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Gastric Ulceration and Expression of Prolactin Receptor in the Brain in Hatano High- and Low-Avoidance Rats

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Recently, prolactin was shown to inhibit the development of stress-induced ulcers. However, the mechanism for suppression of gastric ulcers by prolactin has not been clarified. Hatano high-avoidance (HAA) and low-avoidance (LAA) strains of rats were originally selected and bred from Sprague–Dawley rats based on shuttle-box tasks. The present study focused on the relationships among gastric ulceration and endocrine response with special reference to prolactin secretion and restraint stress in water of HAA and LAA rats. The restraint stress induced an elevation of plasma concentrations of ACTH, corticosterone, and prolactin. Peak levels of plasma ACTH during stressful condition were significantly higher in HAA rats than in LAA rats, while peak levels of prolactin were significantly lower in HAA rats than in LAA rats. The gastric erosion index was significantly higher in HAA rats than in LAA rats 7 h after restraint stress in water. The numbers of prolactin-receptor-positive cells determined by immunohistochemistry in the paraventricular nucleus was significantly increased in LAA rats than in HAA rats 7 h after restraint stress in water. These results indicate that HAA rats were more sensitive than LAA rats to restraint stress in water. The strain differences in gastric ulceration under stress may be involved in peripheral prolactin secretion and central prolactin receptor expression. The expression of prolactin receptor in the paraventricular nucleus may be important in suppressing gastric ulceration.

Key Words: Hatano rats; stress; gastric ulcer; prolactin receptor.

Introduction

Prolactin has a wide range of effects on brain functions including maternal behavior, grooming behavior, sexual behavior, and the stress responses *(1,2)*. Plasma concentrations of prolactin increase in response to acute and chronic stress *(2–5)*. The prolactin receptor has two isoforms, a short-form and a long-form receptor in the brain *(6)*. The expression of the prolactin long-form receptor increases during maternal behavior and stress *(7,8)*. This expression also increased in the choroid plexus of the brain under stress, although prolactin short-form receptor expression dose not change. In addition, prolactin reduces stress-induced gastric ulcers in the rat. Prolactin intracerebroventricular (icv) and intraperitoneal (ip) injection inhibits gastric ulceration under stress *(9)*. In social stress, defender rats show a three- to fourfold increase in plasma prolactin compared with offensive dominant rats *(10)*. These results suggest that prolactin is a protective hormone in stress.

Hatano rat lines have been genetically selected and bred from Sprague–Dawley rats on the basis of their performance in shuttle-box tasks *(11)*. High avoidance animals (HAA) were selected on the basis of the high rate of avoidance response and low avoidance animals (LAA) for the low rate of response. Comparisons of these two Hatano lines in further behavioral tests is used routinely in experimental neurobehavioral teratology and reveals additional differences in activity levels, in maze performance *(11)*, and in behavioral development *(12)*. The effect of acute restraint stress on prolactin was higher in LAA rats than in HAA rats in past studies *(13)*. However, the mechanism of this different stress response in these strains is not clear. To test the hypothesis that Hatano rats show strain differences in prolactin profiles following stress, we used the model of gastric ulceration by

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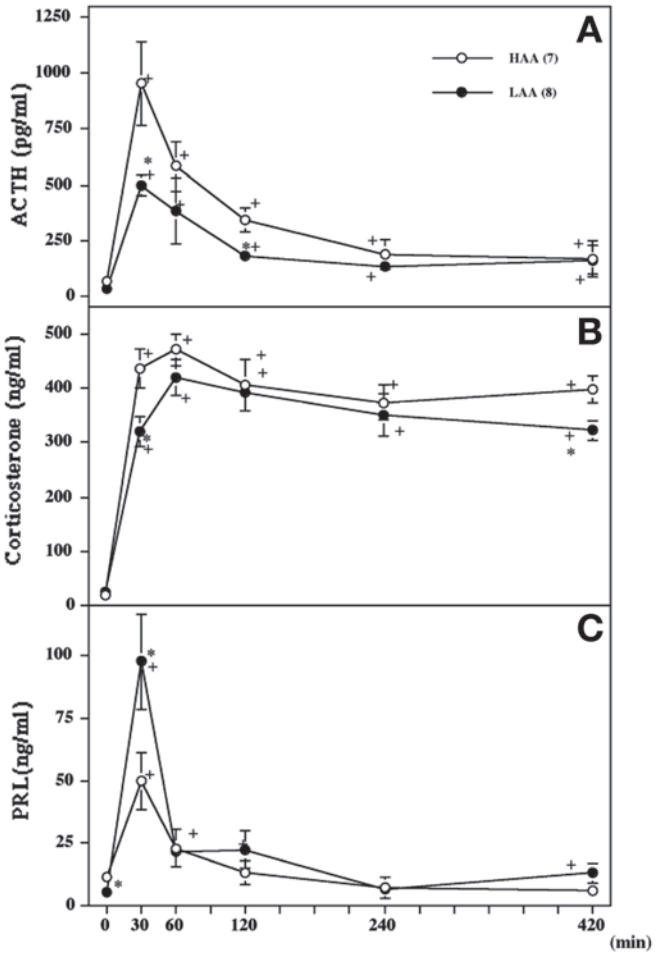

