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Behavioral Changes in Cold-Stressed Mice Related to a Central Calcium-Dependent-Catecholamine Synthesizing System[1]

DEN'ETSU SUTOO, KAYO AKIYAMA AND HITOSHI TAKITA

Department of Pediatrics, Institute of Clinical Medicine, University of Tsukuba, Tsukuba 305, Japan

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SUTOO, D., K. AKIYAMA AND H. TAKITA. *Behavioral changes in cold-stressed mice related to a central calcium-dependent-catecholamine synthesizing system*. PHARMACOL BIOCHEM BEHAV **40**(2) 423–428, 1991.—An investigation was carried out regarding the mechanism of behavioral changes in mice elicited by cold stress. Cold stress was induced in adult male mice by restraining them from free action for 2 h at 4°C. As the control test, mice were restrained from free action for 2 h at room temperature. The locomotor counts in cold-stressed mice were found to be lower than in controls. The counts in cold-stressed mice were increased by IP pretreatment with EDTA or α-methyltyrosine (tyrosine hydroxylase inhibitor), and were further decreased by IP pretreatment with $CaCl_2$. On the other hand, serum calcium and brain calcium levels in cold-stressed mice were increased 15–30 min and 30 min, respectively, after restraint under cold temperatures, and returned to original levels 1 h after restraint. Also, the biochemical and immunohistochemical brain dopamine levels in cold-stressed mice were higher than in control mice. The increment of brain dopamine levels in the control mice was also observed by the administration of $CaCl_2$. Furthermore, the ability of cold stress to enhance the dopamine level in mice brains was attenuated by IP pretreatment with α-methyltyrosine. In light of previous reports that central calcium activates catecholamine-synthesizing enzymes via a calmodulin-dependent system, it is suggested that cold stress enhances the brain calcium level, and then increased calcium enhances dopamine synthesis in the brain through a central calcium-dependent catecholamine synthesizing system. Subsequently, increased dopamine induces behavioral changes.

Cold stress   Calcium-calmodulin-dependent-catecholamine synthesis   Locomotor activity   Microphotometry system
Neostriatal dopamine   Brain calcium

THE relationship between various types of stress, including cold stress, and the metabolism and/or turnover of biogenic amines in the central nervous system has been extensively investigated. A decrease in the cerebral contents of norepinephrine (NE) and epinephrine has been demonstrated during cold-swim stress (19,20), as well as during cold and immobilization stress (12). These changes have been thought to reflect the increased turnover of NE and epinephrine (20). Also, increases in the contents of dopamine (DA) and serotonin (5-HT) in cold stress and electric footshock stress have been reported (9, 14, 29, 30). Increases in tyrosine hydroxylase activity (28,33) and the turnover rates of DA and 5-HT (16) have also been observed in the rat brain. These reports suggest that rises in brain biogenic amine metabolism are involved in stress.

We have investigated the role of central calcium ions in various aspects of physiology, and have suggested that central calcium enhances biogenic amine-synthesizing enzymes, tyrosine hydroxylase and tryptophan hydroxylase, activities through calmodulin-dependent protein kinase and subsequently biogenic amines regulate various physiological functions (22, 24, 26). We therefore postulated that changes in brain biogenic amine synthesis and behavior in stressed mammals may occur through a central calcium-calmodulin-dependent system. This study was carried out to confirm this hypothesis. For this purpose, the effects of cold stress on the locomotor activity and brain DA levels in mice were investigated in comparison with the effects of calcium administration.

METHOD

Animals

Adult male mice of the ddY strain (20–25 g) were provided by Doken Co. Ltd. (Ibaraki, Japan). They were kept in groups of 8–10 in stainless steel cages at room temperature (22±2°C) for more than one week before use in the experiments and were exposed to a 12-h light/dark schedule. Food and water were provided ad lib until the time of experiment and were not given during the restraint. Cold stress was induced in mice by restrain-

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ing them from free action, using plastic holders [3 cm in diameter (∅) × 7 cm] for 2 h at 4°C. As the control test, mice were restrained from free action for 2 h at room temperature.

