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Differential effects of repeated immobilization stress in early versus late postnatal period on stress-induced corticosterone response in adult rats

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ABSTRACT

This study was performed in order to determine how immobilization stress in the early postnatal period or in the late postnatal period affects growth in the developing rat, and the response of the hypothalamus-pituitary-adrenocortical (HPA) axis in adult rats subjected to subsequent novel stresses. In addition, the effects of maternal deprivation (MD) within the same period of exposure to immobilization stress were also examined. We used two different types of immobilization stress and two different types of MD: immobilization stress for 30 min per day from postnatal day 7 (P7) to P13 (IS-E group); immobilization stress for 30 min from P15 to P21 (IS-L group); MD for 30 min per day from P7 to P13 (MD-E group); and MD for 30 min per day from P15 to P21 (MD-L group).

The IS-E group showed a significant reduction in body weight that was maintained until at least P40 when compared with the control group. On the other hand, the IS-L group showed a significant reduction in body weight at only postnatal day (P) 20 when compared with the control group. Furthermore, the IS-E group showed a larger HPA response to novel stress than the IS-L and control groups in adulthood. The MD-E group showed a significant reduction in body weight that was maintained until at least P20 when compared with the control group, but did not show a larger HPA response to novel stress, except at T30 (30 min after exposure to novel stress) than the control group in adulthood. The MD-L group did not show a

significant reduction in body weight or increased HPA response when compared with control rats. These results suggest that repeated immobilization stress, but not MD, in the early postnatal period induces long-term effects on growth and HPA response to novel stress in adulthood.

Keywords: repeated immobilization stress; corticosterone;
hypothalamus-pituitary-adrenocortical axis; rat

In mature rats, stress activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of corticotropin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus (PVN). CRH causes the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to secrete corticosterone [23]. In contrast, mechanisms of hormonal response to stress in immature rats are not fully understood [4]. In particular, mechanisms in early postnatal rats seem to differ from those in mature rats. From postnatal day (P) 4 to P14, rats display a stress hyporesponsive period (SHRP) in the form of a markedly attenuated response of the HPA axis to environmental stress [1].

It has been reported that repeated exposure to stress in adulthood increases the response of the HPA axis to subsequent stresses. For example, adult rats that experienced daily 20-min immobilization stress for 8 days show an exaggerated corticosterone response to forced swimming, a new stress they were subjected to soon after the termination of immobilization stress [7]. In addition, adult rats that experienced daily 3-h immobilization stress for 3 days also show an exaggerated corticosterone response to 2-deoxyglucose injection, a new stress they were subjected to 12 days after the termination of immobilization stress [10].

However, no reports have examined the dependence of age at the time of immobilization stress in the postnatal period on the response of the HPA axis to stresses experienced in adulthood. The purpose of this study was to determine how

immobilization stress in the early postnatal period (SHRP) or in the late postnatal period (post-SHRP) affects the growth of developing rats, and the response of the HPA axis in the adult rats to the subsequent novel stresses.

Male Wistar rats were born 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. Rats were fed commercial rat chow MF, (Oriental Yeast, Tokyo, Japan; 3.6 kcal/g) and tap water ad libitum, unless otherwise stated. Cyclicity of female rats was examined using vaginal smears. The day on which a female rat was found to be sperm-positive was designated as embryonic day 0, and the day of delivery was designated as postnatal day 0 (P0). Two days before the expected day of delivery, pregnant rats were housed individually in polycarbonate cages (36×33×17 cm). Just after delivery, body weights of all pups were measured and the members of a litter were rearranged so as to make the mean body weight of the litters equal. The size of each litter was adjusted to 6 males until weaning (P21). After weaning, pups were reared in individual cages (22×13×5 cm). 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.

Pups were removed from the home cage containing their nursing mother at 10:00 h. Immobilization stress was carried out by taping a plastic board onto the stomach of each rat so that the rat was kept immobile for 30 min. Pups were returned to their home cage at 10:30 h. Ambient temperature and humidity were kept at 33°C and 70%-80% during exposure to immobilization stress.