Fig. 1. Effects of restraint stress in water on plasma concentrations of ACTH (**A**), corticosterone (**B**), and prolactin (**C**) in HAA ($n = 7$) and LAA rats ($n = 8$). Values are means ± SEM. *$p < 0.05$ vs HAA rat, $^{+}p < 0.05$ vs basal levels (two-way ANOVA followed by Tukey–Kramer test).

restraint stress in the water. Furthermore, to test the hypothesis that prolactin inhibits gastric ulceration, we examined prolactin receptor in the choroid plexus and in the paraventricular nucleus (PVN) of the brain.

Results

Changes in Plasma Concentrations of ACTH, Corticosterone, and Prolactin

Plasma concentrations of ACTH increased in both strains during restraint stress in water. Peak concentrations of ACTH were observed 30 min after stress in both strains, but the peak ACTH concentrations were significantly higher in HAA rats than in LAA rats (Fig. 1A). Following restraint stress, plasma concentrations of corticosterone increased and remained high in both strains. However, concentrations of corticosterone tended to be higher in HAA rats than in LAA rats during stress (Fig. 1B). Plasma concentrations of prolactin increased sharply in both strains during stress. The peak levels of plasma prolactin were observed 30 min after stress in both strains, whereas the peak concentrations in LAA rats were significantly higher than in HAA rats (Fig. 1C). After the peak, the prolactin levels in LAA rats maintained the high levels over a basal level.

Table 1
Gastric Erosion Index
after 7 h of Restraint Stress in Water

Strain	Gastric erosion index
HAA ($n = 6$)	75.2 ± 8.0*
LAA ($n = 5$)	25.2 ± 7.3

Values are means ± SEM. Number of estimates is each column. *$p < 0.05$ vs LAA rat (Student's t test).

Gastric Erosion Under Restraint Stress in Water

The incidence and extent of gastric erosion were significantly higher for HAA rats than for LAA rats by 7 h after restraint stress (Table 1).

Immunohistochemical Localization of Prolactin Receptor in the Choroid Plexus and Paraventricular Nucleus

Immunoreactive prolactin receptor was found in cells of the choroid plexus of both strains before and after stress (Fig. 2). Staining intensity of prolactin receptor using U5 and prolactin receptor-1 antibody showed no strain differences before and after stress. Prolactin receptor isotypes in the both rat strains were detected entirely in cytoplasm. Immunopositive cells for prolactin receptor were observed and counted the numbers in the paraventricular nucleus before and after stress. The area of the immunostaining reaction for the prolactin long- and short-form receptors were detected in magnocellular and parvocellular cells in both strains (Figs. 3A–D). The long form of the prolactin receptor in HAA rats was detected in magnocellular cells rather than in parvocellular cells before and after stress (Figs. 3E,G). In LAA rats, the long form of prolactin receptor after stress was detected in especially in parvocellular cells (Figs. 3F,H).

The cell numbers of immunopositive prolactin receptor showed no strain differences before stress. The numbers of cells staining for the prolactin receptor using U5 and prolactin receptor-1 antibody in LAA rats was significantly higher at 7 h after stress than before stress, whereas staining in HAA rats was not chanced by stress (Fig. 4).