Behavioral Test

Behavioral experiments were carried out between 10:00 a.m. and 4:00 p.m. Mice were placed in locomotor activity cages (30×40×40 cm, Animex type S, LKB) for 30 min to allow them to adapt to the environment. They were then removed from the cages and cold stress was induced. After restraint under cold temperature, the mice were returned to the locomotor activity cage and activity counts per minute were measured. All observations were made in an isolated environmental room, maintained at a constant temperature of 22±2°C. Two locomotor activity cages were used and the test animal group was constantly compared with the control group. In the first step of the investigation, the locomotor activities per 10 min in the cold-stressed mice group and the control mice group were measured over 2 h. Next, the effects of pretreatment with ethylenediaminetetraacetic acid (EDTA), α-methyltyrosine (αMPT, inhibitor of tyrosine hydroxylase) or $CaCl_2$ on locomotor activity in cold-stressed mice were analyzed. One μmol/mouse of EDTA was injected IP 1 h before restraint, and 100 mg/kg of αMPT and 40 μmol/kg of $CaCl_2$ were injected IP 24 h and 1 h, respectively, before restraint. The doses of αMPT and $CaCl_2$ were based on previous studies (21,22). The EDTA dose was determined in a pilot experiment. Locomotor counts for each hour were analyzed by the Student's t-test for comparison between the control and stressed groups and by an ANOVA and Dunnett's t-test for multiple comparisons between individual pretreatment groups and the saline-pretreated control group.

Biochemical Test

The effect of cold stress on the brain DA, serum calcium and brain calcium contents in mice were investigated biochemically. High-performance liquid chromatography (HPLC) technique was employed to quantify the DA level in the brain. Five animal groups (7–10 mice/group), i.e., the unstressed control mice pretreated IP with saline (injected just before the restraint), unstressed mice pretreated IP with αMPT (100 mg/kg, injected 24 h before restraint), unstressed mice pretreated IP with $CaCl_2$ (40 μmol/kg, injected just before the restraint), cold-stressed mice pretreated IP with saline, and cold-stressed mice pretreated IP with αMPT, were prepared. After the removal of restraint, the brains were taken out quickly and divided into 2 parts, the cerebrum and rhombencephalon, as described in our previous report (21). Only the cerebrum region was analyzed in the present study. The brain tissue was homogenized with 0.1 M perchloric acid (4:1, v/w) in a glass homogenizer. After centrifuging 10 min at 25,000×g (4°C), the supernatant fluid was diluted 1:500 with distilled deionized water and injected directly into an HPLC. A Tosoh Model HLC-8030 HPLC system automatic catecholamine analyzer was used. In this system, catecholamines in deproteinized brain tissue are purified on the first and second (preparation) columns, then transferred automatically to the third (analytical) column in which DA and NE are resolved. These compounds are then determined fluorometrically with a continuous-flow reaction system by the 1,2-diphenylethylenediamine method (15).

The calcium ions in the serum and brain were measured according to the method of Ray Sarkar and Chauhan (18) using an o-cresolphthalein complexone. Four unstressed control mice groups which were restrained for 15, 30, 60 or 120 min at room temperature, and four cold-stressed mice groups which were restrained for 15, 30, 60 or 120 min under cold temperature, were prepared. The whole brain and blood were obtained from each mice group. The serum was separated quickly and was used for the calcium content assay. The brain was homogenized with 2 ml of saline at 4°C and centrifuged for 1 h at 25,000×g (4°C). The supernatant fluid was assayed.

DA data were analyzed by an ANOVA and Newman-Keuls t-test for subsequent comparisons between each group. Calcium data were analyzed by the Student's t-test for comparison between the control and stressed groups.

