

## Occlusal Disharmony Induces BDNF Level in Rat Submandibular Gland

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*List of abbreviations:* BDNF, Brain-derived neurotrophic factor; ACTH, adrenocorticotrophic hormone

## ABSTRACT

*Objectives:* Brain-derived neurotrophic factor (BDNF), which is produced in rat submandibular gland, is one of the most abundant **neurotrophins** in the central nervous system. It is generally accepted that occlusal disharmony **causes** stress. The purpose of the present study was to investigate whether occlusal disharmony-induced chronic stress affects BDNF levels and morphology in rat submandibular gland.

*Design:* Eight wks old male Wistar rats (n=21) were randomly divided into three groups of 7 rats. In a control (C) group, the rats received no treatment for 8 wks. In a molar cusp-less (OD) group, maxillary molar **cusps** were cut off with a dental turbine at baseline and kept for 8 wks. In a molar cusp-less + recovered cusp (OR) group, maxillary molar **cusps** were cut off and then were recovered after 4 wks using resin material. After the experimental period, expression of BDNF mRNA and protein as well as histological findings **were** evaluated in the submandibular glands. The comparisons between the groups were made using the Mann-Whitney *U* test with Bonferroni correction.

*Results:* The OD group showed a significant increase in submandibular gland BDNF mRNA and protein expression after 8 wks, and plasma adrenocorticotrophic hormone and corticosterone levels increased in a time-dependent manner. There were no

significant differences in BDNF expression in the submandibular glands and in levels of plasma adrenocorticotrophic hormone and corticosterone between the OR and C groups.

*Conclusions:* These results indicate that psychological stress induced by occlusal disharmony reversibly induces BDNF expression in the rat submandibular gland.

## **1. Introduction**

Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the central nervous system and it is closely involved in survival, maintenance, and neural transmission in neural cells.<sup>1</sup> BDNF is produced under various conditions and processes, such as inflammation and allergy.<sup>2</sup> In addition, because BDNF is involved in the mechanism of infarct tolerance in cerebrum, direct intracerebral infusion of exogenous recombinant BDNF protects neural cells in the infarct.<sup>3</sup> Hence, elevation of BDNF level by exogenous BDNF is thought to contribute to the function and protection of neural cells. Furthermore, it is possible that BDNF might have clinical significance as a psychological stress marker. For instance, it has been shown that the production of BDNF in the brain is increased during episodes of psychological stress in order to maintain homeostasis.<sup>4</sup>

BDNF expression has been observed, not only in the central nervous system, but also in non-neural tissues including blood cells,<sup>5</sup> the aorta,<sup>6</sup> heart and lung.<sup>7</sup> Recently, it has been demonstrated that the submandibular gland is one of the sources of circulating BDNF under acute and chronic immobilization stress.<sup>2, 8, 9</sup> These observations suggest that the submandibular gland may play a crucial role in the regulation of psychological stress through the production of BDNF.

Occlusal disharmony is understood to cause chronic psychological stress in animal model studies.<sup>10, 11, 12</sup> Urinary cortisol excretion rates are elevated by the insertion of occlusal splints in monkeys.<sup>10</sup> The placement of acrylic caps on both lower incisors increases plasma corticosterone levels in rats.<sup>11</sup> Therefore, it is possible that chronic psychological stress induced by occlusal disharmony may also induce BDNF expression in the submandibular gland. However, there has been no report regarding a causal relationship between occlusal disharmony and BDNF expression in the submandibular gland.

In the present work, we hypothesized that psychological stress attributed to occlusal disharmony might induce BDNF expression in the submandibular gland. The purpose of the present study was therefore to investigate the effects of occlusal disharmony and of its improvement on BDNF expression in the rat submandibular gland. In addition, the plasma levels of corticosterone and adrenocorticotrophic hormone (ACTH) were measured to evaluate the degree of psychological stress induced.<sup>8</sup>

## **2. Material and Methods**

### 2.1. Animals

Twenty-one male Wistar rats (8-wks-old) were used for this 8-wk-study. The animals were housed under standard conditions and the room was maintained on a cycle of 12 h light and 12 h darkness and at a temperature of 23 °C to 25 °C. The experimental protocol was approved by the Animal Research Control Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. Animals had free access to a powdered diet (Oriental Yeast Co., Tokyo, Japan) and tap water.

