amino acid solution (type AN II and type B; Wako, Osaka, Japan) with distilled water.

Elevated plus-maze test

The elevated plus-maze consisted of a black acrylic cross of two closed arms (27.5 x 5 x 20 cm) and two open arms (27.5 x 5 cm) raised 60 cm above the floor. The open and closed arms were connected by a central platform (5 x 5 cm). For each test, the mouse was placed in the center of the cross facing an open arm and was allowed to explore the maze for 5 min under dim light (70 lux). After each test, the arms and a central platform were cleaned with an ethanol–water solution. In this test, anxiolytic compounds selectively increase the percentage of time spent and/or entries in the open arms while anxiogenic compounds selectively decrease the percentage of time spent and/or entries in the open arms. An entry was counted when all four paws of the mouse entered an open or closed arm.

Open field test

The locomotor activity in a novel environment was recorded employing the open field test. Briefly, animals were individually transferred to an open field area from the home cage. The area was circular (diameter 60 cm and height 35 cm), and made of black takiflex. The test was begun by placing the animals at the center of the area. The behavior of the animals was then observed for 5 min

under dim light (70 lux). After each test, the field was cleaned with an ethanol-water solution. The following behavioral categories were examined as the locomotor activity: distance of path, time the animal spent moving and speed of movement. All behaviors were automatically analyzed as the computer-based video tracking system (AXIS-90, Neuroscience, Inc., Japan).

Statistical analysis

In Experiments 1 and 2, data were analyzed using a one-way analysis of variance. When significant [$P<0.05$] effects were detected, the Dunnett test was used to evaluate the differences from the control. In Experiment 3, data were analyzed by student's t-test. The significant level adopted for statistical test was $p<0.05$. These analyses were performed with StatView Version 5 (SAS Institute Cary, N.C., USA, 1998). Outlying data were eliminated by Thompson's test criterion for outlying observation [$P<0.05$].

Results

Amino acid concentrations in the cerebral cortex and hippocampus

The concentrations of L-ornithine, L-arginine and L-citrulline in the cerebral cortex and hippocampus in Experiment 1 are shown in Figures 1 and 2. Oral administration of L-ornithine significantly increased L-ornithine concentrations in the cerebral cortex [$F(5,39)=41.603$, $P<0.0001$] and hippocampus [$F(5,36)=7.038$, $P<0.0001$] at 30 and 60 min post administration (Figure 1-A and Figure 2-A). In contrast, L-arginine concentrations in the cerebral cortex

[F(5,39)=2.628, P<0.05] and hippocampus [F(5,36)=5.184, P<0.01] were significantly decreased at 60 (only in the hippocampus) and 120 min post administration (Figure 1-B and Figure 2-B). L-Citrulline concentrations in the cerebral cortex [F(5,39)=0.931, P>0.05] and hippocampus [F(5,36)=0.950, P>0.05] were not significantly changed (Figure 1-C and Figure 2-C). L-Lysine concentrations (nmol/g wet tissue) in the cerebral cortex significantly decreased [F(5,39)=3.842, P<0.01] following oral administration of L-ornithine and then returned to control levels (control group, 65.0±4.4; 30 min, 47.7±3.1; 60 min, 43.7±4.7; 120 min, 44.3±3.1; 240 min, 64.1±6.6; and 480 min, 59.7±6.3). L-Lysine concentrations (nmol/g wet tissue) in the hippocampus significantly decreased [F(5,36)=11.149, P<0.0001] following oral administration of L-ornithine and then returned to control levels (control group, 318±9; 30 min, 277±22; 60 min, 210±12; 120 min, 210±13; 240 min, 292±22; and 480 min, 323±18).

Percentage of time spent and entries in the open arms in the elevated plus-maze test

The effect of orally administered L-ornithine on the percentage of time spent and entries in the open arms of the elevated plus-maze test for 5 min is shown in Figure 3. Significant increases in the percentage of time spent [F(3,52)=5.071, P<0.01] (Figure 3-A) and entries [F(3,52)=3.505, P<0.05] (Figure 3-B) in the open arms were observed following 0.75 mmol of L-ornithine group compared to the control group.

Locomotor activity in the open field test

Figure 4 shows locomotor activity in the open field during a 5 min period. There were no significant difference between the control group and 0.75 mmol of L-ornithine group in moving distance [P=0.55] (Figure 4-A), moving time [P=0.81] (Figure 4-B) and moving speed [P=0.26] (Figure 4-C) in the area.