Immunohistochemical Test

As demonstrated in the Results section, the cerebrum DA levels in cold-stressed mice were higher than in the brains of control mice, as well as in the brains of mice pretreated IP with $CaCl_2$. Therefore, the effect of cold stress on the immunohistochemical distributions of DA in the mice brains was investigated in detail as compared with the effect of calcium administration. Immunohistochemical distributions and the amounts of DA in the mice brains were measured using a fluorescence microphotometry system which we developed (25). This system surpasses by two orders the quantitative linearity of an image analyzer used with high-sensitivity TV cameras and it also surpasses by three orders the sensitivity of HPLC with an electrochemical detector. Three animal groups (ten mice/group), i.e., the unstressed control mice, the unstressed mice pretreated IP with $CaCl_2$ (40 μmol/kg, injected 1 h before use) and the cold-stressed mice, were prepared. These mice were anesthetized with pentobarbital sodium (40 mg/kg, IP), and perfused intracardially with 50 ml solution of 0.1 M cacodylate and 1% sodium *meta*-bisulfite containing 2.5% glutaraldehyde (pH 7.5). The whole brain was removed and postfixed with the same solution for 30 min, then frozen on dry-ice and kept at −80°C until use, which was within 1 day. The frozen brain was sectioned coronally and continuously at 20 μm in a cryostat. For analysis of the distribution and amount of DA, brain slices located approximately 5 mm rostral from the interaural line (Fig. 3) were chosen in light of previous report (26) that DA levels in the neostriatum and nucleus accumbens septi regions of this section were increased by the intraventricular (IVT) administration of $CaCl_2$.

The brain slices were stained immunohistochemically for DA as described in our previous reports (26,27). The stained brain slices were analyzed by a fluorescence microphotometry system. The microphotometer was calibrated by uranium glass through a 6 μmϕ pin hole (25), and brain slices were measured through the same spot. Therefore, the data in the present study are indicated relatively for fluorescence intensity per 6 μmϕ of area compared with the intensity of uranium glass. The stained slices were measured stepwise at 100 μm intervals. The total fluorescence intensity value in each measuring point had the background value subtracted. The background value was obtained from slices treated with the same procedure without DA antibody. The microphotometry system's operating conditions were as follows: excitation range, 420–490 nm; interference filter, 530 nm; photomultiplier voltage, 850 V; and objective, 20×/0.75 (magnification/numerical aperture). The distributions of fluorescence intensity originating from DA in brain slices were obtained and are indicated in Fig. 3. The actual fluorescence intensities are indicated in Table 2. The average fluorescence intensity in each region was analyzed by an ANOVA and Newman-Keuls t-test for subsequent comparisons of three groups (8–14 slices/group).

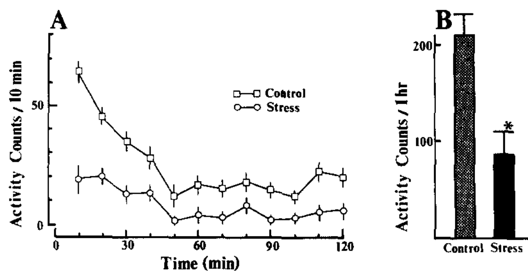

FIG. 1. The time-course of locomotor activity after the removal of restraints. Mice were restrained from free action for 2 h at 4°C (cold-stressed group) or 22°C (control group). Activity counts at 10-min intervals for the 2-h recording period are provided in A. Total counts over the first 1-h period are provided in B. Each value represents the mean ± SEM of 10 tests. *$p<0.01$ when compared with control group by Student's t-test.