### 2.2. Experimental design

The rats were randomly divided into three groups of 7 rats each: a control (C) group, which received no treatment for 8 wks; an occlusal disharmony (OD) group, in which all maxillary molar crowns were cut off for 8 wks, and an occlusion recovery (OR) group, in which all maxillary molar crowns were cut off for 4 wks and were then recovered with resin materials for the remaining 4 wks. In the OD group, the maxillary molar crowns were cut off at the gingival margin using a dental turbine.<sup>13</sup> In the OR group, preliminary trays and the silicon-base impression material (Speedex; Coltene, Altstätten, Switzerland) were prepared and were used to make an impression of the

maxillary teeth of each rat following which the maxillary molar crowns were cut off using the same method as for the OD group. After 4 wks, the resin (Superbond C&B; Sun Medical, Shiga, Japan) was carefully put on the crown-less teeth using the trays attached with the impression, in order to recover occlusion. All treatments were done under general anesthesia by inhalation of 2–4% isoflurane delivered in an O<sub>2</sub> gas.

### 2.3. Preparation of Plasma

Plasma samples were collected from the tail vein at baseline and at 4 wks, and from the heart at 8 wks, between 7:00 and 9:00, in tubes containing EDTA (TERUMO, Tokyo, Japan). The tubes were immediately placed on ice and then centrifuged at 2000 × g for 10 min at 4 °C. The supernatants were collected and stored at -80 °C before use.

### 2.4. Measurement of Plasma ACTH and Corticosterone Levels

Plasma ACTH and corticosterone levels were determined using an ELISA (Phoenix Pharmaceuticals, Burlingame, California), and an EIA kit (Yanaihara Institute Inc, Shizuoka, Japan), respectively according to the manufacturers' instructions. These measurements were performed in duplicate and both intra- and inter-assay coefficients of variation were <5%.

### 2.5. Histological Analysis

The animals were sacrificed under general anesthesia after 8 wks. Samples of the left



submandibular glands were resected from each rat and were immediately fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The fixed tissue samples were embedded in paraffin and were stained with hematoxylin and eosin. Three discontinuous sections were randomly selected per rat, and the mean of the three histological data was calculated for each rat.

## 2.6. Immunohistochemistry

Immunohistochemical analysis was performed using the Simple stain MAX-PO (R) kit (Nichirei, Tokyo, Japan). The sections were incubated with an anti-BDNF rabbit polyclonal antibody (sc-546, 1:2000, Santa Cruz Biochemistry, Santa Cruz, CA, USA) for 24 h at 4 °C. The number of striated and intercalated ducts from three randomly selected gland regions of a fixed size/area (1.0 mm<sup>2</sup>) was counted.<sup>14</sup> A mean value representing BDNF positive ducts per unit area (1.0 mm<sup>2</sup>) in each gland section was calculated.

## 2.7. Determination of BDNF mRNA Levels

The right submandibular glands were harvested, were immediately frozen, and were kept at -80 °C until they were processed for real time reverse transcription–polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from the submandibular gland biopsy samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according

to the manufacturer's instructions. The isolated RNA was quantified by measurement of the absorbance at 260 nm and its purity was determined by the 260/280 nm absorbance ratio. Samples with a ratio of >1.8 were used in the procedure described below.<sup>15</sup> Total RNA (2 µg) was reverse transcribed by AMV Reverse Transcriptase (TAKARA) at 42 °C for 30 min.<sup>16</sup> The cDNA prepared was diluted 10-fold with yeast RNA (10 µg/mL). Real-time PCR was performed using TOYOBO SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan) and the LightCycler™ (Roche Applied Science, Mannheim, Germany) system, for 45 cycles of 95 °C for 20 s, the appropriate annealing temperature for 4–5 s and 72 °C for 20 s. The primer sequences were 5'-CAGGGGCATAGACAAAAG-3' and 5'-CTTCCCCTTTTAATGGTC-3'.<sup>2</sup> The primers used to detect the internal control marker, GAPDH, were 5'-GTATTGGGCGCCTGGTCACC -3' and 5'-CGCTCCTGGAAGATGGTGATGG -3'.

