

## The Effects of Immobilization Stress on the Synthesis and the Catabolism of Dopamine and Norepinephrine in the Rat Hypothalamus<sup>†</sup>

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**= Abstract = Immobilization stress was adopted as a prototype stress model for studying the effects of stress on dopaminergic and noradrenergic neuronal activity in the rat hypothalamus. Norepinephrine content was significantly increased after 4 hours of immobilization stress. Also, the turnover rates of both dopamine and norepinephrine were found to be significantly increased after the stress, providing the evidence of increased synthesis rates of both neurotransmitters. These findings were consistent with the increase in plasma corticosterone and catecholamine levels. The activities of tyrosine hydroxylase and dopamine- $\beta$ -hydroxylase, the synthesizing enzymes for catecholamines, were significantly increased after the stress, while that of monoamine oxidase, the catabolizing enzyme, did not change to a significant degree at all. Kinetic analysis of tyrosine hydroxylase, the rate-limiting step in catecholamine biosynthesis, revealed that  $V_{max}$  was significantly increased after the stress without significant change of  $K_m$ . These findings suggest that dopamine and norepinephrine may play a significant role in mediating stress responses by increasing their neuronal activities.**

*Key Words: Immobilization stress, Dopamine, Norepinephrine, Turnover rate, Tyrosine hydroxylase, Dopamine- $\beta$ -hydroxylase, Monoamine oxidase*

### INTRODUCTION

Decades of research have shown that stress induces significant changes in our bodily processes and consequently acts as a

possible cause of physical illnesses as well as mental ones. It is well known that the central nervous system plays an essential role in mediating stress responses. However, the exact mechanism of the central nervous system in mediating stress responses has not been clarified sufficiently as yet.

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Studies on stress effects historically started with the adrenal glands and revealed that stress causes increased secretion of adrenal hormones such as cortisol and catecholamines (CAs). And the majority of the studies have been focused on the peripheral effects of stressful stimuli (Kvetnansky and Mikulaj 1970; McCarty and Kopin 1978). With the advent of neuroscience and the development of sophisticated technology, researchers on

stress began to shift their zone of interest to the central nervous system(Roth *et al.* 1982).

Among the studies done on the central nervous system, the effects of stress on the hypothalamo-pituitary-adrenal axis have received the most consistent attention. It has been revealed that stressful stimuli activate the axis in humans as well as in animals(Hennessy and Levine 1979; Sourkes 1983). It is generally recognized that the adrenal cortical function changes during short-term and long-term adaptation responses to stressful stimuli. These responses are known to be mediated by the hypothalamo-pituitary-adrenal axis, with adrenocorticotrophic hormone(ACTH) secretion regulated by corticotropin-releasing factor(CRF) (Weiner and Ganong 1978). CRF secretion is known to be regulated by neurotransmitters and the CRF structure has been identified(Vale *et al.* 1981). The relationship between neurotransmitters and the hypothalamo-pituitary-adrenal axis has been widely studied: monoamines and the circadian rhythm of corticosteroid(Lee *et al.* 1982), serotonin and the hypothalamo-pituitary-adrenal axis(Suh and Park 1983; Suh *et al.* 1983), and CAs and serotonin and ether stress(Suh *et al.* 1986).

However, the studies on neurotransmitters and stress so far have not produced consistent findings. For example, regarding stress effects on dopamine(DA), there have been conflicting results of increased content(Goldstein *et al.* 1980), decreased content(Herve *et al.* 1979), and no change(Gordon *et al.* 1966; Bliss *et al.* 1968; Carr and Moore 1968; Gibson *et al.* 1969). The effects of stress on norepinephrine(NE) is no exception with results of decreased content (Bliss *et al.* 1968; Carr and Moore 1968; Palkovits *et al.* 1975) and increased content (Roth *et al.* 1982).