Discussion

The present study clearly demonstrated differences between high vs low avoidance rats in gastric ulcerogenesis and prolactin receptor expression in the hypothalamus (PVN) after restraint stress. Prolactin has been shown to alter neuronal activity in different regions of the hypothalamus *(14–16)*. Vagal nerve activity decreases gastric acid secretion and gastric mucosal blood flow, and decreases mucus secre-

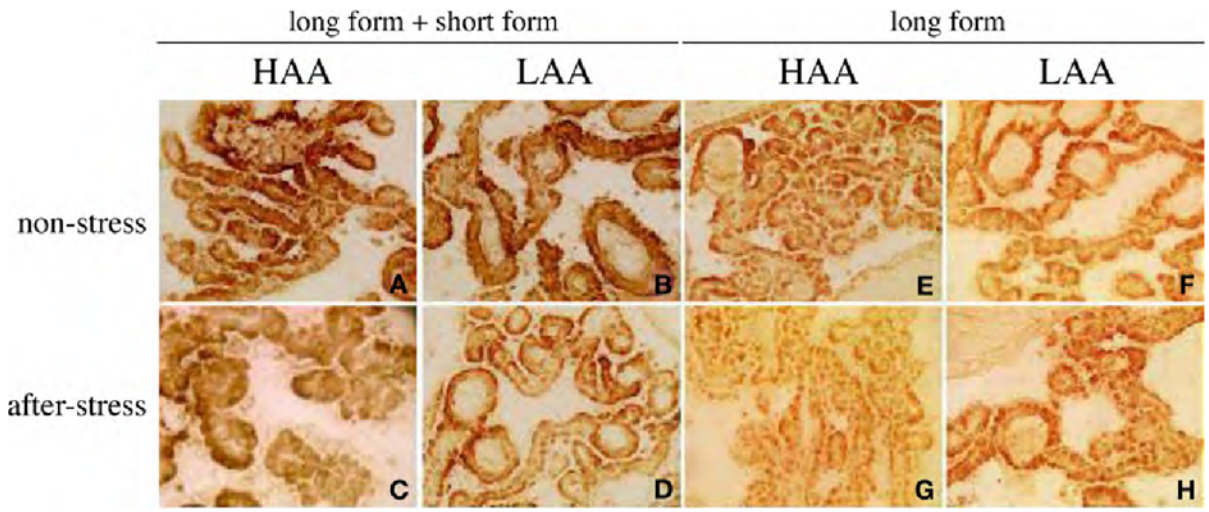

Fig. 2. Photomicrographs of histological sections from brain of HAA and LAA rats before and 7 h after restraint stress in the water. Tissue sections were immunostained for prolactin receptor long and short form using U5 antibody (**A**–**D**) and for prolactin long form using prolactin receptor-1 antibody (**E**–**H**) in the choroid plexus. The upper panel represents choroid plexus before stress (**A**, **B**, **E**, **F**). The bottom panel represents choroid plexus after 7 h restraint stress in the water (**C**, **D**, **G**, **H**). Magnifications ×400.