<sup>17</sup> BDNF gene expression was expressed in terms of the relative copy number ratio of BDNF to GAPDH for each sample.

## 2.8. Determination of BDNF Protein Levels in Submandibular Glands and Plasma

BDNF concentrations in the submandibular gland and plasma were assayed using ELISA kit (Promega Co., Madison, WI, USA) according to the manufacturers' instructions.<sup>9</sup> Submandibular gland tissue samples were homogenized in ice-cold cell

lysis buffer (Cell Signaling Technology Japan, Tokyo, Japan) with 1mM phenylmethylsulfonyl fluoride. Both submandibular glands and plasma samples were treated by adding 1 N HCl until the pH was <3.0 to enhance the signal. Total protein concentrations in the submandibular glands were measured by spectrophotometry using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

## 2.9. Statistical Analysis

All data analysis was done using a statistical software package (SPSS 15.0J for Windows; SPSS Japan, Tokyo, Japan). Comparisons between the groups were made using the Mann-Whitney *U* test with Bonferroni correction. The level of significance was set at  $p < 0.016$ .

### 3. Results

There were no significant differences in food consumption among the three rat groups over the experimental period. Body weights [mean (SD)] for the C, D and R groups were 415.3? (18.4), 392.1 (22.5) and 396.4 (23.0) g at 4 wks and 507.1 (30.6), 482.7 (36.9) and 491.1 (27.5) g at 8 wks, respectively. There was no significant difference in body weight between the any groups.

Plasma level of corticosterone in the D group increased in a time-dependent manner and the value was higher than that in the C and R groups at 4 and 8 wks ( $p < 0.016$ ) (Fig. 1). No significant difference in the value was detected between the C and the R groups.

The level of A $\beta$ 42 was higher in the D group than

The level of BDNF mRNA expression was higher in the OD group than in the C or the OR groups at 8 wks (215% and 190% higher, respectively) ( $p < 0.016$ ) (Fig. 2). There was no significant difference in BDNF mRNA expression between the C and the OR groups.

#### 3.5. Levels of BDNF in Submandibular Glands and Plasma by ELISA

There were no significant differences in the levels of BDNF in submandibular glands between the any groups, although the levels of BDNF in the OD group had a tendency

to increase compared to those in the control group ( $p = 0.037$ ) (Fig. 4). The levels of plasma BDNF were undetectable except for some rats in the OR group (data not shown).

### 3.6. Pathologic Evaluation of the Submandibular Glands

No pathological changes were observed in the submandibular glands of any of the three rat groups (data not shown). Vacuolization of the acinar cells and inflammatory changes in the submandibular glands were also not observed.

#### **4. Discussion**

In the present study, we investigated if there might be a causal relationship between occlusal disharmony and BDNF expression in the rat submandibular gland. The results showed that 8 wks of occlusal disharmony induced BDNF expression in the submandibular gland. In addition, occlusal disharmony increased the plasma levels of corticosterone and ACTH in a time-dependent manner. Corticosterone and ACTH are accepted indicators of psychological stress,<sup>9</sup> and it is known that immobilization stress increases BDNF expression in the submandibular gland.<sup>2, 8, 9</sup> Therefore, it is feasible that occlusal disharmony could stimulate BDNF expression in the submandibular gland by increasing psychological stress. Consistent with this theory, improvement of occlusal disharmony by the use of resin materials diminished the OD-induced increase in BDNF expression in the submandibular gland, as well as resulting in decreased plasma levels of corticosterone and ACTH at 8 wks compared to the OD group. The decrease in psychological stress as a result of the recovery of occlusion may suppress BDNF expression in the submandibular gland.