These contradictory conclusions seem to have been derived from limitations in the methodology adopted in the studies reviewed. First, despite the fact that content changes can be reflections of various factors involved in catecholamine(CA) synthesis and catabolism and do not always reflect the activity of the

involved nervous system per se, simple measurement of content change was mainly adopted in the previous studies. Secondly, stress induction methods have varied greatly, such as surgery(Van Loon *et al.* 1971), electric shock including footshock(Thierry *et al.* 1976; Fadda *et al.* 1978; Lavielle *et al.* 1978; Herve *et al.* 1979; Herman *et al.* 1982), cold stress (Zigmond *et al.* 1974), drugs(Lidbrink *et al.* 1972; Dairman and Udenfriend 1970), insulin-induced hypoglycemia(Weiner and Mosimann 1970), food deprivation(Knott *et al.* 1973), subcutaneous injection of formalin(Palkovits *et al.* 1975), ether stress(Hedge *et al.* 1976; Smythe *et al.* 1983), and cold swimming(Hedge *et al.* 1976; Roth *et al.* 1982; Smythe *et al.* 1983). Stress effects may depend on the method of induction, stressor intensity, and duration of the applied stressor.

Considering these factors, we in this study tried to further elaborate the mediating mechanisms of stress by the CA system, determining the CA(DA and NE) turnover rates and the activities of CA synthesizing enzymes, tyrosine hydroxylase(TH) and dopamine- $\beta$ -hydroxylase (DBH), and CA catabolizing enzyme, monoamine oxidase(MAO), as well as the simple measurement of CA contents. As a prototype stress model, we employed 4-hour immobilization in our study.

## MATERIALS AND METHODS

### Animals

Male Wistar rats(wt. 200-250 g), raised under controlled conditions at Seoul National University Laboratory Animal Service, were used. The animals were housed five in a cage in a constant temperature room(20-25°C) with a 12 hour light-dark cycle(lights on from 7:00 am to 7:00 pm) and were given commercial ratchow and tap water ad libitum. They were allowed to acclimatize to the conditions of a quiet laboratory for at least 1 hour before starting experimental procedures. They were exposed to minimum stimuli during transport. We started all experiments between 1:00pm and 2:00pm.

Forced immobilization was adopted as stressor, i.e., binding four legs on a hard board in the supine position for 4 hours. After that, the rats were sacrificed by neck dislocation and decapitation. Blood for measuring plasma corticosterone was collected from severed neck blood vessels into a heparinized tube. The whole brain was immediately extracted and the hypothalamus dissected out at 0°C (Glowinski and Iversen 1966).

#### **Determination of corticosterone and CA contents in plasma, and DA and NE contents in the hypothalamus**

Plasma corticosterone was measured with the spectrofluorometric method of Zenker and Bernstein (1958). Plasma CA content and hypothalamic DA and NE contents were measured by fluorometric assay as described by Ansell and Beeson (1968).

#### **DA and NE turnover rate measurements in the hypothalamus**

CA turnover rates were determined using alpha-methyl-para-tyrosine, a selective antagonist of TH, as an inhibitor of CA synthesis. Rats were sacrificed by decapitation at pre-defined time intervals (0, 1, 2, 4 hours) after injection of alpha-methyl-para-tyrosine (250 mg per kg, intraperitoneal). The hypothalami were rapidly removed and frozen on dry ice and kept at -25°C until used. The rate constant ( $\text{hr}^{-1} \pm \text{SE}$ ) and turnover time ( $\text{hr} \pm \text{SE}$ ) were calculated using the exponential decline slope described by Brodie *et al.* (1966). The synthesis rate was calculated by multiplying steady state level and rate constant of amine loss.

#### **Determination of TH, DBH, and MAO activities**

Hypothalamic tissue was weighed and homogenized. After centrifugation at 6,000xg for 10 minutes, the supernatant was used for assaying TH and DBH. The precipitated pellet was dissolved again and used for measuring MAO activity. Protein in the hypothalamic tissue was measured as described by Lowry *et al.* (1951).

TH activity was measured radiochemically

by the method of Reis *et al.* (1975). TH activity was defined by the amount of dihydroxyphenylalanine (DOPA) converted from tyrosine per minute. For measuring DBH activity, dual-wavelength spectrophotometry described by Kato *et al.* (1974) was used. DBH activity was defined as the amount of octopamine converted from tyramine per minute.

