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The Ventral Ascending Noradrenergic Bundles Are Involved in the Stress Response to Immobilization in Rats

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Abstract

Stressful stimuli induced by immobilization are perceived as acute stress in rats. This acute stress activates corticotropin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus (PVN), resulting in stimulation of the hypothalamic-pituitary-adrenal (HPA) axis. The ventral ascending noradrenergic bundles (V-NAB) from the brainstem innervate the PVN. To investigate the relationship between the response of the HPA axis and the V-NAB, we examined changes in plasma corticosterone, the final output of the HPA axis, and extracellular noradrenaline (NA) in the PVN following immobilization stress in rats that received bilateral 6-hydroxydopamine (6-OHDA) lesions of the V-NAB. 6-OHDA microinjection into the V-NAB reduced the magnitude of the responses of plasma corticosterone and extracellular NA in the PVN following immobilization stress. Our results suggest that V-NAB innervation of the PVN is involved in immobilization stress-induced activation of the HPA axis.

Keywords

Hypothalamus-Pituitary-Adrenal Axis, Immobilization Stress, 6-Hydroxydopamine, Ventral Ascending Bundles of Noradrenergic Neurons, Rat

1. Introduction

Stressful stimuli activate neurons in the hypothalamic paraventricular nucleus (PVN), which produce and release corticotropin-releasing hormone (CRH) [1]. CRH induces the anterior pituitary to secrete adrenocorticotropic

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hormone (ACTH), which in turn stimulates the adrenal cortex to secrete plasma corticosterone as the final output of the hypothalamic-pituitary-adrenal (HPA) axis. The PVN plays an important role in the regulatory mechanisms of the endocrine and autonomic systems and in adaptive behavior to a stressful environment [2].

Noradrenaline (NA)-containing cell bodies innervating the PVN are located in the medulla oblongata (A1 and A2 cell groups) and the locus coeruleus (A6 cell group) [3]. The ventral ascending noradrenergic bundles (V-NAB) include fibers from all three brainstem groups and constitute a major noradrenergic pathway that supplies the PVN [3].

Many studies have examined the role of the V-NAB in the response of the HPA axis to some types of stress [4]-[6]. For examples, medullary catecholamine innervation of the hypothalamus plays an important role in modulating the stimulatory effect of alcohol stress on the HPA axis [4]. And a conditioned emotional (fear) stress-induced increase in plasma corticosterone concentration is mediated by catecholaminergic nerves into the PVN [5] [6]. On the other hand, a differential involvement of the hypothalamic noradrenergic innervation upon the HPA axis according to the nature of stress conditions has been reported [7]. We previously reported that immobilization affects the HPA axis in the rat, indicating that immobilization is perceived as acute stress [8]-[10]. However, no reports appear to have examined the relationship between changes in plasma corticosterone levels and NA levels in the PVN following immobilization stress or the relationship between these changes and the V-NAB. It is important to examine this relationship in understanding the effect of immobilization stress on the cardiovascular, neuroendocrine, behavioral and metabolic responses.

In this study, we investigated the role of the V-NAB in the response of the HPA axis to immobilization stress using 6-hydroxydopamine (6-OHDA), a specific and long-lasting catecholamine neurotoxin.

2. Materials and Methods

2.1. Animals

Male Wistar rats were bred and reared in our animal facility in which environmental conditions were controlled as follows: temperature, 22˚C ± 1˚C; humidity, 60% ± 5%; and a 12-h light: dark cycle (lights on from 06:00 to 18:00). Light intensity at the cage surface was approximately 100 lux. Just after delivery, the body weights of all pups were measured to standardize the weight of each litter. The size of each litter was adjusted to six males until weaning. Rats were fed commercial rat chow MF (Oriental Yeast, Tokyo, Japan; 3.6 kcal/g) and tap water ad libitum. After weaning, rats were reared in individual cages (22 × 13 × 15 cm). The body weight of each rat was 300 - 400 g at the beginning of the experiment. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Hokkaido University Graduate School of Dental Medicine.