Discussion

We previously demonstrated that intracerebroventricular injection of L-ornithine caused sedative and hypnotic effects under social isolation stress in neonatal chicks.[7,10,16,17] However, whether orally administered L-ornithine can reduce stress was unknown. To attenuate the stress response, orally administered L-ornithine must first be transported into the brain. In Experiment 1, it was confirmed that orally administered L-ornithine was transported into the brain as shown by elevated L-ornithine levels in the cerebral cortex and hippocampus at 30 and 60 min post administration. Moreover, L-ornithine levels in the cerebral cortex and hippocampus were the highest values at 30 min post administration. These concentrations returned to control values at 240 min post administration (Figure 1-A and Figure 2-A). L-Ornithine has previously been shown to be rapidly metabolized in the brain[5] and thus its levels decrease over 4 h. In contrast, L-arginine showed different responses without increases (Figure 1-B and Figure 2-B).

These changes reflect the L-ornithine levels in the blood. L-Ornithine and L-arginine transport across the blood-brain barrier is mediated exclusively by

cationic amino acid transporter 1 (system y^+).[12] Thus, increases in blood L-ornithine concentrations would compete with L-arginine for the system y^+ transporter. L-Lysine transport across the blood-brain barrier is also mediated exclusively by system y^+.[12,18] L-Lysine concentrations decreased significantly following oral administration of L-ornithine and then returned to control levels. The values were significantly decreased at 60 and 120 min post administration as were the levels of L-arginine. The changes in L-lysine further supported the conclusion that the system y^+ transporter was mainly used for transport of L-ornithine.

L-Arginine, L-ornithine and L-citrulline are critical intermediates of urea cycle in the liver. L-Citrulline transport across the blood-brain barrier is mediated by the large neutral amino acid transporter 1 (system L)[11,18], instead of the system y^+ of L-ornithine and L-arginine. Brain L-citrulline, a nitric oxide (NO) co-product, is an index of brain NO generation via NO synthase (NOS).[19] However, the intracerebroventricular injection of L-arginine had no effects on NOx (NO_2 + NO_3), an index of NO production, in several brain sites.[9] In the present study, L-arginine levels were decreased in both brain sites by orally administered L-ornithine. The Km value of NOS is around 2.9 μM.[20] When an enzyme has a low Km value, such as NOS, the catalytic proficiency reaches the maximum value when the substrate concentrations are low. The L-arginine levels in the L-ornithine-treated groups were sufficient such that no changes in L-citrulline concentrations in the cerebral cortex and hippocampus (Figure 1-C and Figure 2-C) were observed in the present study.

Putrescine, a metabolite of L-ornithine, exerts an antidepressant-like effect in both the forced swimming test and tail suspension test in mice.[21] Zomkowski et al.[21] suggested that the antidepressant-like effect of putrescine in the forced swimming test was mediated by the inhibition of the polyamine site of *N*-Methyl-*D*-aspartate (NMDA) receptors. In the present study, we only investigated the effect of L-ornithine itself for any effect on the stress response, not its metabolite. According to Kurauchi et al.[10], the L-ornithine metabolite putrescine was less effective at attenuating the stress response, since L-ornithine had both sedative and hypnotic effects while putrescine had only a sedative effect. Therefore, we speculate that orally administrated L-ornithine can most effectively reduce stress when L-ornithine concentrations in the brain were increasing.

In Experiment 2, significant increases in the percentage of time spent and entries in the open arms in the elevated plus-maze test were observed in the 0.75 mmol of L-ornithine group compared to the control group at 30 min post administration of L-ornithine. The elevated plus-maze test has a strong predictive validity for screening anxiolytic drugs; anxiolytic drugs increase while anxiogenic drugs decrease the percentage of entries into the open arms and the time spent there.[15,22] The dose-response curve, however, had an inverted U shape with both higher and lower doses being ineffective. Significant changes in the percentage of time spent and entries in the open arms in the elevated plus-maze test were not observed in mice receiving 3, 6 or 12 mmol of L-ornithine (our unpublished data). Furthermore, we examined whether an anxiolytic-like effect observed in 0.75 mmol of L-ornithine group was caused by hyperlocomotion