There have been other studies that have investigated the effects of occlusal disharmony on plasma levels of corticosterone. For instance, it was reported that the mean plasma levels of corticosterone in rats with incisal caps were significantly higher

than those in rats without incisal caps at 90, 120, and 150 min after the cap placement.<sup>18</sup>

It has also been reported that rats in a molarless group exhibited a significantly higher level of corticosterone than those in the control group at 24 wks of age.<sup>19</sup> These observations are consistent with the present results, which showed that occlusal disharmony caused increased plasma levels of corticosterone.

Some previous reports demonstrated that chronic stress does not significantly modulate BDNF gene expression in the brain.<sup>20, 21</sup> These reports suggest that chronic stress has little effect on BDNF gene expression in the brain. On the other hand, a previous study showed that rats which had received chronic immobilization stress expressed the BDNF gene at an approximately 7-fold higher level in their submandibular glands than the controls.<sup>9</sup> The current results also demonstrated that chronic stress induced by occlusal disharmony induced a mRNA expression of BDNF in the submandibular gland that was approximately 200% higher than that of the controls. Although further studies are needed, BDNF gene expression in submandibular glands may be more sensitive to chronic stress than that in the brain.

In this study, the experimental model for occlusal disharmony involved the cutting off of all of the maxillary molar crowns. It is therefore possible that local inflammation in the injured maxillary regions might also have affected BDNF expression in the

submandibular gland. In the rat model, experimental periodontitis was shown to induce vacuolization of acinar cells in the submandibular gland at 8 wks.<sup>15</sup> However, in the present study, occlusal disharmony did not induce any pathological changes in the submandibular gland at 8 wks. This result suggests that, in our model, inflammation of the injured maxillary regions had little effect on BDNF expression in the submandibular gland.

The role of increased BDNF expression in the submandibular glands is still unclear in our model system. BDNF has been shown to protect the central nervous system following a variety of insults.<sup>22</sup> Previous studies have demonstrated that occlusal disharmony in aged mice induces hippocampal neuron loss,<sup>23</sup> and hippocampal expression of glucocorticoid receptors and glucocorticoid receptor mRNA.<sup>24</sup> Therefore, increased BDNF expression in the submandibular glands would be expected to elevate the circulating level of BDNF,<sup>8</sup> and this increased circulation of BDNF may play crucial roles in protecting the central nervous system from chronic stress. However, there were no significant differences in plasma BDNF between the all groups in our study. Because the level of stress by occlusal disharmony seems to be lower than that by immobilization,<sup>8,9</sup> the further studies with longer experimental periods or greater level of stress will be necessary to clarify this issue.



There were no significant differences in the levels of BDNF protein per total protein in submandibular glands between the all groups, although the number of BDNF positive ducts cells per unit area was significantly higher in the OD group than in the C and the OR groups. This discrepancy may be due to the difference in evaluation methods. The BDNF expression in the immunohistochemical analysis was evaluated only in the unit area, but not in the whole specimen. On the other hand, the BDNF expression by the ELISA method reflects the concentration of BDNF protein against total protein in the whole submandibular glands. Therefore, the BDNF concentration in case of ELISA might not reach the significant differences between the all groups in our study.

One limitation of our study was that, in the occlusion recovery group, we were unable to confirm the occlusal height in each rat. Therefore, it is unclear whether occlusion was recovered correctly in our model. However, the current results showed no significant differences in plasma levels of corticosterone or ACTH between the control and the occlusion recovery groups. These findings indicate that occlusion must have been recovered to a level at which psychological stress was not induced.

In conclusion, occlusal disharmony increased plasma levels of corticosterone and ACTH and induced BDNF expression in the submandibular gland. In addition,

recovering occlusal disharmony diminished the increased plasma levels of corticosterone and ACTH and decreased BDNF expression in the submandibular gland. These results suggest that psychological stress induced by occlusal disharmony affects BDNF expression in rat submandibular gland.