2.2. Blood Sampling and Determination of Corticosterone

Blood sampling was performed as described previously [11]. A small incision was made at the tip of the freely moving rat tail using a razor blade 1 h before the beginning of blood sampling. About 50 µl of blood was collected from the incision into a heparinized capillary tube. Blood sampling was finished within 0.5 min after the first touch of the rat's tail. Snipping the tail was only necessary for the first blood sample. Separated plasma was frozen at −80˚C until the corticosterone assay. Plasma corticosterone levels were determined using the competitive protein binding assay using 10 µl of the sample. The minimum detectable level of corticosterone was 0.125 ng (1.25 µg/dl plasma). Intra- and inter-assay variabilities were 3.2% and 6.5%, respectively.

2.3. Sampling of Extracellular Fluid and Determination of Noradrenaline

Extracellular fluid near the PVN was collected from freely moving rats using *in vivo* microdialysis. Two days before the start of sampling, each rat was anesthetized with pentobarbital sodium (5.0 mg/kg body weight ip), and a guide cannula was implanted just above the PVN and fixed to the skull with dental resin. The stereotaxic coordinates were 3.3 mm posterior to the bregma, 0.5 mm lateral from the midline, and 5.7 mm below the brain surface. Dialysis probes were constructed as described previously [12]. On the day of sampling, the dummy probe was replaced with a dialysis probe that was continuously perfused with Ringer's solution at a flow rate of 1.0 µl/min using an infusion pump (EP-60, Eicom, Kyoto, Japan). The dialysate was collected into sampling tubes containing 100 µl 0.1 N perchloric acid at 1-h intervals. Samples were stored at −80˚C until the NA assay.

The NA concentration was determined with high-pressure liquid chromatography with electrochemical detec-

tion as reported previously [12]. The column was 4.5 × 150 mm (Eicopack CA-50DS, Eicom, Kyoto, Japan), and the mobile phase was 0.1 M phosphate buffer (pH 6.1) containing 400 mg sodium 1-octanesulfonate, 12 mg/l EDTA, and 0.6% methanol. The minimum detectable amount of NA was 1.0 pg.

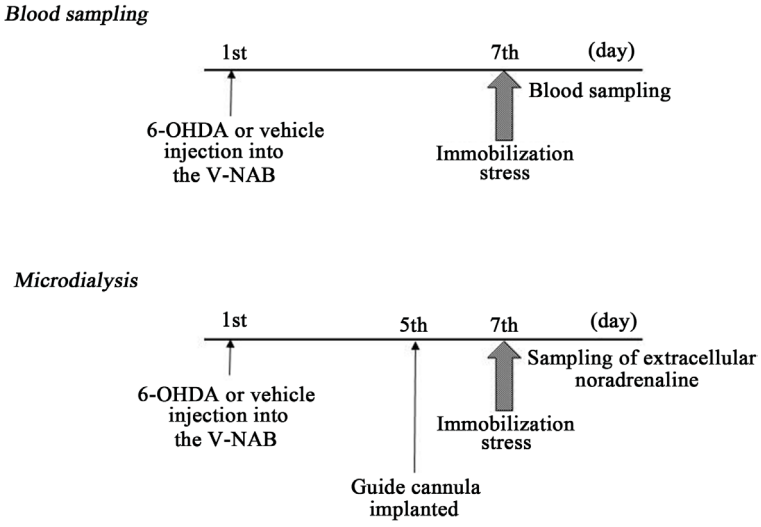
2.4. Microinjection of 6-Hydroxydopamine

Microinjection of 6-OHDA (Sigma, St. Louis, MO) into the V-NAB in the midbrain was performed stereotaxically using an infusion pump. Each rat was anesthetized with pentobarbital sodium (5.0 mg/kg body weight ip), and a small hole was opened on the skull with a dental drill. A 30-gauge needle was inserted into the V-NAB in the midbrain, and 6-OHDA (6 µg dissolved in 0.2 µl physiological saline solution containing 0.1% ascorbic acid) was injected at a flow rate of 1.0 µl/min. The stereotaxic coordinates were 7.5 mm posterior to the lambda, 0.5 mm lateral from the midline, and 8.5 mm below the brain surface. Bilateral microinjection was performed during the light period. Vehicle was injected into the other experimental group using exactly the same procedure.