MAO activity was determined radiochemically with the method described by Fowler *et al.* (1979). MAO activity was defined as the amount of phenylacetic acid converted from phenylethylamine per minute.

#### **Statistical analysis**

Student's t-test (paired) was used in comparing plasma corticosterone level and CA content, hypothalamic DA and NE contents and turnover rates, and activities of TH, DBH and MAO, between the non-stressed control and the stressed groups.  $V_{\text{max}}$  and  $K_m$  were determined using the least-squares method in linear regression. All measured values were expressed as mean  $\pm$  SE.

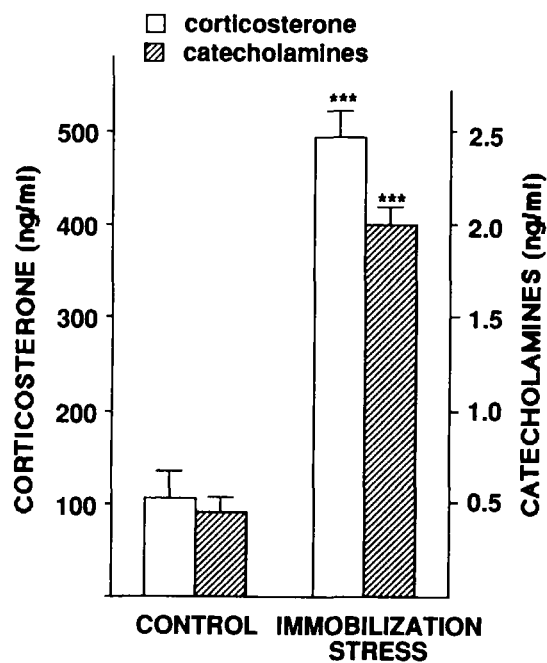
## **RESULTS**

#### **Change of plasma corticosterone and CA contents after immobilization stress**

In stressed rats, the plasma corticosterone content showed an increase to  $494.0 \pm 27.9$  (mean  $\pm$  SE) ng/ml of plasma compared to  $104.9 \pm 30.2$  ng/ml of plasma in the controls, with a 4.7 fold increase. Comparison of the control and the stressed groups showed a 4.4 fold increase of plasma CA content in the stressed group ( $1.997 \pm 1.102$  ng/ml of plasma vs.  $0.452 \pm 0.080$  ng/ml of plasma) as shown in Fig. 1 ( $p < 0.01$  by Student's t-test, paired).

#### **Change of DA and NE contents and turnover rates in the hypothalamus after stress**

Immobilization stress did not cause a significant change in hypothalamic DA content from the control level ( $1.017 \pm 0.090$   $\mu\text{g/g}$  of tissue vs.  $0.974 \pm 0.101$   $\mu\text{g/g}$  of tissue) (N.S. by Student's t-test, paired). However, NE content



**Fig. 1.** Plasma corticosterone and catecholamines after immobilization stress in the rat. Results are expressed as ng per ml of plasma(mean  $\pm$  SE). Groups consisted of 10 animals, assayed individually in duplicate samples.

\*\*\* P<0.001 compared to non-immobilized control rats

increased significantly in the stressed group ( $1.836 \pm 0.136 \mu\text{g/g}$  of tissue vs.  $1.578 \pm 0.123 \mu\text{g/g}$  of tissue)(Table 1,  $p < 0.01$  by Student's t-test, paired).

**Table 1.** Levels of dopamine(DA) and norepinephrine(NE) in the rat hypothalamus before and after immobilization stress

|                       | DA                | NE                     |
|-----------------------|-------------------|------------------------|
| Control               | $0.974 \pm 0.101$ | $1.578 \pm 0.123$      |
| Immobilization stress | $1.017 \pm 0.090$ | $1.836 \pm 0.136^{**}$ |

Results are expressed as  $\mu\text{g/g}$  of tissue(mean  $\pm$  SE). Groups consisted of 10 animals, assayed in duplicate samples.