A rat in an individual cage was transferred to a new cage of the same size, which contained fresh wood chips at 10:00 h. For 1 week before novel stress exposure, the home cage was not cleaned. A rat transferred into a new cage immediately showed exploring behaviors such as sniffing, wandering and standing.

Blood sampling was undertaken 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 2.5 min after the first touch of the rat cage. It was only necessary to snip the tail for the first blood sampling. Separated plasma was frozen at -80°C until corticosterone assay.

Plasma corticosterone levels were determined by competitive protein binding assay using 10 μl of sample [22]. The minimum detectable level of corticosterone was 0.125 ng (1.25 μg/dl plasma). Intra- and inter-assay variances were 3.0% and 5.9%, respectively.

Experimental protocols are illustrated in Figure 1. Thirty male pups born on the same day were divided into the control group (n=6) and the experimental group (n=24). The experimental group was divided into 4 sub-groups (n=6 each) according to age at which immobilization stress was performed or the age at which maternal deprivation (MD) was performed: immobilization stress for 30 min per day from P7 to P13 (IS-E group); immobilization stress for 30 min per day from P15 to P21 (IS-L group); MD for 30 min per day from P7 to P13 (MD-E group); and MD for 30 min per day from P15 to P21 (MD-L group). The control group was not subjected to immobilization stress or MD, and was left undisturbed.

At 10:00 on the day when pups were considered to have reached adulthood (P60), they were exposed to novel stress. Blood was sampled at 10:00 (before the exposure to novel stress, [T0]), 10:30 (T30), 11:00 (T60), 11:30 (T90), 12:00 (T120), 12:30 (T150) and 13:00 (T180). Body weight of each rat was measured every week.

Data were analyzed using SPSS Version 10 (SPSS Inc., Chicago, IL). Body weights were analyzed by repeated-measures ANOVA, with group (IS-E group, IS-L group, MD-E group, MD-L group and control group) as the between-subjects variable and postnatal days (P0, P10, P20, P30, P40, P50 and P60) as the within-subjects variable. Corticosterone levels were analyzed by repeated-measures ANOVA, with group (IS-E group, IS-L group, MD-E group, MD-L group and control group) as the between-subjects variable and time after the exposure to novel

stress (T0, T30, T60, T90, T120, T150 and T180) as the within-subjects variable. Post-hoc comparisons were made using Bonferroni testing.

Figure 2 shows changes in body weight in the IS-E, IS-L, MD-E, MD-L and control groups. All groups gained weight over time, as shown by the significant effect of postnatal days ($F_{6,150}$=1854.13, $p < 0.01$), group ($F_{4,25}$=6.28, $p < 0.01$) and postnatal days × group interaction ($F_{4,150}$=17.70, $p < 0.05$). Body weights were significantly lower in the IS-E group than in the control group at P10, P20, P30 and P40 (p=0.01, p<0.01, p<0.01 and p=0.05, respectively). Body weights were significantly lower in the IS-L group than in the control group at P20 (p=0.01). Body weights were significantly lower in the MD-E group than in the control group at P10 and P20 (p<0.01, respectively). No significant differences in body weight were seen between the MD-L group and the control group. Body weights were significantly lower in the IS-E group than in the IS-L group at P10, P20 and P30 (p=0.03, p<0.01 and p<0.01, respectively).

Figure 3 shows stress-induced levels of plasma corticosterone in the IS-E, IS-L, MD-E, MD-L and control groups. All groups experienced significant variations in plasma corticosterone levels, as shown by the significant effect of time after exposure to novel stress ($F_{6,150}$=704.78, $p < 0.01$), group ($F_{4,25}$=11.67, $p < 0.01$), and time after exposure to novel stress × group interaction ($F_{4,150}$=15.47, $p < 0.05$). Plasma corticosterone levels in each group increased gradually, peaking at T30 or

T60, before decreasing. Plasma corticosterone levels in the IS-L, MD-L, MD-E and control groups returned to baseline levels (T0) at T120 or T150. However, plasma corticosterone levels in the IS-E group remained higher at T150 than at T0 (p=0.04). Plasma corticosterone levels were significantly higher in the IS-E group than in the control group at T30, T60, T90, T120 and T150 (p=0.02, p<0.01, p=0.01, p=0.02, and p=0.03, respectively). Plasma corticosterone levels were significantly higher in the IS-L group than in the control group at T60 (p=0.01). Plasma corticosterone levels were significantly higher in the MD-E group than in the control group at T30 (p=0.05). No significant differences in plasma corticosterone levels were seen between the MD-L group and the control group. Plasma corticosterone levels were significantly higher in the IS-E group than in the IS-L group at T90, T120 and T150 (p=0.03, p<0.01 and p<0.01, respectively).