The effects of 6-OHDA were examined by measuring the NA concentration in the PVN immediately after completion of the experiment. Brain tissues were obtained following decapitation, which was completed within 1 min of the first touch of the rat cage. Immediately after decapitation, the whole brain was removed from the skull within 30 sec and frozen on a glass plate placed on a dry ice block with the ventral surface of the brain up. The portion of the brain containing the PVN was sliced into 800-µm-thick sections with a freezing microtome (CM1850, Leica, Tokyo, Japan), and the nuclei were bilaterally punched out with a needle puncher (inner diameter = 1.2 mm). The punched out tissues were immediately homogenized in homogenizing glass tubes containing 100 µl 0.1 M $HClO_4$ solution containing 1.0 µg 3, 4-dihydroxybenzylamine as an internal standard. Duplicate 10-µl samples of the homogenate were reserved for determination of the protein concentration with the Lowry method [13]. The homogenate was centrifuged (12,000 rpm, 45 min, 4˚C), and the supernatant was stored at −80˚C until the NA assay.

2.5. Experimental Protocol

In this study, we used 18 male rats bred from two mothers on the same day that were randomly divided into three groups (n = 6 each). These three groups included the unmanipulated control group (n = 6) and the experimental group (n = 12), which was divided into two sub-groups (n = 6 each) that were injected with 6-OHDA or vehicle. The experiment lasted for 7 days (**Figure 1**). On day 1, 6-OHDA or vehicle was microinjected into the V-NAB. At 10:00 on Day 7, immobilization stress was performed by taping the body of each rat to a plastic board so that the rat remained immobile for 30 min. Blood was sampled at 10:00 (before the start of immobilization stress, T0), 10:30 (T30), 11:00 (T60), 11:30 (T90), 12:00 (T120), 12:30 (T150), and 13:00 (T180). Body



6-OHDA, 6-hydroxydopamine; V-NAB, ventral ascending noradrenergic bundles.

Figure 1. Experimental protocol.

weight and food consumption were measured at 10:00 on each day of the experiment except Day 7. To measure concentrations of extracellular NA in the PVN, a microdialysis experiment was performed using another 12 male rats and the same experimental procedures (Figure 1). Extracellular fluid in the PVN in the three groups was sampled at 10:00 (before the start of immobilization stress, T0), 11:00 (T60), 12:00 (T120), and 13:00 (T180).

2.6. Statistical Analysis

Data were analyzed using SPSS Version 10 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) with post-hoc Bonferroni testing was used for comparison of the two mean values from different groups. Corticosterone and NA levels were analyzed with repeated-measures ANOVA, with the group (rats injected with 6-OHDA, rats injected with 6-OHDA or vehicle, and unmanipulated control rats) as the between-subjects variable and time after the exposure to immobilization stress (corticosterone levels: T0, T30, T60, T90, T120, T150, and T180; NA levels: T0, T60, T120, and T180) as the within-subjects variable. Post-hoc comparisons were made using Bonferroni testing.

3. Results

Figure 2 shows the NA concentration in the PVN in rats injected with 6-OHDA or vehicle and in the unmanipulated control rats. Six days after bilateral 6-OHDA microinjection into the V-NAB, the NA concentration in the PVN was significantly decreased by 77.5% and 77.1% compared with vehicle microinjection and the control, respectively (8.67 ± 2.75 pg/μg protein for rats injected with 6-OHDA vs. 38.83 ± 5.83 pg/μg protein for rats injected with vehicle vs. 37.83 ± 3.59 pg/μg protein for the unmanipulated control; $p < 0.01$; one-way ANOVA with post-hoc Bonferroni testing).

As shown in Table 1, 6-OHDA microinjection did not induce changes in body weight gain or food intake. The body weight gain and food intake in the three groups did not significantly differ at any stage of the experiment.