\*\*  $p < 0.01$  compared to control

Regarding DA turnover, rate constant in the stressed group significantly increased to  $0.993 \pm 0.113$  per hour compared to  $0.731 \pm$

$0.047$  per hour in the controls, with an increase of 35.8%. Calculated turnover time decreased significantly to  $1.007 \pm 0.095$  hours by 26.4% in the stressed group compared to  $1.368 \pm 0.142$  hours in the controls. Synthesis rate increased to  $1.010 \pm 0.134 \mu\text{g/g/hr}$  by 41.9% in the stressed group compared to the controls(Table 2,  $p < 0.01$  by Student's t-test, paired).

**Table 2.** Kinetic parameters of DA turnover in the rat hypothalamus before and after immobilization stress

|                       | rate constant<br>( $\text{hr}^{-1} \pm \text{SE}$ ) | turnover time<br>( $\text{hr} \pm \text{SE}$ ) | synthesis rate<br>( $\mu\text{g/g/hr} \pm \text{SE}$ ) |
|-----------------------|---|--|--|
| Control               | $0.731 \pm 0.047$                                   | $1.368 \pm 0.142$                              | $0.712 \pm 0.069$                                      |
| Immobilization stress | $0.993 \pm 0.113^{**}$<br>(135.8%)                  | $1.007 \pm 0.095^{**}$<br>(73.6%)              | $1.010 \pm 0.134^{**}$<br>(141.9%)                     |

The values represent mean  $\pm$  SE of 5 animals of two different experiments and were measured from the decline of DA after alpha-methyl-para-tyrosine administration(250 mg/Kg).

( ): % of control

\*\*  $p < 0.01$  compared to control

Rate constant of NE in the stressed group increased by 60.6% compared to the controls ( $0.342 \pm 0.037$  per hour vs.  $0.213 \pm 0.042$  per hour). Turnover time decreased significantly after stress by 37.3% to  $2.928 \pm 0.311$  hours compared to  $4.669 \pm 0.392$  hours in the controls. Synthesis rate increased significantly by 85.5% to  $0.627 \pm 0.090 \mu\text{g/g/hour}$  in the stressed group compared to  $0.338 \pm 0.045 \mu\text{g/g/hour}$  in the controls(Table 3,  $p < 0.01$  by Student's t-test, paired).

**Change of TH, DBH and MAO activities in the hypothalamus after stress**

In the stressed group, TH activity increased markedly by 63% to  $48.22 \pm 4.51$  pmoles/mg protein compared to  $29.58 \pm 3.76$  pmoles/mg protein in the controls, as shown in Fig. 2( $p < 0.01$  by Student's t-test, paired). DBH also showed an increased activity after stress to  $112.2 \pm 6.51$

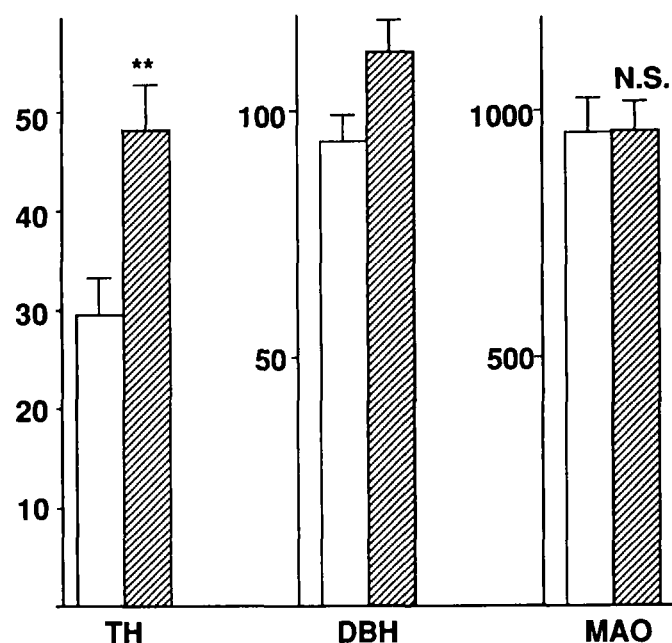
**Table 3.** Kinetic parameters of NE turnover in the rat hypothalamus before and after immobilization stress

|                       | rate constant<br>(hr <sup>-1</sup> ± SE) | turnover time<br>(hr ± SE) | synthesis rate<br>(μg/g/hr ± SE) |
|-----------------------|--|----------------------------|----------------------------------|
| Control               | 0.213 ± 0.042                            | 4.669 ± 0.392              | 0.338 ± 0.045                    |
| Immobilization stress | 0.342 ± 0.037**<br>(160.6%)              | 2.928 ± 0.311**<br>(62.7%) | 0.627 ± 0.090**<br>(185.5%)      |

The values represent mean ± SE of 5 animals of two different experiments and were measured from the decline of NE after alpha-methyl-para-tyrosine administration(250 mg/Kg).