The present study demonstrated that repeated immobilization stress in the early postnatal period induces long-term effects on growth and the response of the HPA axis to novel stress in adulthood. Rats subjected to repeated immobilization stress for 30 min from P7 to P13 (IS-E group) showed a significant reduction in body weight that was maintained until at least P40, as compared with the control group. On the other hand, rats subjected to repeated immobilization stress for 30 min from P15 to P21 (IS-L group) showed a significant reduction in body weight only at P20, as compared with the control group. Furthermore, the IS-E group showed a larger HPA

response to novel stress than the IS-L group and the control group in adulthood. The present study thus also indicated that a critical age exists in the postnatal period during which repeated immobilization stress will induce these effects.

Early life stress has been shown to be associated with enhanced anxiety-like behavior and dysfunction of the HPA axis [8]. In particular, early MD is an animal model of early life stress that permanently modifies neurobiological and behavioral parameters [27]. Among those modifications, the characteristics of the HPA axis have been the most extensively explored. Twenty-four hour MD enhances and/or prolongs stress-induced corticosterone release in young and adult male rats, particularly if deprivation is performed during the postnatal period [24]. In this study, the rat is removed from the home cage that contains the nursing mother and is immobilized. Thus, exposure to immobilization stress in the postnatal period might also induce effects associated with MD. Therefore, in this study, growth and stress response in rats subjected to immobilization stress were compared with rats subjected to MD. Rats subjected to repeated MD for 30 min from P7 to P13 (MD-E group) showed a significant reduction in body weight that was maintained until at least P20, as compared with the control group; however, they did not show a larger HPA response to novel stress than the control group in adulthood, except at T30. In addition, rats subjected to repeated MD for 30 min from P15 to P21 (MD-L group) did not show a significant reduction in body weight or increased HPA response when

compared with control rats. These results suggest that repeated immobilization stress, but not MD, in the early postnatal period induces long-term effects on growth and HPA response to novel stress in adulthood. The reason why repeated MD had no effect on HPA response to novel stress in this study is likely due to the short periods (30 min) of MD. These results are consistent with our previous reports showing that short periods (less than 3 h) of MD had little effect on growth and HPA response to novel stress [29]. An alternative explanation for the lack of an effect of MD in this study is related to age (from P7 to P13) of the pups at the time of MD, as the effects of MD are most prominent when MD is imposed in the first postnatal week [16].

The IS-E group showed a significant reduction in body weight that was maintained until P40 when compared with rats in the other 4 groups. Interestingly, body weight in the MD-E group also showed a significant reduction that was maintained until P20, but was similar to that in the control group at P30, although this group was denied maternal care for 30 min for 7 days along with the IS-E group. These results indicate that the reduced body weight gain in the IS-E group cannot be explained by decreased milk intake from the mother.

In the early postnatal period when the thermoregulatory system is not fully developed [13], an important source of heat in terms of thermoregulation of newborn rats is brown adipose tissue (BAT) [6]. The thermogenetic activity of BAT is regulated by the sympathetic nervous system [17]. Immobilization stress is reported

to increase sympathetic activity [5]. Therefore, increased sympathetic activity could be involved in the long-term effects of repeated immobilization stress on body weight changes in the present study. However, body weight in the IS-E group should catch up with that of rats in other groups after the termination of immobilization stress due to rebound hyperphagia [14]. Thus, it is unlikely that the long-term effects on body weight gain result from increased metabolism of BAT in the early postnatal period.