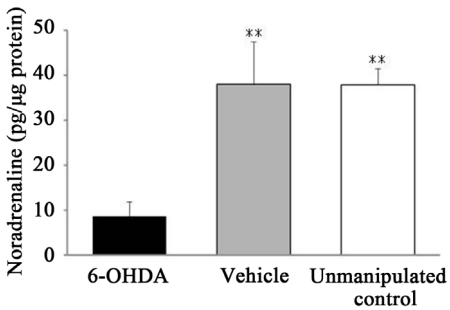


Figure 2. NA concentration in the PVN in rats injected with 6-OHDA or vehicle and in the unmanipulated control rats. Values are the means ± SEM (n = 6 per group). $^{**}p < 0.01$ vs. rats injected with 6-OHDA.

Table 1. Body weight and food intake in rats injected with 6-OHDA or vehicle and unmanipulated control rats.

	Body weight (%)			Food intake (g/100 g body weight/day)		
	6-OHDA	Vehicle	Unmanipulated control	6-OHDA	Vehicle	Unmanipulated control
Day 1	100	100	100	8.51 ± 1.41	8.95 ± 1.41	11.57 ± 1.00
Day 2	100.65 ± 0.80	100.69 ± 0.83	102.31 ± 2.17	8.24 ± 0.79	9.01 ± 1.46	11.21 ± 1.09
Day 3	101.57 ± 1.17	101.57 ± 1.17	103.81 ± 2.71	9.20 ± 0.74	9.99 ± 0.99	11.01 ± 0.72
Day 4	103.11 ± 0.89	103.40 ± 0.76	105.19 ± 2.03	11.32 ± 0.97	10.43 ± 1.34	10.87 ± 1.08
Day 5	104.78 ± 0.60	105.19 ± 0.81	106.78 ± 1.75	10.61 ± 1.22	10.58 ± 0.93	10.35 ± 1.53
Day 6	106.70 ± 1.25	107.13 ± 1.58	108.54 ± 1.78	-	-	-

Values are the means ± SEM (n = 6 per group). For example, food intake on Day 3 indicates food consumption from 10:00 on Day 3 to 10:00 on Day 4. Body weights were normalized to weights on Day 1.

Figure 3 shows the stress-induced plasma corticosterone levels. All groups experienced significant variations in plasma corticosterone levels as shown by the significant effect of time after exposure to immobilization stress ($F_{6,90} = 789.20$, $p < 0.01$), group ($F_{2,15} = 5.98$, $p < 0.01$), and time after immobilization stress × group interaction ($F_{2,90} = 1.80$, $p = 0.04$). Plasma corticosterone levels in rats injected with 6-OHDA or vehicle increased gradually, peaked at T30, and then decreased. Plasma corticosterone levels in rats injected with 6-OHDA returned to baseline levels (T0) at T120. However, plasma corticosterone levels in rats injected with vehicle remained higher at T180 than at T0 ($p = 0.04$). Plasma corticosterone levels were significantly lower in rats injected with 6-OHDA than in rats injected with vehicle at T30, T60, T150, and 180 ($p = 0.02$, 0.01, 0.02, and 0.02, respectively). Plasma corticosterone levels were significantly higher in rats injected with 6-OHDA than in unmanipulated control rats at T30 and T60 ($p = 0.05$ and 0.04, respectively). Plasma corticosterone levels were significantly higher in rats injected with vehicle than in unmanipulated control rats at T30, T60, T90, T120, T150, and T180 ($p < 0.01$, < 0.01, $= 0.02$, $= 0.03$, < 0.01, and $= 0.03$, respectively).

Figure 4 shows stress-induced NA levels in the PVN. All groups experienced significant variations in NA levels as shown by the significant effect of time after exposure to novel stress ($F_{3,45} = 5.87$, $p < 0.01$), group ($F_{2,15} = 7.92$, $p < 0.01$), and time after immobilization stress × group interaction ($F_{2,45} = 2.47$, $p = 0.05$). NA levels in rats injected with 6-OHDA or vehicle increased gradually, peaked at T60, and then decreased. NA levels returned to baseline levels (T0) at T120 in rats injected with 6-OHDA and at T180 in rats injected with vehicle. NA levels were significantly lower in rats injected with 6-OHDA than in rats injected with vehicle at T120 ($p = 0.05$). NA levels were significantly higher in rats injected with 6-OHDA than in unmanipulated control rats at T60 ($p = 0.05$). NA levels were significantly higher in rats injected with vehicle than in unmanipulated control rats at T60 and T120 ($p < 0.01$ and $= 0.03$, respectively).