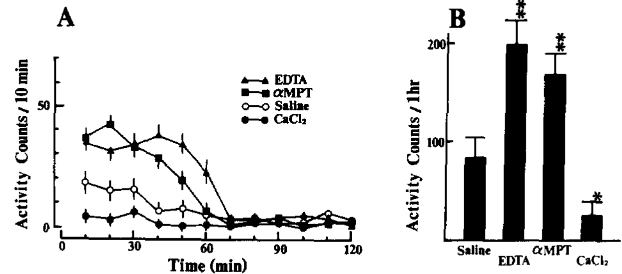

FIG. 2. Time-course of locomotor activity after removal of restraints in mice pretreated with saline, EDTA, αMPT or $CaCl_2$. Mice were pretreated IP with saline, EDTA (1 μmol/mouse), αMPT (100 mg/kg) or $CaCl_2$ (40 μmol/kg) 1 h, 1 h, 24 h or 1 h, respectively, before restraint for 2 h at 4°C. Activity counts at 10-min intervals for the 2-h recording period are provided in A. Total counts over the first 1-h period are provided in B. Each value represents the mean ± SEM of 10 tests. *$p<0.05$, **$p<0.01$ when compared with the saline group by the Dunnett's t-test.

RESULTS

Locomotor Activity

The locomotor activities in cold-stressed and control mice were compared for each 10-min period over 120 min, as seen in Fig. 1. Immediately after the removal of restraint, control mice moved hurriedly, though cold-stressed mice crouched on the floor and moved only a little. The locomotor activity in the first 1-h period in cold-stressed mice was lower by approximately 60% ($p<0.01$) than in the control mice. In the second 1-h period, however, the locomotor activity in control mice decreased to the same level as that of the cold-stressed mice. Also, the locomotor activities in cold-stressed mice with IP pretreatment of EDTA, αMPT and $CaCl_2$ were compared with those pretreated with saline as shown in Fig. 2. The lower locomotor activity induced by cold stress was improved significantly by pretreatment with EDTA or αMPT, however, it was further reduced by pretreatment with $CaCl_2$.

Biochemical DA Levels

The cerebrum DA levels in various treated mice groups are shown in Table 1. The cerebrum DA levels in cold-stressed mice were higher by 15% ($p<0.01$) than in the control mice. Similar results were observed in unstressed mice pretreated with $CaCl_2$, i.e., the cerebrum DA level was increased by the administration of $CaCl_2$ by 11% ($p<0.05$) as compared with the control group. The cerebrum DA levels in cold-stressed mice were not changed significantly as compared to those of unstressed mice pretreated with $CaCl_2$.

In contrast, the ability of cold stress to enhance DA levels in mice brains was attenuated by IP pretreatment with αMPT, while the DA level itself was reduced in control mice. The cerebrum DA levels in unstressed mice pretreated with αMPT and in cold-stressed mice pretreated with αMPT were lower by 21% ($p<0.01$) and 24% ($p<0.01$), respectively, as compared with the unstressed control mice pretreated with saline and cold-stressed mice pretreated with saline. The brain DA levels in cold-stressed mice pretreated with αMPT were higher than in unstressed mice pretreated with αMPT, but they were not significantly changed.

Immunohistochemical DA Distributions

The distributions of brain DA are seen in Table 2. The neostriatum showed the highest fluorescence intensity in control mice, followed by the nucleus accumbens septi, cortex cerebri area cinguli, tractus diagonalis, cortex cerebri area frontoparietalis and nucleus septi lateralis. In contrast to the control group, the fluorescence intensities of DA in the neostriatum and nucleus accumbens septi in mice pretreated IP with $CaCl_2$ were increased by 16–20% ($p<0.05$–0.01) and 22% ($p<0.01$), respectively. The DA levels in other brain regions were not changed significantly by the IP administration of $CaCl_2$. On the other hand, the DA levels in the neostriatum and in the nucleus accumbens septi regions in cold-stressed mice were higher by 29–32% ($p<0.01$) and 34% ($p<0.01$), respectively, than in the brains of the control mice, as well as in the brains of mice pretreated IP with $CaCl_2$. The DA levels in the neostriatum and in the nucleus accumbens septi regions in cold-stressed mice were not changed significantly as compared to those of unstressed mice pretreated with $CaCl_2$. The changes in the DA levels in these regions are displayed in Fig. 3 along with changes in other regions.