An alternative hypothesis is that repeated immobilization stress suppresses levels of circulating growth hormones. It is known that exposure to stress suppresses food intake [2]. Furthermore, increased sympathetic activity induced by stress suppresses feeding and drinking [26]. Malnutrition in the early postnatal period reportedly reduces the concentration of insulin-like growth factor I in adult rats [12]. Lower levels of growth hormones may affect development of rats subjected to repeated immobilization stress.

Although central mechanisms involved in the stress-induced inhibition of food intake have not been fully elucidated, stress-induced anorexia has been attributed to activation of the CRH and/or serotonin (5-hydroxytryptamine) pathways. Both of these transmitters are elevated in response to stress in a number of brain areas, including those that are involved in the regulation of feeding behavior [20].

Stress-induced corticosterone levels in adulthood were significantly higher in the IS-E group than in controls during the experiment, except at T180. Furthermore,

stress-induced corticosterone levels in the IS-E group did not return to baseline levels until at least 150 min after exposure to novel stress. However, temporal variations in stress-induced corticosterone levels in the IS-L group did not significantly differ from those in the control group, except at T60. These results suggest that repeated immobilization stress in the early postnatal period, as opposed to repeated immobilization stress in the late postnatal period, has a long-term effect on the responsiveness of the HPA axis to novel stress.

Although the mechanisms for this long-term effect on stress-induced corticosterone levels by repeated immobilization stress in the early postnatal period are unknown, modification of negative feedback regulation of the HPA axis may be involved. Glucocorticoid receptors in the hippocampus mediate the negative feedback effects on the HPA axis [15]. It has been shown that repeated stress produces structural and functional changes in the brain, particularly in the hippocampus [18]. The long-term effects of repeated immobilization stress in the early postnatal period on the HPA axis may be mediated by increased corticosterone secretion in rats subjected to repeated immobilization stress, modifying the expression of glucocorticoid receptors in the hippocampus and thereby decreasing negative feedback regulation of the HPA axis [19].

Gilles et al. reported that MD within the first postnatal week affects the HPA axis of developing rats [9]. Plotsky et al. reported that the first 2 weeks of

postnatal life in infant rats represent a critical period, with the HPA axis components involved in stress response becoming susceptible to modulation such as MD [25]. The results of these studies and the present investigation suggest that repeated immobilization stress and periodic MD from about the first week to the second week of the postnatal period have long-term effects on stress response of the HPA axis in rats.

Repeated exposure to a particular stress can result in habituation of the hormonal response to that same (homotypic) stress [28], or sensitization of the hormonal response to a novel (heterotypic) stress [21]. Both perceived stressfulness and stress adaptation have been characterized as being tightly coupled to relative activity of the HPA axis [3]. The findings seen in the present study are thought to indicate a type of sensitization. However, it should also be noted that there are differential effects of repeated immobilization stress in the early versus late postnatal period on stress-induced response in adulthood. Further studies on the strength of repeated immobilization stress and novel stress, age of the rat at which these stresses are applied, and time interval between these stresses are necessary.

In conclusion, repeated immobilization stress from P7 to P13 affected the growth of developing rats and response of the HPA axis to novel stress in adulthood. Conversely, repeated immobilization stress from P15 to P21 had little effect.

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LEGENDS

Figure 1: Experimental protocols.

Figure 2: Body weight changes in rats subjected to immobilization stress or maternal deprivation (MD) under various conditions; IS-E (●), IS-L (○), MD-E (■), MD-L (□) and control group (△). Values are expressed as means ± standard error of the mean (SEM) (n=6 in each group). *p<0.05 and **p<0.01; IS-E group vs. control group. †p<0.05; IS-L group vs. control group. ‡‡p<0.01; MD-E group vs. control group. #p<0.05 and ##p<0.01; IS-E vs. IS-L.

Figure 3: Basal and stress-induced levels of plasma corticosterone in adult rats subjected to immobilization stress or MD under various conditions; IS-E (●), IS-L (○), MD-E (■), MD-L (□) and control group (△). Values are expressed as means ± standard error of the mean (SEM) (n=6 in each group). Black horizontal bar on the abscissa indicates the period of immobilization stress.