4. Discussion

In the present study, 6-OHDA microinjection into the V-NAB decreased the levels of plasma corticosterone and extracellular NA in the PVN in response to immobilization stress. These results suggest that the V-NAB innervating the PVN are involved in immobilization stress-induced activation of the HPA axis.

Previously, we demonstrated that immobilization stress increases plasma corticosterone in rats [8]-[10]. Consistent with these previous studies, rats injected with vehicle and unmanipulated control rats in our current study

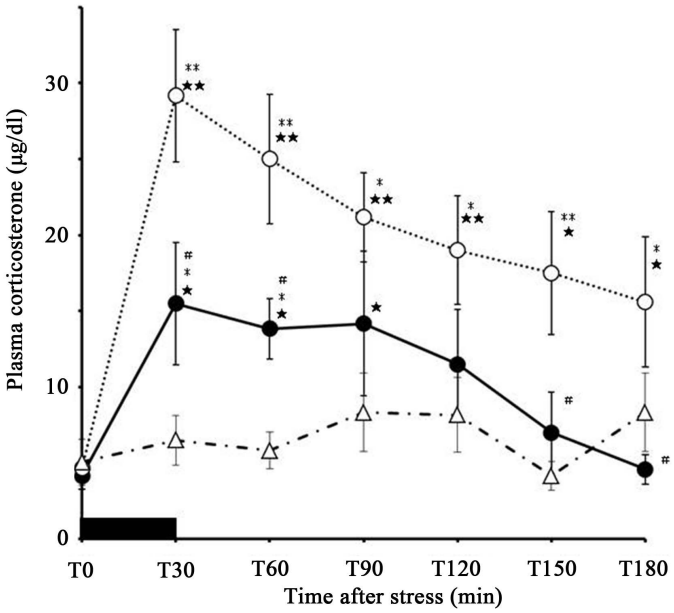


Figure 3. Plasma corticosterone levels in rats injected with 6-OHDA (●), rats injected with vehicle (○), and unmanipulated control rats (Δ). Values are the means ± SEM (n = 6 per group). ★$p < 0.05$; ★★$p < 0.01$ vs. concentration at T0. *$p < 0.05$; **$p < 0.01$ vs. unmanipulated control rats; #$p < 0.05$ vs. rats injected with vehicle. The black horizontal bar on the abscissa indicates the period of immobilization stress.

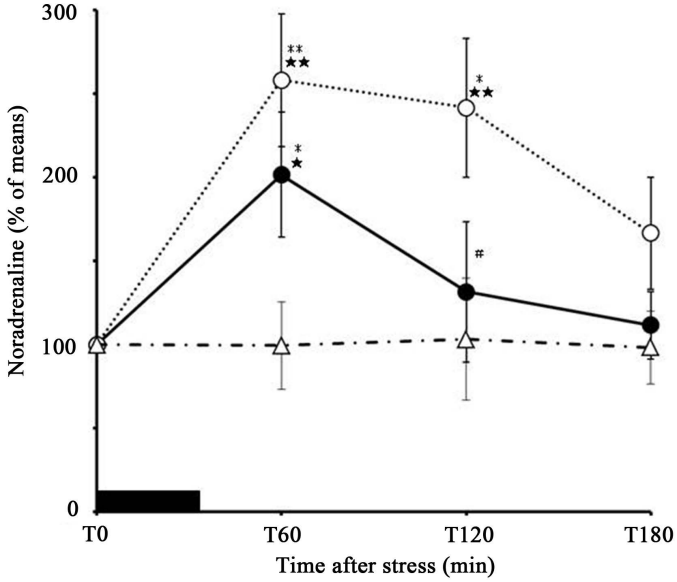


Figure 4. Noradrenaline levels in the PVN in rats injected with 6-OHDA (●), rats injected with vehicle (○), and unmanipulated control rats (△). Values are the means ± SEM (n = 6 per group). ★$p < 0.05$, ★★$p < 0.01$ vs. concentration at T0; *$p < 0.05$; **$p < 0.01$ vs. unmanipulated control rats; #$p < 0.05$ vs. rats injected with vehicle. The black horizontal bar on the abscissa indicates the period of immobilization stress.