Calcium Ion Levels

Serum and brain calcium ion levels in unstressed control mice and cold-stressed mice are shown in Table 3. Serum and brain

TABLE 1

CEREBRUM DOPAMINE CONTENTS IN COLD-STRESSED MICE AND UNSTRESSED MICE

Animal	Treatment	Dopamine Content [ng/mg wet wt. ± SEM (n)]
Unstressed Mice	Saline	1.75 ± 0.04 (10)
	αMPT	1.38 ± 0.06 (8)†
	$CaCl_2$	1.94 ± 0.05 (9)*
Cold-stressed Mice	Saline	2.01 ± 0.05 (10)†
	αMPT	1.52 ± 0.06 (7)‡§

Cold stress was induced by restraining from free action for 2 h at 4 °C. Unstressed control mice were restrained from free action for 2 h at 22 °C. Saline or $CaCl_2$ (40 μmol/kg) were injected IP just before the restraint, and αMPT (100 mg/kg) was injected IP 24 h before restraint.

*$p<0.05$, †$p<0.01$ compared with unstressed saline group.

‡$p<0.01$ compared with cold-stressed saline group.

§Not significant compared with unstressed αMPT group.

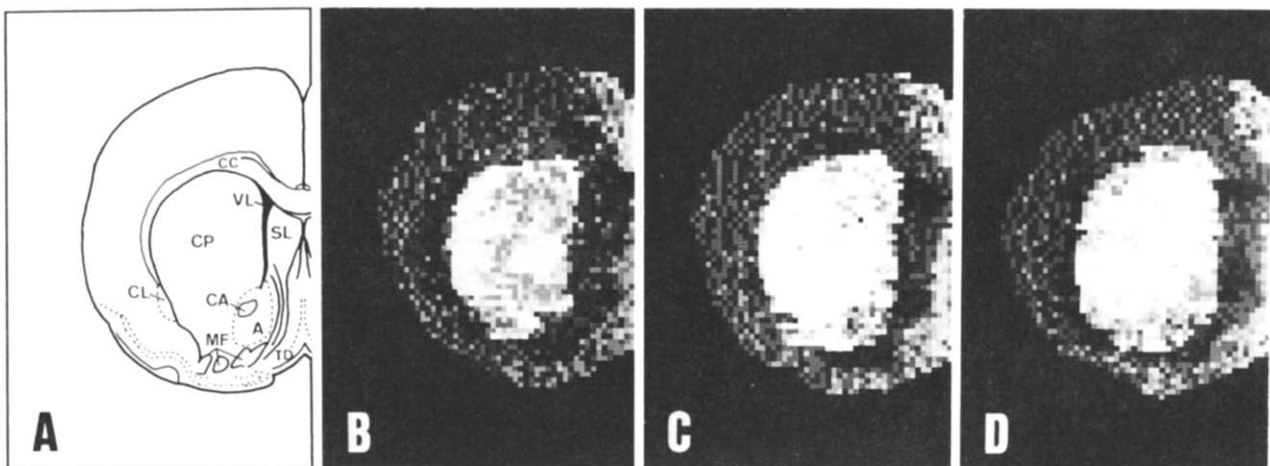

FIG. 3. Drawing of a coronal section approximately 5 mm rostral from the interaural line in mice (A). The effects of cold stress and IP administration of $CaCl_2$ on the immunohistochemical DA distributions in this mouse brain section were compared by a fluorescence microphotometry system. A, nucleus accumbens septi; CA, commissura anterior; CC, corpus callosum; CL, claustrum; CP, nucleus caudatus-putamen; MF, median forebrain bundle; SL, nucleus septi lateralis; TD, tractus diagonalis; VL, ventriculus lateralis. The photo shows an example of the immunohistochemical fluorescence distribution of DA in the brain of control mice (B), cold-stressed mice (C) and unstressed mice pretreated IP with 40 μmol/kg of $CaCl_2$ (D). The fluorescence intensity values of each point had the background value subtracted, and were classified into fifteen ranks and indicated by lightness. The actual fluorescence intensity values in each brain region are indicated in Table 2.

calcium ion levels in cold-stressed mice were increased temporarily and returned to original levels by restraint under cold temperature. Serum calcium level 15–30 min after and brain calcium level 30 min after restraint under cold temperature were higher by 7–9% ($p<0.05$) and 12% ($p<0.05$), respectively, when compared to the control level. However, serum calcium and brain calcium levels 60 min after restraint under cold temperature were not significantly changed when compared to the control level.