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## Figure Legends

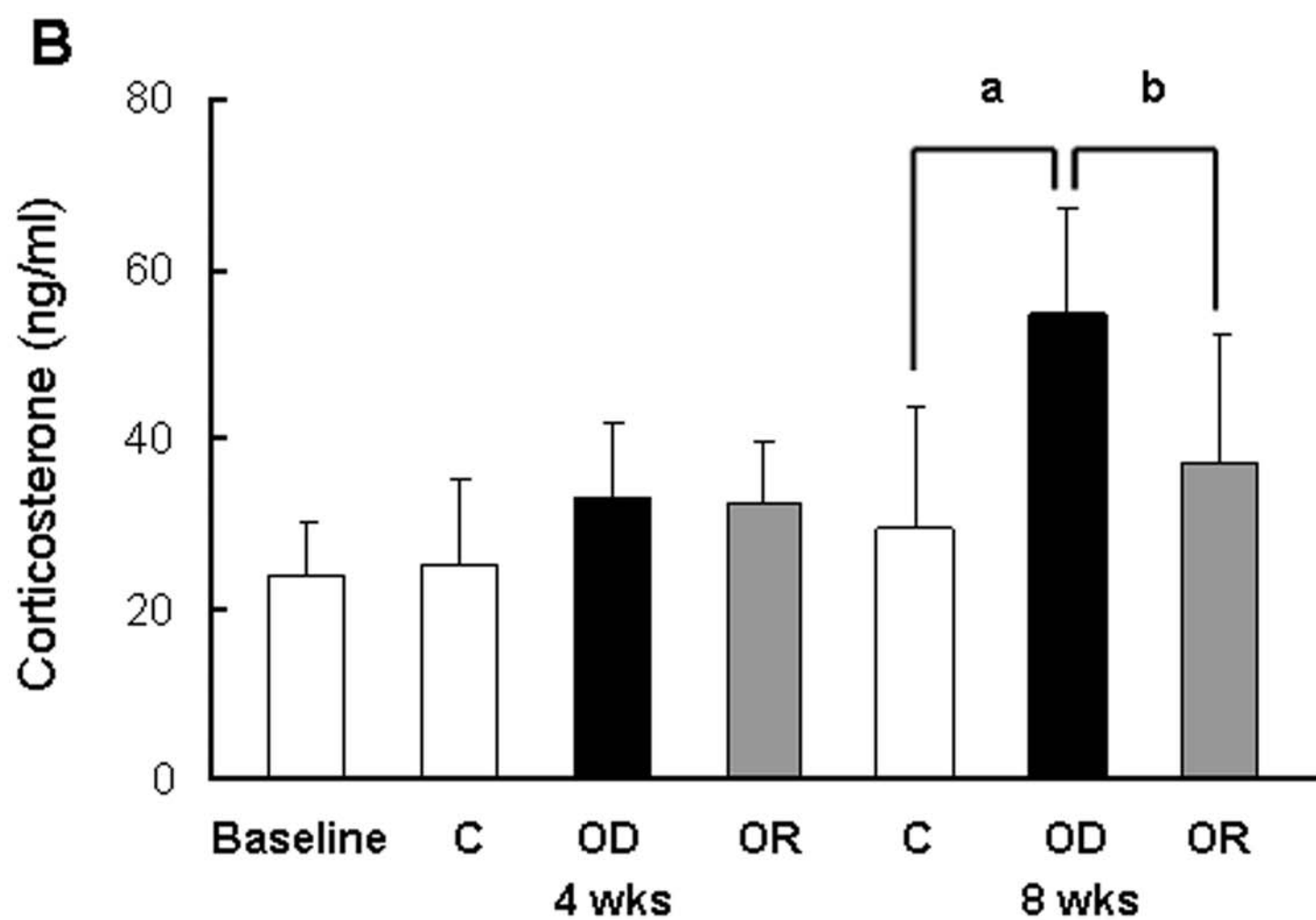
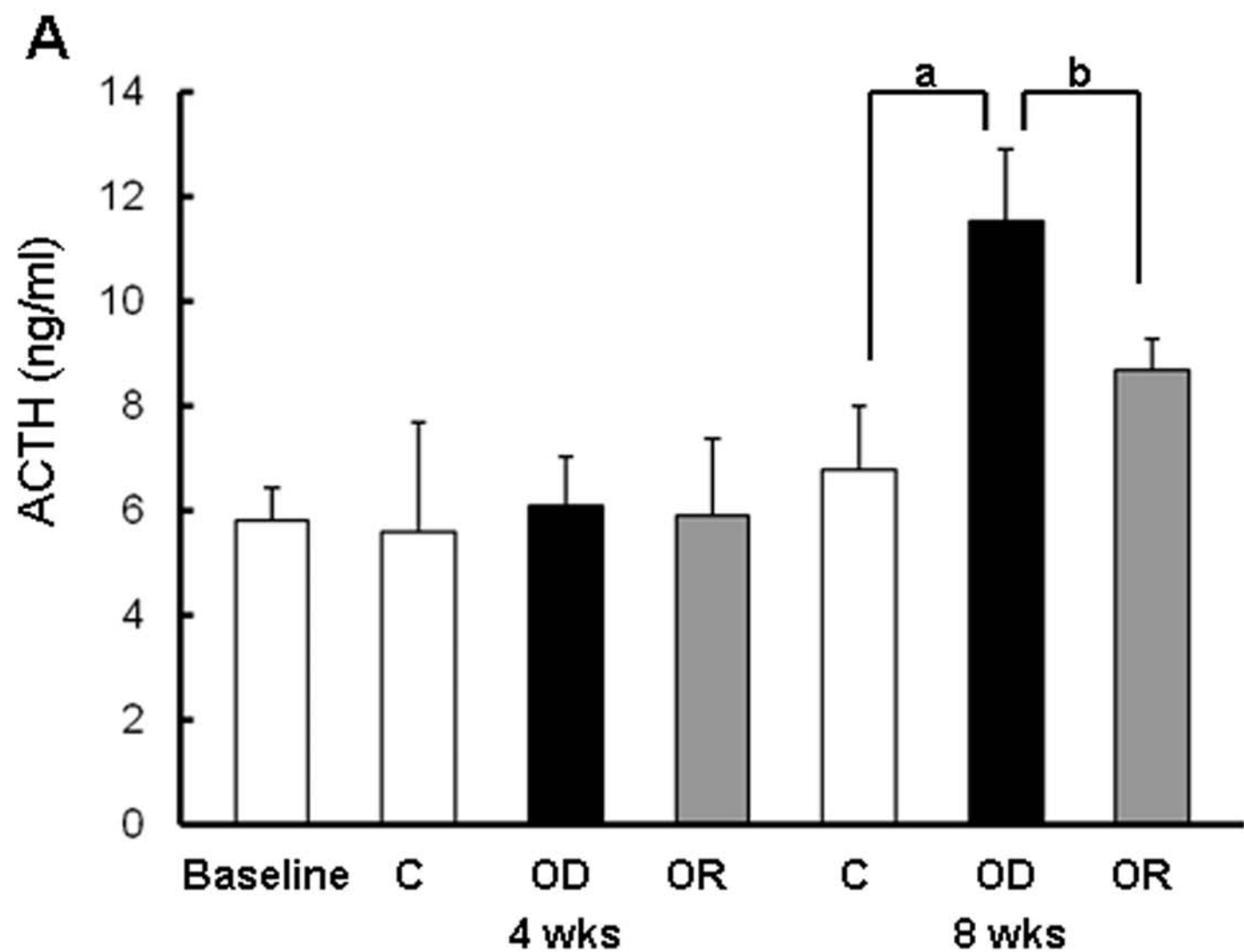
**Figure 1.** Effect of occlusal disharmony on plasma levels of adrenocorticotrophic hormone (ACTH) and corticosterone. Plasma levels of ACTH (A) and corticosterone (B) were measured using an ELISA and an EIA respectively, in rats at baseline and at 4 and 8 wks after initiation of occlusal disharmony. The values of the occlusal disharmony (OD) group increased in a time-dependent manner and these values were higher than those of the control (C) group at 8 wks (<sup>a</sup> $p < 0.016$ ). The values for the OR group were lower than those for the OD group (<sup>b</sup> $p < 0.016$ ) and no significant differences in these values were detected between the C and OR groups at 8 wks. Data are expressed as means  $\pm$  SD (n=7).