*p<0.05 and **p<0.01; IS-E group vs. control group. †p<0.05; IS-L group vs. control group. ‡p<0.05; MD-E group vs. control group. #p<0.05 and ## p<0.01; IS-E vs. IS-L. Statistical comparisons between each value and baseline levels (T0) within groups are described in the text.

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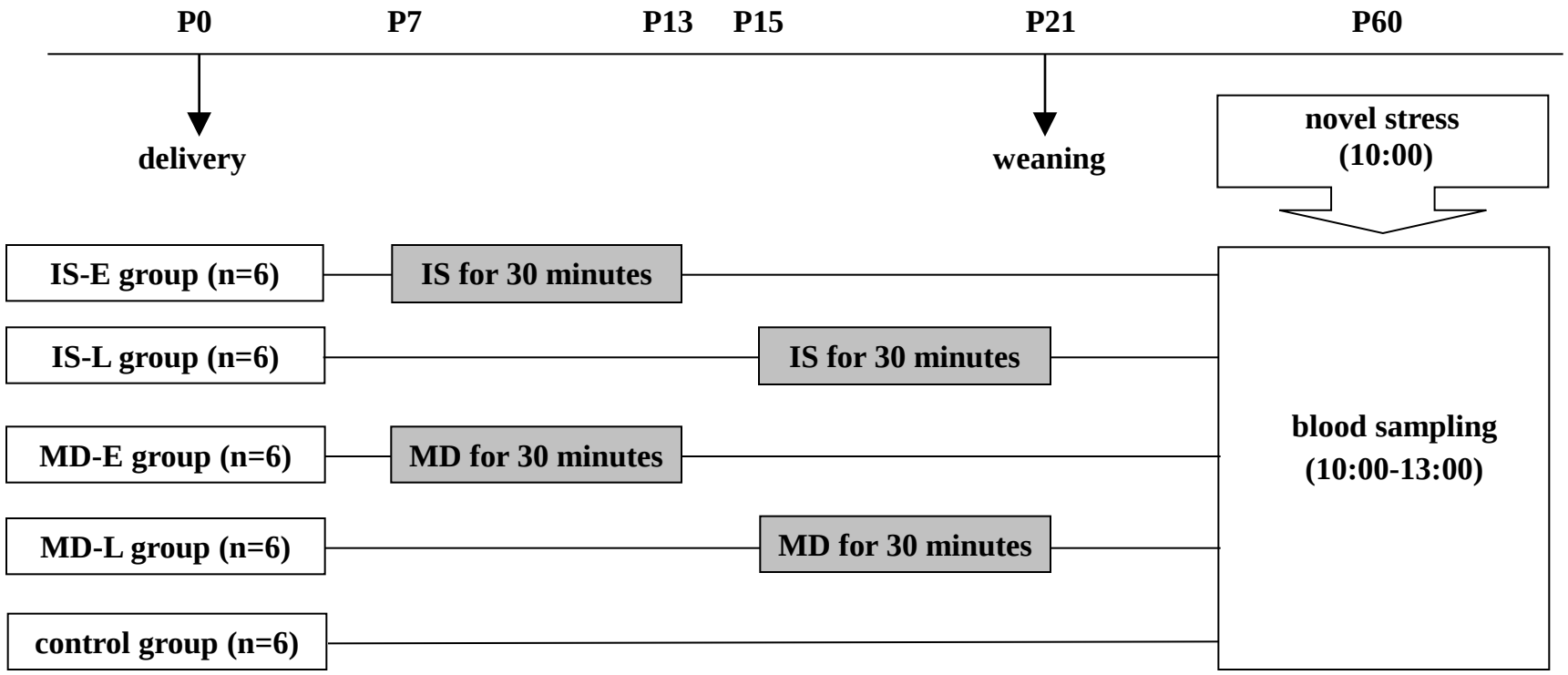
Figure 1.



P : postnatal day
IS : immobilization stress
MD : maternal deprivation

IS-E group ; immobilization stress for 30 min per day from P7 to P13
IS-L group; immobilization stress for 30 min per day from P15 to P21
MD-E group; MD for 30 min per day from P7 to P13
MD-L group; MD for 30 min per day from P15 to P21
control group; No immobilization stress or MD was subjected.