also demonstrated increased plasma corticosterone and NA levels after immobilization stress. On the other hand, plasma corticosterone and NA levels in the rats injected with 6-OHDA were transiently affected and returned to basal levels 120 min after immobilization stress. The neurons of the medulla oblongata (A1 and A2 cell groups) and the locus coeruleus (A6 cell group) send important excitatory projections to the PVN [4] [14], which is the primary hypothalamic regulator of HPA axis function. NA released from these neurons activates CRH neurons in the PVN [14]. Our previous and current studies suggest that the PVN is associated with the regulation of pituitary-adrenocortical outflow and that the V-NAB from these three cell groups play an excitatory role in the PVN during the perception of acute stress such as immobilization stress.

The effect of changes in eating behavior and body weight gain by 6-OHDA injection are dependent on the site of injection [15]. For example, significant reductions in food intake and body weight throughout the period examined after 6-OHDA injection into either the lateral hypothalamic nucleus or ventromedial hypothalamic nucleus are well documented [16] [17]. On the other hand, a transient reduction [18] and no significant change [19] in food intake and body weight were found after 6-OHDA injection into the PVN. Furthermore, we previously reported no significant differences in food intake after 2-day food deprivation between rats injected with 6-OHDA and those injected with vehicle into the V-NAB [20]. In our current study, body weight gain and food intake in the three groups also did not differ significantly at any stage of the experiment. The transient reduction in food intake in rats injected with 6-OHDA or vehicle into the V-NAB compared with unmanipulated control rats found in this study may be due to damage caused by the surgery.

The involvement of the central stimulatory action of NA in the control of the HPA axis is well known. Noradrenergic neurons facilitate the electrical activity of tuberoinfundibular neurons of the PVN [21] and secretion of CRH [14]. Catecholaminergic stimulation is involved in the stress-induced release of ACTH [22]. Furthermore, loss of noradrenergic innervation of the hypothalamus with local microinjection of 6-OHDA leads to strong inhibition of stress-induced activation of the HPA axis after ether inhalation [23], photic, acoustic, or sciatic nerve stimulation [24], or emotional stress [6]. Our current study indicates that the V-NAB input to the PVN is associated with a stimulatory role of immobilization stress-induced activation of the HPA axis, similar to the above stressors or stimulations.

Compared with vehicle microinjection, the NA concentration in the PVN was reduced by about 70% with bilateral 6-OHDA microinjection into the V-NAB. Similar depletion of NA concentrations in the PVN has been reported in other studies [24]. The reason that 30% of the NA remained in this study is unknown. Insufficient

diffusion of the 6-OHDA solution and/or the presence of other noradrenergic neurons that project to the PVN such as the dorsal ascending bundles of noradrenergic neurons (D-VAB) to the PVN [25] [26] are possibilities. In fact, 6-OHDA microinjection into both the V-NAB and D-VAB induces an 85% reduction in NA concentration in the PVN [7].

Most of the V-NAB that project to the PVN originate from A1 (68%) and A2 (26%) cell groups in the medulla oblongata, and the rest are from the A6 cell group (6%) of the locus coeruleus [27]. Electrophysiological stimulation of A1 and A2 cell groups induces excitation of CRH neurons, whereas stimulation of the A6 cell group inhibits their activity [28]. On the other hand, a study using 6-OHDA microinjection showed that the locus coeruleus plays a different role in the HPA axis during acute stress or chronic stress [26]. Whether the stimulatory role of NA that is derived from the V-NAB on the HPA axis that was found in the present study is induced by each cell group or by interactions among these three cell groups is unknown. Further study is needed to examine which type of innervation (A1, A2, or A6) by these common noradrenergic bundles is important in immobilization stress-induced activation of the HPA axis.

5. Conclusion

In conclusion, our current study suggests that the V-NAB project to the PVN in the hypothalamus plays a major stimulatory role in the mechanism involved in stress-induced activation of the HPA axis.

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