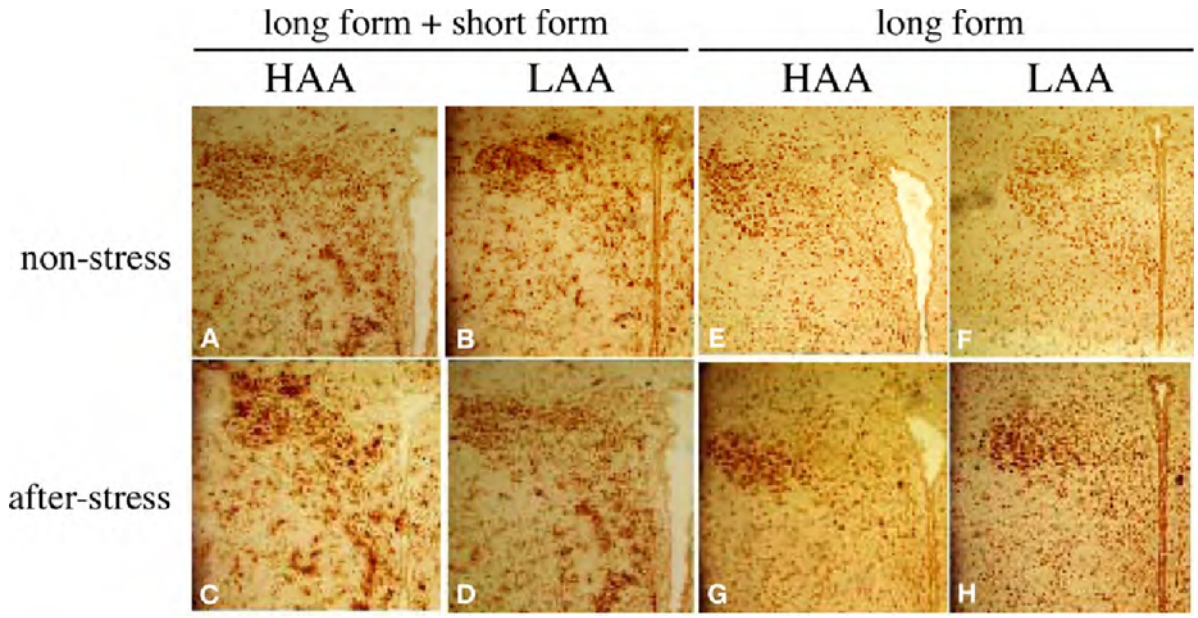

Fig. 3. Photomicrographs of histological sections from brain of HAA and LAA rats before and 7 h after restraint stress in water. Tissue sections were immunostained for prolactin receptor long and short form using U5 antibody (**A**–**D**) and for prolactin long form using prolactin receptor-1 antibody (**E–H**) in the paraventricular nucleus (PVN). The upper panel represents PVN before stress (**A**, **B**, **E**, **F**). The bottom panel represents PVN after 7 h restraint stress in water (**C**, **D**, **G**, **H**). Magnifications ×100.

tion, making the stomach more prone to damage by gastric acid. Lactation, which induces high circulating prolactin levels, is associated with increased vagal activity and inhibits gastric ulcerogenesis *(17)*. Hyperprolactinemia, as induced by pituitary homografts under the kidney capsule, is accompanied by inhibition of the development of gastric ulcers *(18)*. Previous reports also suggest that prior administration of prolactin inhibits gastric erosion *(19)*. Passive immunization against prolactin in the rat increases gastric ulcerations *(36)*. Prolactin molecules, which do not cross the blood–brain barrier, are present in cerebrospinal fluid *(20–23)*. The increase in the circulating prolactin levels results in increased prolactin-binding sites in the choroid plexus and enhanced transport of prolactin from blood to cerebrospinal fluid *(23)*. The long form of the prolactin receptor in the choroid plexus appears to function in the transport of prolactin molecules from blood to cerebrospinal fluid during stress *(19)*. Prolactin is viewed as being transported to the brain, increasing prolactin receptor long-form expression in the paraventricular hypothalamic nucleus (PVN),

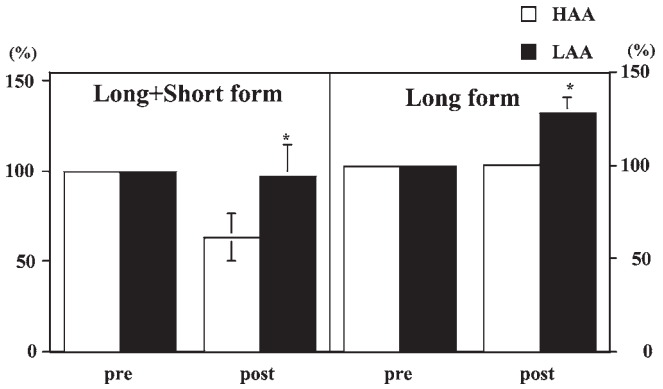

Fig. 4. Percentage increases of prolactin receptor positive cell numbers in paraventricular nucleus in HAA and LAA rats before and 7 h after restraint stress in water. Values are means ± SEM. *$p < 0.05$ vs HAA rat (Student's t test).