activity in the open field test. However, the locomotion activity was not significantly changed between the control group and 0.75 mmol of L-ornithine group. Thus, these results suggest that orally administrated L-ornithine, within a limited dose range, had an anxiolytic-like effect.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the CNS. Biochemical and electrophysiological studies have shown that benzodiazepine anxiolytics (e.g. diazepam) facilitate GABAergic transmission in the mammalian CNS via positive allosteric modulation of the $GABA_A$ receptor complex.[23,24] L-Ornithine was shown to attenuate corticotropin-releasing factor-induced stress responses acting at $GABA_A$ receptors in neonatal chicks.[16] In the previous study, it was not determined whether L-ornithine acted as a $GABA_A$ receptor agonist, a GABA reuptake inhibitor or if it elevated GABA levels in the brain. In the present study, GABA levels in the cerebral cortex were not significantly [$F(5,39)=0.344$, $P=0.8833$] different (control group, 522±37; 30 min, 472±43; 60 min, 477±44; 120 min, 474±35; 240 min, 525±53; 480 min, 478±51). GABA levels in the hippocampus were not also significantly different [$F(5,36)=0.751$, $P=0.5907$] (control group, 2879±121; 30 min, 2739±148; 60 min, 2598±98; 120 min, 2650±116; 240 min, 2658±124; 480 min, 2820±97). Therefore, we speculate that elevated cerebral cortex and hippocampus L-ornithine levels did not elevate GABA synthesis in the brain. In this study, however, we investigated only content, and not release, in the cerebral cortex and hippocampus. Further studies using microdialysis are needed to determine if L-ornithine can induce GABA release.

In conclusion, increased cerebral cortex and hippocampus L-ornithine levels by oral administration of L-ornithine had an anxiolytic-like effect in the elevated plus-maze test. The present study suggested that oral administration of L-ornithine can possibly have beneficial effects on brain function related to anxiety-like behavior.

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Figure Legends

Figure 1. Effect of orally administrated L-ornithine on the concentrations of L-ornithine (A), L-arginine (B) and L-citrulline (C) in the cerebral cortex at various time. Results are expressed as mean±S.E.M. The number of samples used for analysis was 5-6 (L-ornithine group) or 15 (control (Cont) group).

L-Ornithine versus control at $P<0.05$.

Figure 2. Effect of orally administrated L-ornithine on the concentrations of L-ornithine (A), L-arginine (B) and L-citrulline (C) in the hippocampus at various time. Results are expressed as mean±S.E.M. The number of samples used for analysis was 4-6 (L-ornithine group) or 15 (control (Cont) group).

L-Ornithine versus control at $P<0.05$.

Figure 3. Effect of orally administrated L-ornithine on the percentage of time spent (A) and the entries (B) in the open arms in the elevated plus-maze test. Results are expressed as mean±S.E.M. The number of mice used in each group was 14.

L-Ornithine versus control at $P<0.05$.

Figure 4. Effect of orally administrated 0.75 mmol of L-ornithine on the moving distance (A), moving time (B) and moving speed (C) in the open field test. Results are expressed as mean±S.E.M. The number of mice used in each group

was 8-9.