( ): % of control

\*\* P < 0.01 compared to control



**Fig. 2.** Tyrosine hydroxylase(TH), dopamine-β-hydroxylase(DBH) and monoamine oxidase(MAO) activities in the rat hypothalamus before and after immobilization stress. Results are expressed as pmoles/mg protein/min. Values are means ± SE for group of 10 rats.

□ control group

▨ stress group

\* p < 0.05 compared to control

\*\* p < 0.01 compared to control

N.S. non-significant

pmoles/mg protein compared to 94.0 ± 5.45 pmoles/mg protein in the controls, but with only a moderate increase of 19.4% (Fig. 2, p < 0.05 by

Student's t-test, paired).

On the contrary to the increased activities of synthesizing enzymes, the activity of MAO, the catabolizing enzyme, did not show significant stress effects (959.7 ± 60.8 pmoles/mg protein vs. 956.2 ± 69.7 pmoles/mg protein) (Figure 2).

### Change of kinetic parameters of TH in the hypothalamus after stress

No significant change of Km was observed in the kinetic analysis of hypothalamic TH activity in the stressed group compared to the controls. On the contrary, Vmax showed a significant increase of 55.8% in the stressed group compared to the controls (Table 4, p < 0.01 by Student's t-test, paired).

**Table 4.** Kinetic parameters of tyrosine hydroxylase activity in the rat hypothalamus

|                       | Km<br>(μM)   | Vmax<br>(pmoles/mg/min)   |
|-----------------------|--------------|---------------------------|
| Control               | 257.5 ± 27.2 | 94.2 ± 5.7                |
| Immobilization stress | 264.0 ± 39.1 | 146.8 ± 5.3**<br>(155.8%) |

The values represent mean ± SE of 10 animals.

( ): % of control

\*\* p < 0.01 compared to control

## DISCUSSION

Considerable progress has been made in the past two decades on the elucidation of the role of neurotransmitters in mediating stress effects. Among the neurotransmitters widely studied, we now know that serotonin content changes in stress situations: it has been shown that stress increases 5-hydroxyindoleacetic acid content and the turnover rate of brain serotonin (Bliss *et al.* 1968; Curzon and Green 1969; De Souza and Van Loon 1986). The role of CA in modulating neuroendocrine functions in the hypothalamus has also been studied (George and Van Loon 1982). However, many details still remain to be clarified regarding the role of neurotransmitters in mediating stress responses.

We have noted that most studies have put emphasis on the simple content change of neurotransmitters with little attention paid to further elaboration of the related mechanisms. As indicated in the literature reviewed and the inconsistent findings observed, simple measurements of content change do not reveal sufficient information regarding the activity of a specific neuronal system. For example, even when synthesis is slowed down, if secretion is decreased further, the net content change could be measured as increased and vice versa.

Also, stress induction methods and duration of the stressor applied have not been standardized across the studies. The variability of stress induction methods may have affected metabolism by the particular method per se. For example, it would be difficult to determine which one contributes more between drug effect causing biochemical change in the brain and physical stimuli of being injected. Electrical stimuli may potentially affect neuronal functions and may not be considered as a neutral stimulus. Therefore, stress induction methods possibly affect the study outcome and it should be taken into account as an important variable when interpreting the findings (Hedge *et al.* 1976; Smythe *et al.* 1983). Even with the same stressor, duration of application of the stressor may also confound the outcome (Roth *et al.* 1982), making comparisons of the research outcome difficult. It has been already noted that content change can vary depending on the duration of the stressor applied (Curzon and Green 1969; Curzon and Green 1971; Kvetnansky *et al.* 1977).