DISCUSSION

We have previously reported that the IP, IV and IVT administration of $CaCl_2$ as well as the IVT administration of DA, NE and 5-HT enhances ethanol-induced sleeping time, and that the ability of calcium ions is antagonized by the administration of calmodulin antagonists, αMPT and p-chlorophenylalanine (inhibitor of tryptophan hydroxylase) (21–23). We also confirmed biochemically and immunohistochemically that the IV and IVT administration of $CaCl_2$ brought on rises in the cerebral DA level through a calmodulin-dependent system (21,26). These studies suggested that calcium ions activate tyrosine hydroxylase and tryptophan hydroxylase through an intracerebral calmodulin-dependent system. We have developed these studies to elucidate the mechanisms of stress. In the present study, the action of calcium ions in cold-stressed mice was investigated by using a behavioral, biochemical and immunohistochemical techniques.

In this study, the biochemical cerebrum DA levels and the immunohistochemical DA levels in the neostriatum and nucleus accumbens septi regions in mice were enhanced after restraint

TABLE 2

THE EFFECT OF COLD STRESS OR $CaCl_2$ ADMINISTRATION ON THE IMMUNOHISTOCHEMICAL DISTRIBUTIONS OF DOPAMINE IN THE MICE BRAINS

Brain Region	Fluorescence Intensity (Measured Value × 10)		
	Control	Stress	$CaCl_2$
Cortex cerebri, area cinguli	0.97 ± 0.10 (12)	0.91 ± 0.13 (14)	0.92 ± 0.11 (8)
Cortex cerebri, area frontoparietalis	0.70 ± 0.11 (12)	0.71 ± 0.08 (14)	0.68 ± 0.13 (8)
Neostriatum, pars medialis	4.51 ± 0.16 (12)	5.82 ± 0.20 (14)†	5.25 ± 0.19 (8)*
Neostriatum, pars lateralis	4.73 ± 0.18 (12)	6.25 ± 0.19 (14)†	5.67 ± 0.21 (8)†
Nucleus accumbens septi	4.28 ± 0.14 (12)	5.73 ± 0.17 (14)†	5.24 ± 0.16 (8)†
Nucleus septi lateralis	0.65 ± 0.10 (12)	0.62 ± 0.12 (14)	0.68 ± 0.09 (8)
Tractus diagonalis	0.95 ± 0.11 (12)	0.88 ± 0.10 (14)	0.90 ± 0.16 (8)

Each value represents the mean ± SEM (number of slices) of relative fluorescence intensity per 6 μmϕ area. Cold stress was induced in mice by restraining them from free action for 2 h at 4 °C. $CaCl_2$ (40 μmol/kg) was injected intraperitoneally to unstressed mice 1 h before death. Uranium glass (ϕ = 100 μm) was used as the fluorescence intensity standard (25).

*$p<0.05$, †$p<0.01$ compared with control group by the Newman-Keuls t-test.

TABLE 3

SERUM AND BRAIN CALCIUM CONTENTS IN COLD-STRESSED MICE AND UNSTRESSED MICE

Animal	Restraining Time (min) 15	30	60	120
	Serum Calcium (μg/ml)			
Unstressed Mice	105.5 ± 2.4 (9)	105.3 ± 2.3 (9)	104.1 ± 2.2 (9)	106.3 ± 1.8 (9)
Cold-stressed Mice	113.0 ± 2.2 (8)*	114.4 ± 2.5 (8)*	106.4 ± 1.7 (8)	104.2 ± 2.0 (9)
	Brain Calcium (ng/mg wet wt.)			
Unstressed Mice	52.4 ± 1.7 (9)	52.6 ± 1.8 (9)	53.3 ± 1.6 (9)	52.2 ± 1.8 (9)
Cold-stressed Mice	55.4 ± 2.1 (8)	58.8 ± 1.4 (8)*	54.6 ± 1.8 (8)	51.9 ± 1.2 (9)