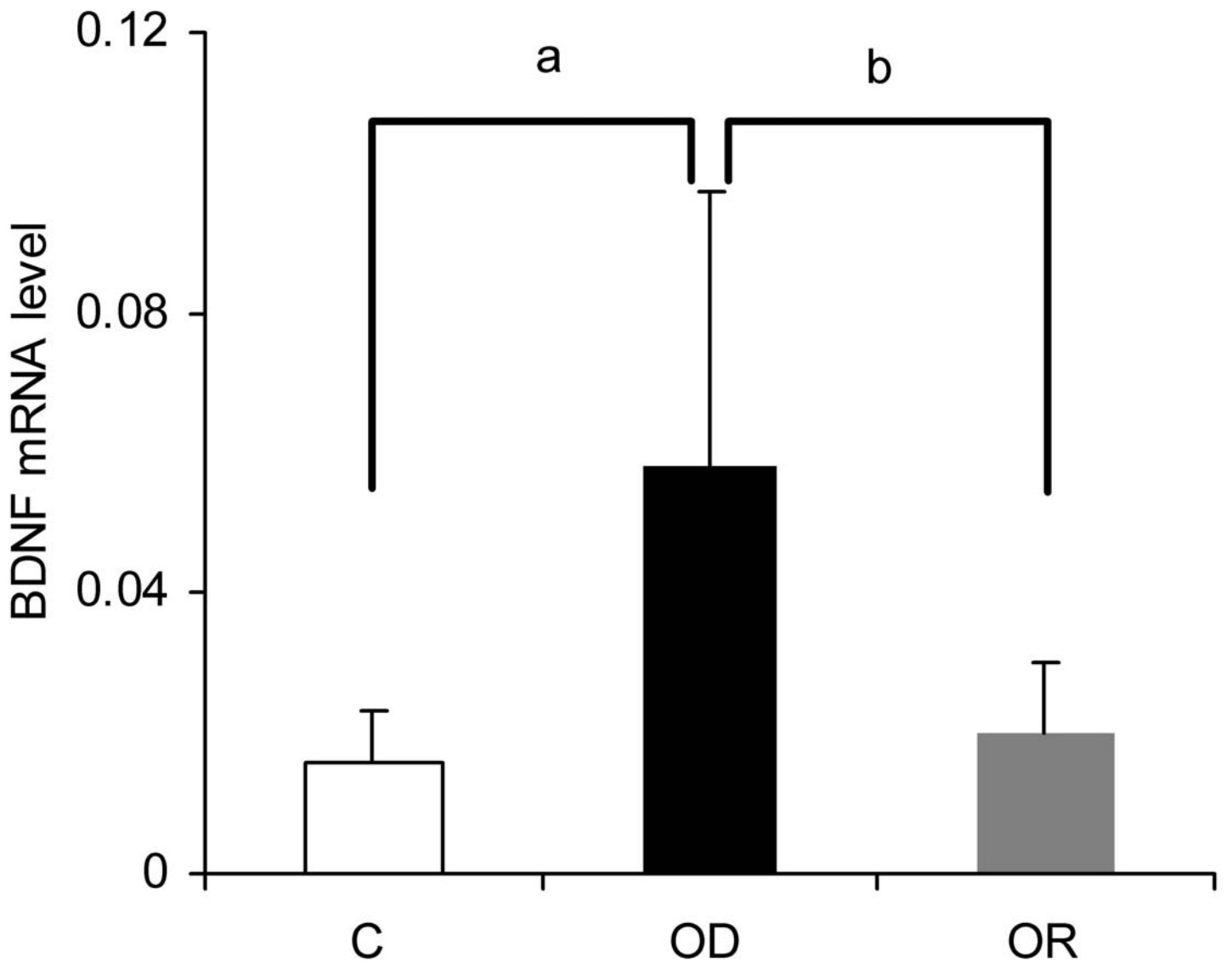
**Figure 2.** Effect of occlusal disharmony on brain-derived neurotrophic factor (BDNF) mRNA levels in rat submandibular glands. The BDNF/GADPH mRNA ratio, of rat submandibular glands 8 wks after initiation of occlusal disharmony was obtained using RT-PCR. The values for the OD group were higher than those for the C and the OR groups (<sup>a</sup> $p < 0.016$ ). No significant differences between these values were detected between the C and the OR groups. Data are expressed as means  $\pm$  SD (n=7).