Figure 2.

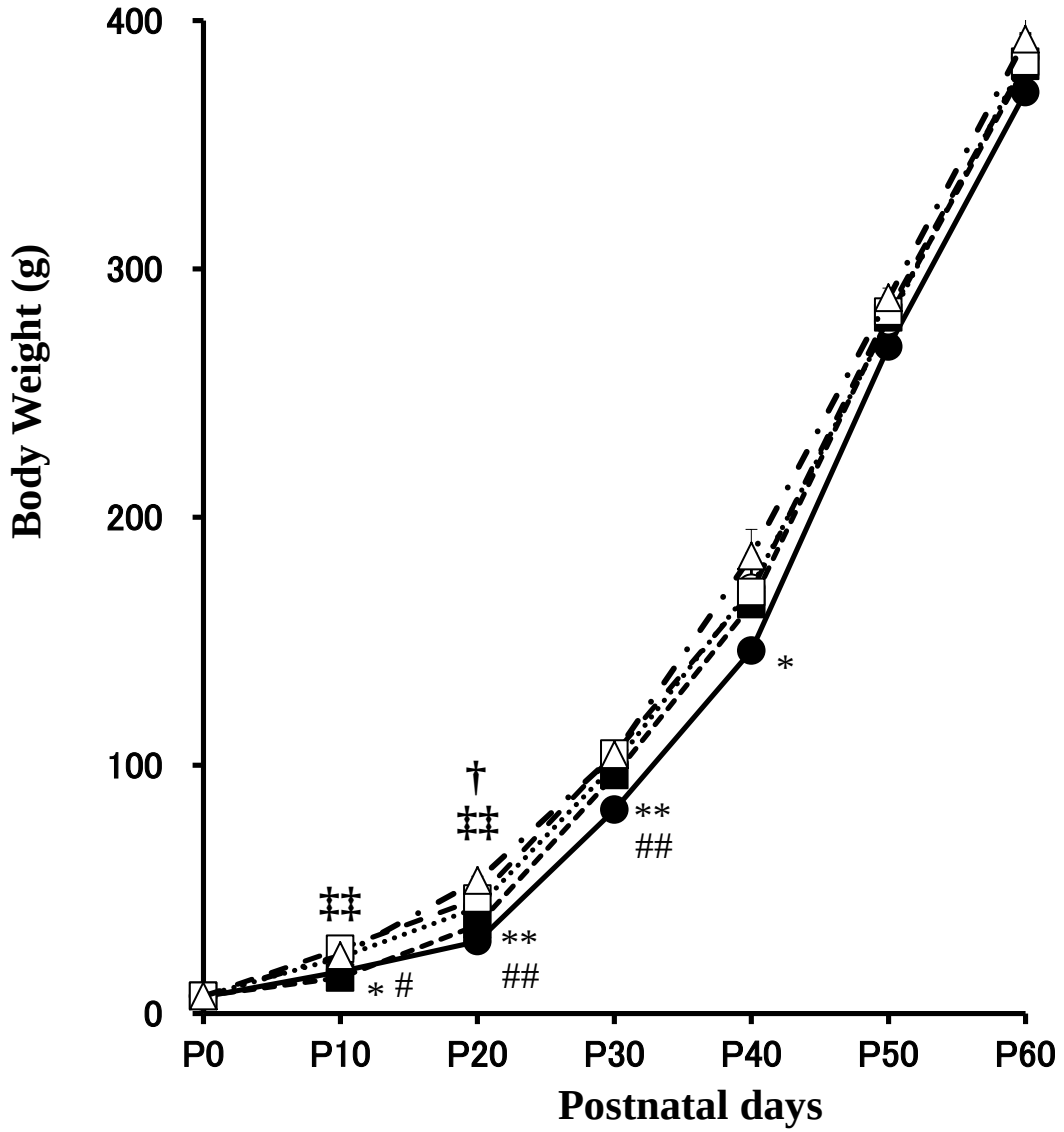

Body Weight (g)
400
300
200
100
0
P0
P10
P20
P30
P40
P50
P60
Postnatal days
* #
‡‡
†
‡‡
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