and then regulating corticotropin-releasing hormone (CRH) neurons to produce an antistress effect. The present study clearly shows that plasma concentrations of prolactin were higher in LAA rats compared with HAA rats. The mRNA levels of prolactin long-form receptor in choroid plexus increased 7 h after restraint stress in water *(19)*. The peak of mRNA expression was 2 h after restraint stress, and then decreased *(19)*. The protein levels of prolactin long-form receptor in PVN increased 7 h after restraint stress in water *(24)*. In the present study, the prolactin receptor positive paravocellular cells in LAA rats also increased in the PVN 7 h after restraint stress in the water. The gastric ulcerations were lower in the LAA rats compared with HAA rats. In addition, the mRNA expression of the long-form prolactin receptor increased in the choroid plexus 30 min after stress *(19)*. Prolactin receptor immunoreactivity in the PVN was increased by ip or icv injections of rat prolactin *(19)*. Moreover, gastric erosion was inhibited by microinjection of rat prolactin into PVN *(19)*. In contrast, administration of a prolactin receptor morpholio-antisense oligonucleotide in the PVN increased gastric erosion *(24)*. The present results of LAA rats in conjunction with previous findings indicate that the expression of prolactin long-form receptor in the parvocellular cells of PVN may be important in protecting for gastric ulcerogenesis.

CRH and vasopressin synthesized and secreted into the pituitary portal circulation by parvocellular neurons in the PVN are the main regulators of ACTH secretion in the pituitary corticotroph *(25)*. In Hatano rats, the adrenal response of corticosterone release to ACTH secretion during restraint stress was higher in LAA rats than in HAA rats *(13)*. The response of corticosterone in the stress is important to avoid gastric ulcers. Some studies have shown that in human *(26–28)* and animals *(29)* high doses of exogenous glucocorticoids increase the incidence of gastric ulcers. Weiss found that the degree of ulceration after stress correlated positively with the level of plasma corticosterone *(30)*. However, opposing findings have also been reported. A significant negative correlation was found between adrenocortical activity and the degree of stress-induced gastric erosion *(31)*. Gastric erosion increases when corticosterone secretion was inhibited or glucocorticoid receptors were occupied by an antagonist. The icv administration of CRH and corticosterone treatment inhibited gastric ulceration *(32–34)*. The anti-ulcerogenetic effects of prolactin may be mediated via the CRH system *(36)*. The gastric ulcer is formed with various factors of autonomic nerve and endocrinological conditions. Therefore, it is difficult to investigate a direct causal relationship between the prolactin responses and the gastric ulcerations. Further studies are needed to clarify the mechanism of action of prolactin on the prevention of gastric ulcerogenesis in response to stress.

It is also known that prolactin, and dopamine that controls prolactin, are involved in acquisition of avoidance responses in the shuttle-box. Hyperprolactinemic rats reduced to freezing and facilitated acquisition of active avoidance responses in the shuttle-box *(18)*. This effect is reduced either by dopamine or opioid receptor antagonists and does not decrease in long-term hyperprolactinemic rats. Dopamine-deficient rats, caused by 6-hydroxydopamine injection, reduced shuttle-box avoidance acquisition *(35)*. The strain difference of prolactin regulation in the brain may be due to behavioral phenotype.

In conclusion, the results of the present study indicate that HAA and LAA rats exhibit marked differences in their response of gastric ulcerogenesis 7 h after restraint stress in water. These Hatano rats may be useful animal models in studying the role of prolactin in gastric ulceration.

Materials and Methods

Animals

Adult male rats (13–15 wk) from each strain, HAA rats ($n = 15$) and LAA rats ($n = 15$) were used. Animals were maintained under a 12-h light–dark cycle (light period from 07:00 h to 19:00 h), at a temperature of 21–25°C and relative humidity of 45–75%. Food (CE-2, Clea Japan, Inc., Tokyo, Japan) and water were available *ad libitum*. The experimental protocol was approved in accordance with the Animal Care and Use Committee at the Hatano Research Institute of Food and Drug Safety Center.

Restraint Stress in Water and Sampling Protocol

A silicone cannula was inserted under sodium pentobarbital anesthesia into the right atrium through a jugular vein 1 d before the restraint experiment. Rats were exposed to stress as described previously *(36)*. Briefly, rats were put into individual small restraining wire net cages and immersed in water to the chest. The temperature of the water was kept constant at 22.5–23°C. After 0.5, 1, 2, and 4 h of stress, blood samples from unanesthetized animals (0.8 mL) were taken from a jugular vein catheter under restraint stress. After 7 h of stress, rats were removed from cages and

decapitated. Five sham-operated control rats from each strain were decapitated under non-stress conditions. All experimental manipulations were performed between 09:00 and 16:00 h. Blood was collected into heparinized tubes containing aprotinin and centrifuged immediately. Plasma was separated and stored at –20°C until assayed for ACTH, corticosterone, and prolactin. The cerebrum was removed and was quickly frozen in dry ice and stored at –80°C until used.