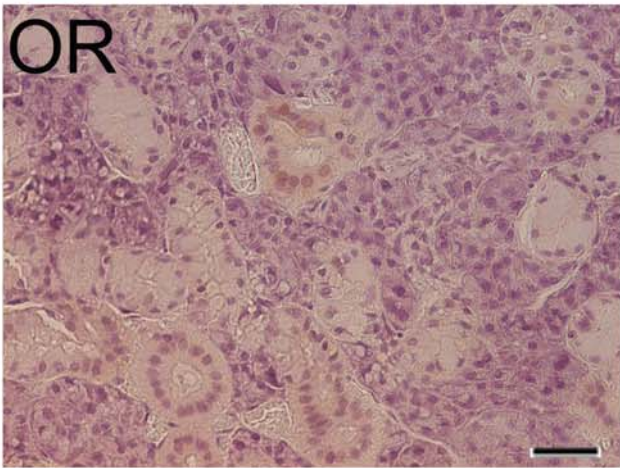
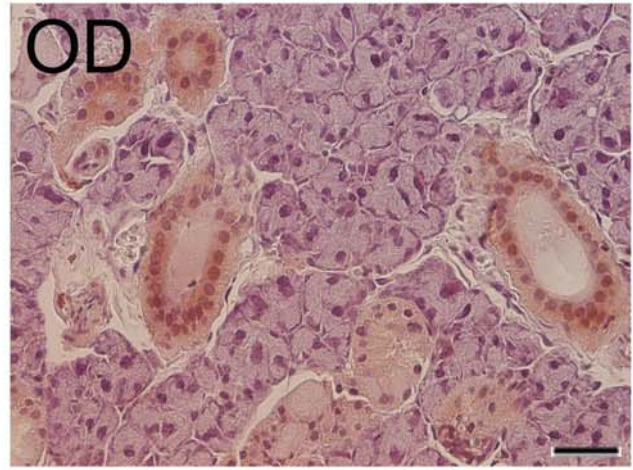
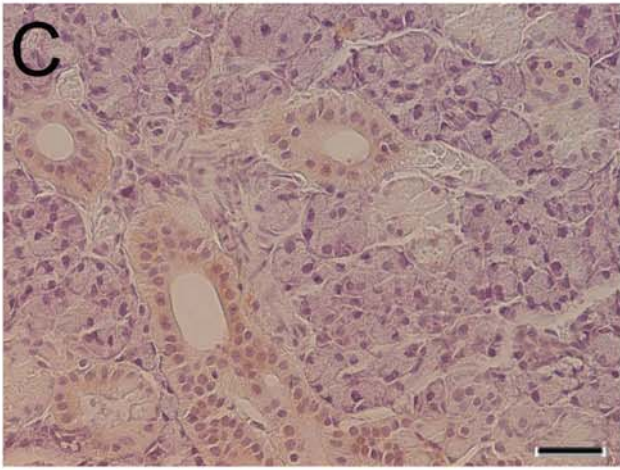
**Figure 3.** Immunohistochemical analysis of brain-derived neurotrophic factor (BDNF) in the submandibular glands of the different rat groups. Photomicrographs show the immunohistochemical localization of the BDNF protein in the indicated rat groups (A). BDNF protein expression was observed in duct cells in the OD group. In contrast, both the C and the OR groups exhibited weak BDNF expression in duct cells. The number of BDNF positive ducts cells in the OD group was higher than the number in the C and the OR groups (<sup>a</sup>  $p < 0.016$ ) and no significant differences between these values were detected for the C and the OR groups (B). Data are expressed as means  $\pm$  SD (n=7). Bar=50  $\mu$ m.

**Figure 4.** Brain-derived neurotrophic factor (BDNF) protein levels in rat submandibular glands. There were no significant differences in BDNF protein between the any groups. Data are expressed as BDNF protein per total protein in tissue extracts and means  $\pm$  SD (n=7).





# A



# B