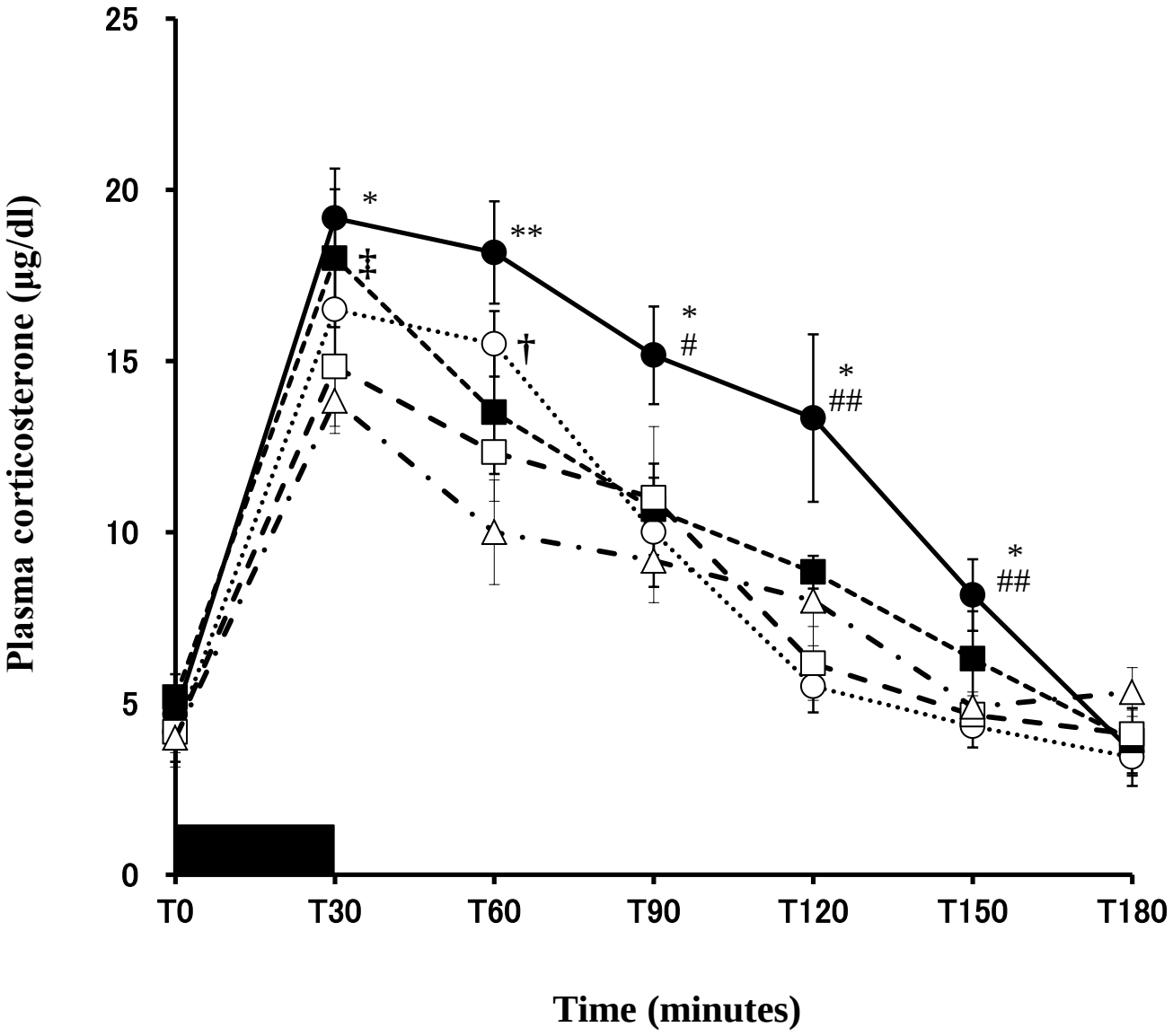
Figure 3.


Plasma corticosterone (μg/dl)
25
20
15
10
5
0
T0
T30
T60
T90
T120
T150
T180
Time (minutes)
*
**
‡
†
*
#
*
##
*
##