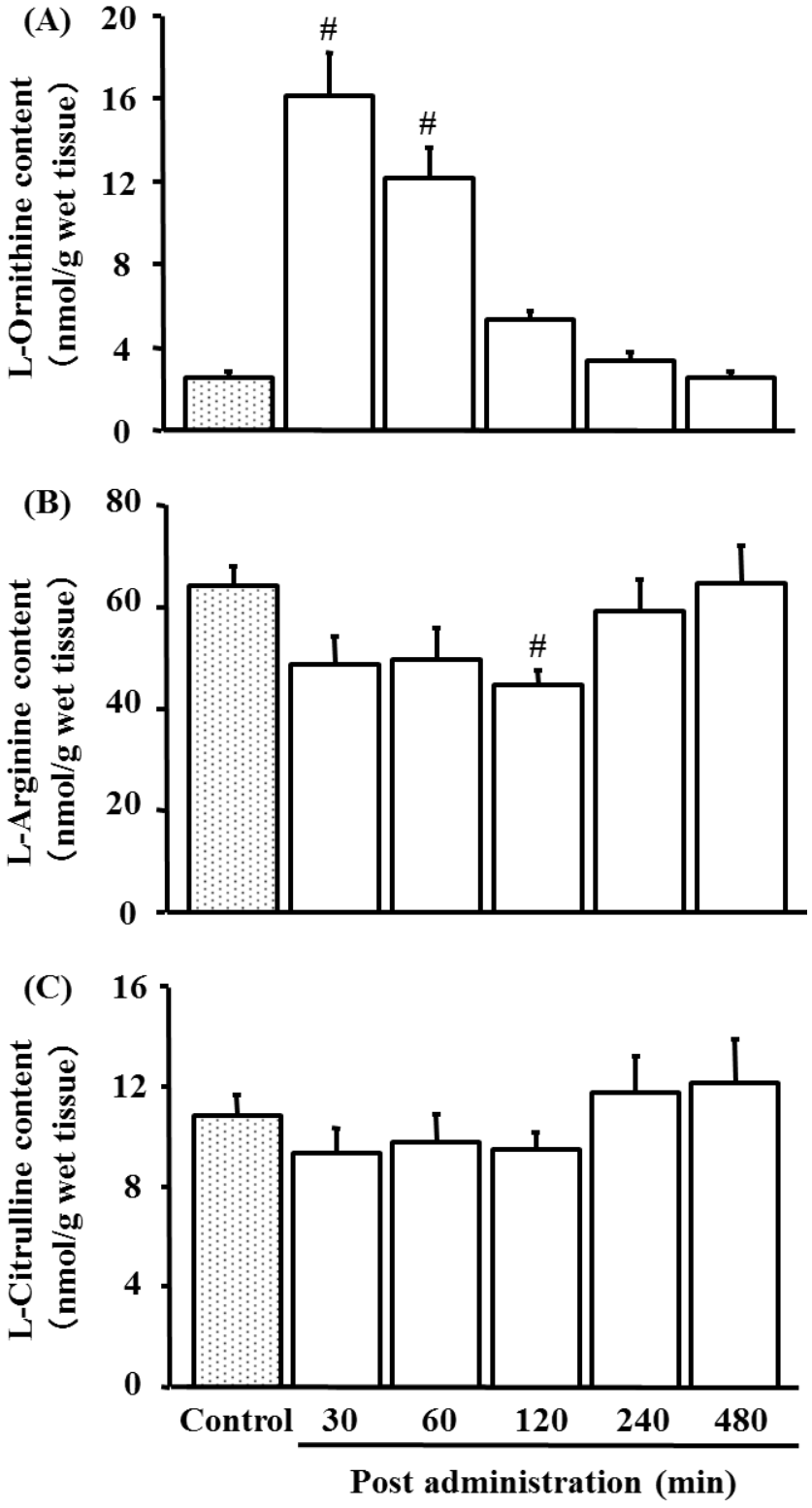
(A)
L-Ornithine content
(nmol/g wet tissue)
20
16
12
8
4
0
#
#
(B)
L-Arginine content
(nmol/g wet tissue)
80
60
40
20
0
#
(C)
L-Citrulline content
(nmol/g wet tissue)
16
12
8
4
0
Control
30
60
120
240
480
Post administration (min)