Radioimmunoassay (RIA)

Concentrations of ACTH *(37)* and corticosterone *(38)* in plasma were measured by double-antibody RIAs using ^{125}I-labeled radioligands as described previously. Synthetic rat ACTH 1-39 (Sigma Chemical Co., St. Louis, MO, USA) was used as reference standard. The intra- and interassay coefficients of variation were 11.3% and 11.9% for ACTH and 9.5% and 16.4% for corticosterone. Concentrations of prolactin in plasma were measured using NIDDK kits for rat prolactin. Hormones for iodination were rat prolactin-I-5. The antisera used were anti-prolactin-S-9. Results were expressed in terms of NIDDK rat prolactin-RP-2. The intra- and interassay coefficients of variation were 3.4% and 5.2% for prolactin, respectively.

Gastric Erosion Index

After 7 h of stress, rats were decapitated. The stomachs were removed and inflated by injection of 10% formalin, and then immersed in 10% formalin solution for more than 30 min. The fixed stomachs were then opened along the greater curvature and examined for gastric lesions. The gastric erosions obtained from this stress model were in shape of a point or a line, and no wide areas appeared. The gastric lesion index was calculated as the cumulative length of gastric lesions *(36)*.

Immunohistochemistry for Prolactin Receptor in Choroid Plexus and Paraventricular Nucleus

The frozen brain tissues in HAA rats (n = 7) and LAA rats (n = 7) were cut into coronal sections serially (12 μm thickness) in a cryostat and mounted on silanized slides. The sections on slides were fixed with 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB) for 30 min at room temperature. The tissue sections were acetylated with 0.25% acetic anhydride in 0.1 *M* triethanolamine, passed through a graded series of alcohol, and prepared for immunohistochemical staining using the Vectastain ABC kit Elite (Vector Laboratories, Burlingame, CA, USA). The two primary antibodies against prolactin receptor were mouse anti-rat monoclonal antibody U5 (MA 1-610, Affinity Bioreagents, Inc., Golden, CO, USA) for the long and short form of the receptor *(39)* and polyclonal antiserum against a synthetic peptide corresponding to amino acids 29–46 (prolactin receptor-1 peptide) *(40)*. The prolactin receptor-1 antibody was kindly provided by Dr. F Talamantes (Department of Molecular, Cell and Developmental Biology, University of California, Santa Cruz, CA, USA). Nonspecific protein binding was blocked by incubating the section with 10% normal horse serum (NHS) or 10% normal goat serum (NGS) and 0.3% Triton X in 0.01 *M* PBS for 2 h. The mouse monoclonal prolactin receptor antibody U5 was used at an optimal concentraion of 10 μg/mL in 0.01 *M* PBS containing 2% NHS. The prolactin receptor-1 antiserum was diluted 1:500 in 0.01 *M* PBS containing 2% NGS. Tissue sections were incubated for 18 h at room temperature. Biotinylated horse anti-mouse IgG or goat anti-rabbit IgG 7.5 μg/mL was diluted to 0.01 *M* PBS containing 2% NHS or 2% NGS for 3 h. The tissue sections were incubated in the second antiserum for 3 h. The tissue sections were incubated for 3 h in the ABC reagent diluted 20 times in 0.01 *M* PBS for 3 h. The primary antibody bound to the sections was visualized by treatment with 3,3'-diaminobenzidine tetrachloride (Sigma FAST DAB Tablet, Sigma Chemical Co., St. Louis, MO, USA). The sections were washed between steps in 0.01 *M* PBS. Specificity of the antibodies was examined using NHS or NGS instead of the primary antibody, using the same concentrations of protein as the primary antibody. Eight serial sections per rat were analyzed.

Statistical Analysis

All values are expressed as means ± SEM. Time course data were for effects of stain and time by two-way analysis of variance (ANOVA) followed by Tukey–Kramer test. The significance of differences between HAA rats and LAA rats was analyzed by Student's t test. $p < 0.05$ was considered statistically significant.

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