Therefore, we were interested in studying the following points in this experiment: First, in order to elaborate the role of CA in the hypothalamus, which is known to react sensitively to stressors, we did not rely solely on the simple measurement of CA contents. We also included measurement of CA turnover rates, which contribute very significantly to the content change. Second, we also measured all the major enzymes involved in the synthesis and the ca-

tabolism of CA, i.e., TH, the synthesizing enzyme, DBH, the enzyme involved during the conversion of DA into NE, and MAO, the major CA catabolizing enzyme. Third, the immobilization stressor was adopted as a prototype model stressor (Zebrowska-Lupina *et al.* 1990; Hirano *et al.* 1991), in order to isolate stress effects from unnecessary confounding effects. Duration of the stressor applied was determined as 4 hours depending on the preliminary study. We also confirmed the validity of immobilization as a potent stressor by showing the increase of corticosterone and CA in plasma in the stressed group vs. the controls.

NE content was significantly increased after stress, while DA showed no significant content change after stress. One may simply speculate then that NE activity is much more facilitated under stress than DA activity (Van Loon *et al.* 1971; Ganong *et al.* 1976). However, there seem to be certain factors which need to be considered. It should be taken into account that NE neurons are more densely distributed in the hypothalamus than DA neurons and are probably more available to react to stress. Also, the duration of stress, i.e., 4 hours, may be long enough to permit a substantial amount of DA to be converted to NE (Stone 1973).

The advantage of measuring turnover rates is evident with the observation in the stressed group that both DA and NE turnover rates were increased significantly while the steady state content change was noted only in NE. Therefore, it is certain that turnover rate measurement can provide us with more reliable information regarding the neuronal activity of the neurotransmitter system involved.

Further elaboration of turnover rate changes by measuring the enzyme activities involved showed that TH activity, the physiological rate-limiting step in CA synthesis (Nagatsu *et al.* 1964), and DBH activity, were enhanced under immobilization stress. The increased TH activity is thought to be an adaptation process to the stressor, increasing CA output without decreasing steady-state CA content. DBH activity increase is also regarded as an adaptation mechanism

for rapid conversion of NE from DA. However, as shown in Fig. 2, TH seems to have a dominant role over DA.

Measurements of Vmax (representing maximal specific activity of enzyme) and Km (representing substrate affinity) of TH provided further elaboration on the catecholaminergic mechanism of mediating stress responses. Using the kinetic concepts, increased TH activity observed could be interpreted in two steps: 1) TH increasing its affinity to cofactor (co-substrate) under stress situation; 2) TH increasing the absolute amount of enzyme under stress situation. The first step seems to occur in short-term adaptation to stress and the second one in long-term adaptation (Joh *et al.* 1973; Fluharty *et al.* 1985). It seems that the choice of a certain step depends on the stress-induction method and the stress duration. In this study, considering the duration of the stressor applied, i.e., 4 hours, it is probable that at the initial phase increased affinity for cofactor was adopted. However, as the process went on, the second step, the increase of the absolute amount of the enzyme took over and became the major mechanism involved. This speculation is supported by the finding in our study that a significant increase of Vmax was observed without a significant change of Km.

Depending on the observation that MAO activity did not show a significant change after stress, it may be possible that MAO does not play a significant role in stress adaptation. Adaptation to stress seems to involve an active increase of synthesizing enzyme activity and consequently an increased amount of synthesis rather than a decreased catabolic rate by decreasing catabolic enzyme activity (Stone 1973).

In summary, we observed findings supporting the presence of DA and NE synthetic changes in the hypothalamus under immobilization stress. Increases in turnover rates of DA and NE were evidently observed. More specifically, synthesizing enzyme activities (TH and DBH), major determinants of turnover rate, were found to be significantly increased. Finally, kinetic

analysis of TH revealed increased synthesis of enzyme amount acting as a mediating mechanism. Catabolizing enzyme (MAO) did not seem to be a major contributing factor to stress adaptation. These observations provide further elaboration on the activity of the DA and NE neuronal systems under a stress situation.

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