Figure 1

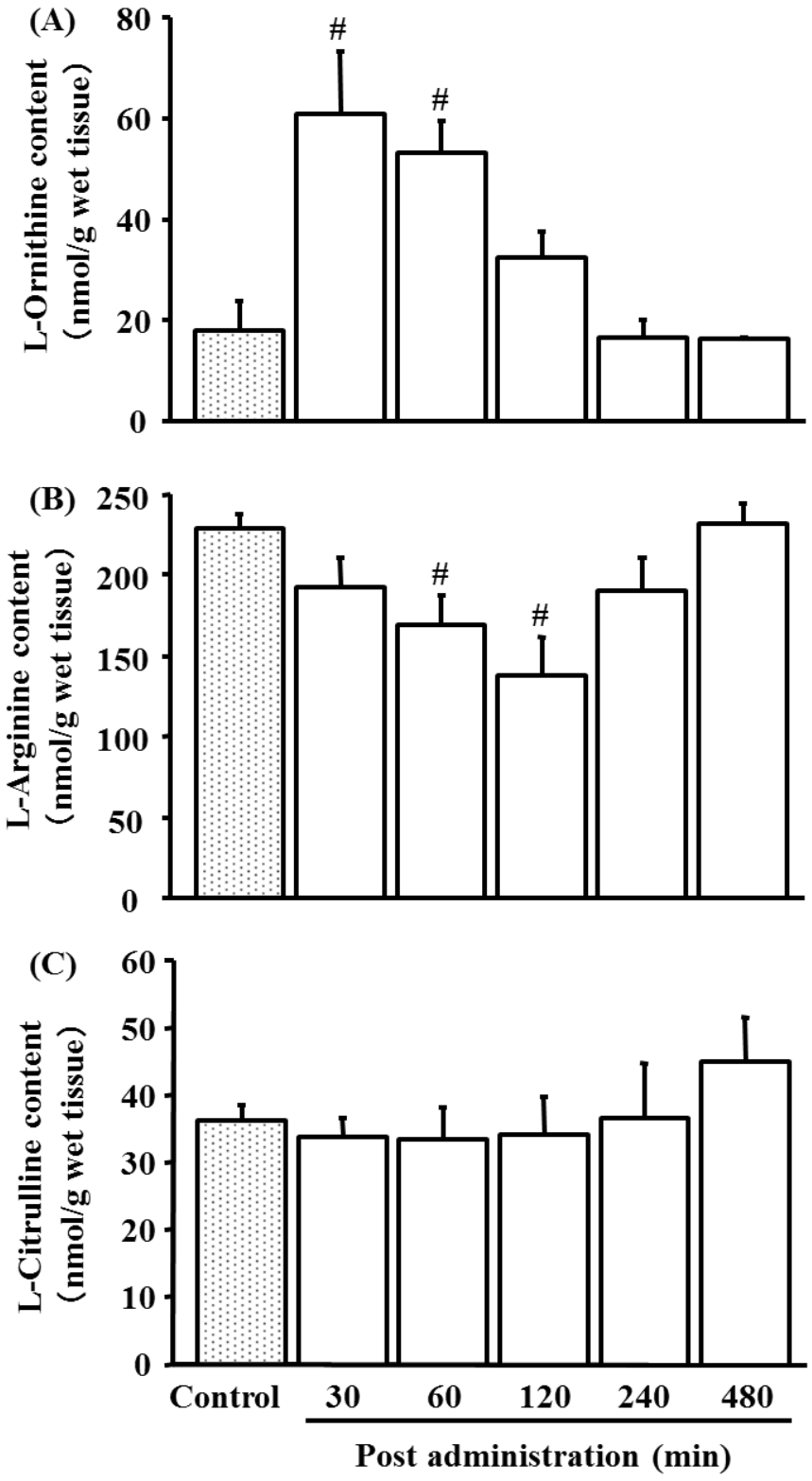
(A)
L-Ornithine content (nmol/g wet tissue)
80
60
40
20
0
#
#
(B)
L-Arginine content (nmol/g wet tissue)
250
200
150
100
50
0
#
#
(C)
L-Citrulline content (nmol/g wet tissue)
60
50
40
30
20
10
0
Control
30
60
120
240
480
Post administration (min)

Figure 2

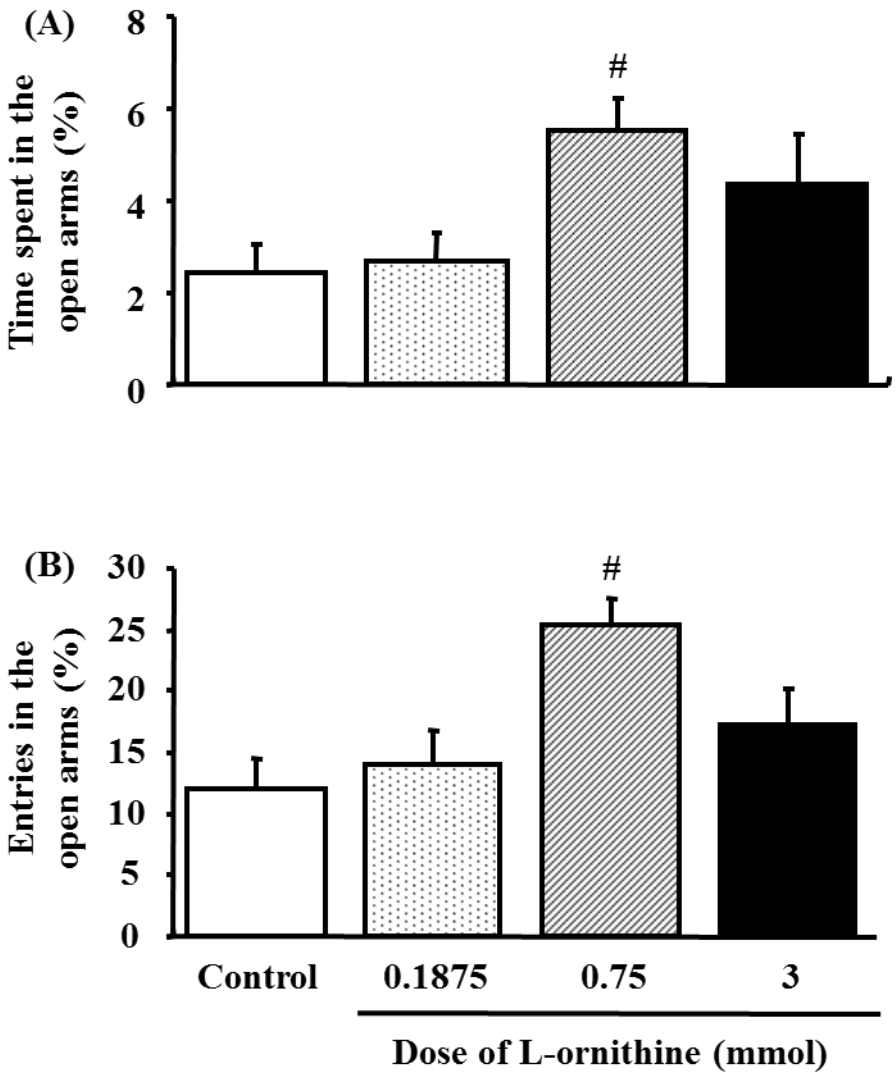
(A)
Time spent in the open arms (%)
8
6
4
2
0
#
(B)
Entries in the open arms (%)
30
25
20
15
10
5
0
#
Control
0.1875
0.75
3
Dose of L-ornithine (mmol)