Values are expressed as mean ± SEM (n).
Cold-stressed mice groups were restrained for 15, 30, 60 or 120 min under cold temperature, and unstressed mice groups were restrained for 15, 30, 60 or 120 min at room temperature.
*$p<0.05$ compared with unstressed control mice group by the Student's *t*-test.

under cold temperature. Also, the ability of cold stress to enhance brain DA levels in mice was attenuated by pretreatment with αMPT. This effect of cold stress on the brain DA levels is consistent with previous reports. Increased amounts of DA in various brain regions of repeated cold-stressed rats (9) and cold-stressed mice (29) were observed. Increments in tyrosine hydroxylase activity (28,33) and in the turnover rate of DA (16) were also observed in the cold-stressed rat brain. Moreover, after treatment with αMPT, the neostriatal DA level in electric foot-shock stressed mice was significantly higher than it was in the control mice (14).

In this behavioral test, the locomotor counts in cold-stressed mice were observed to be lower than those in controls. This finding is in agreement with previous results (30). Also, cold stress-dependent locomotor suppression was attenuated by the IP administration of αMPT in the present study. A number of researchers have shown that the administration of DA (31), L-tyrosine (catecholamine precursor) (2) or agonists (10) increases locomotor activity in normal animals. However, the locomotor response by the administration of catecholamine was often reversed in cold-stressed mice and in other abnormal animals. For example, though locomotor activity in unstressed mice was increased by supplementing the diet with L-tyrosine, that in cold-stressed mice was decreased (29). Our study indicates that the locomotor counts in cold-stressed mice were lower than those in controls, and were attenuated by the reduction of the catecholamine level. Combining these behavioral findings with the above biochemical and immunohistochemical findings, we suggest that cold stress enhances DA synthesis in the brain and subsequently increased DA suppress locomotor activity.

On the other hand, we also confirmed that the biochemical cerebrum DA levels and immunohistochemical DA levels in the neostriatum and nucleus accumbens septi regions were significantly increased by the IP administration of $CaCl_2$. This result indicates that the effect of cold stress on brain DA levels closely resembles the effect of the administration of $CaCl_2$. Also, cold stress-dependent locomotor suppression was attenuated by the IP administration of EDTA, and was further aggravated by $CaCl_2$. These results suggest that the calcium level is increased in cold-stressed mice and that increased calcium may involve the central calcium-calmodulin-dependent catecholamine synthesizing system. This theory is supported by the biochemical findings in this study and in previous reports (11,17) that the calcium level in the serum and brain is increased following cold stress. Also, Mooney et al. (13) have reported increased fluctuating bone asymmetry in neonates as a function of prenatal cold, heat and audiogenic stress. We think that the conclusion drawn in this study, i.e., calcium-dependent activity is enhanced in cold stress, may be applicable for elucidating on a wide range of stress diseases because calcium regulates various functions in organisms. This idea is supported by reports that cold stress-dependent gastric lesions are attenuated by calcium-channel blockers (8,32). Several investigators have observed that various types of stress accelerate the turnover of DA in mice and rats brains (3, 4, 7, 16), concurrently others have reported that they retard it (1, 5, 6, 14). Further study in this area is required, as the conclusion in the present study must be confirmed from the standpoint of the DA turnover rate in cold-stressed animals. In addition, the effect of cold environment on the periphery, e.g., muscular tone and blood circulation, should be confirmed.

In conclusion, we suggest that cold stress enhances the calcium level and then the increased calcium enhances DA synthesis in the brain through a central calcium-dependent catecholamine synthesizing system and successively increased DA induces behavioral changes.

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