Figure 3

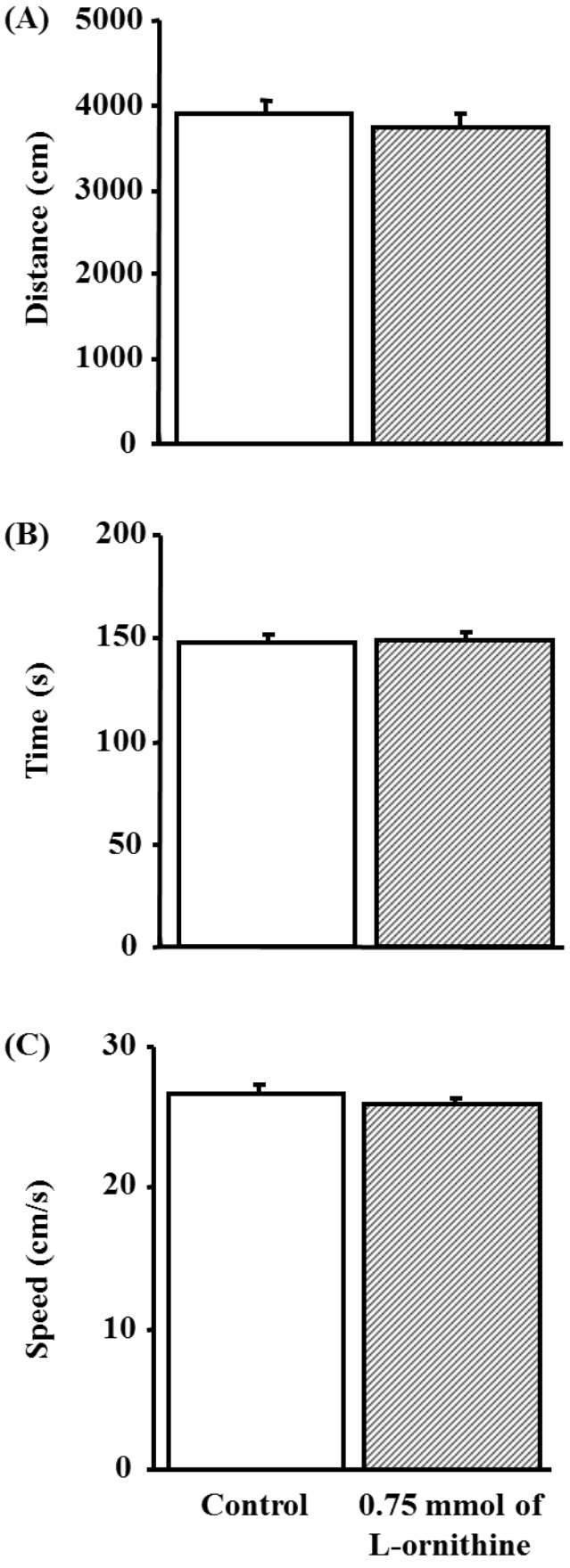
(A)
5000
4000
3000
2000
1000
0
Distance (cm)
(B)
200
150
100
50
0
Time (s)
(C)
30
20
10
0
Speed (cm/s)
Control
0.75 mmol of
L-ornithine

Figure 4