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Orosensory deprivation alters taste-elicited c-Fos expression in the parabrachial nucleus of neonatal rats

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Abstract

In the present study we examined the effects of neonatal orosensory deprivation on taste-elicited gustatory activity in the rat parabrachial nucleus (PBN) using the functional anatomical marker c-Fos. Animals in three groups (GG, GO and GM) received gastric cannula implantation surgery on postnatal day 9 (P9). Animals in the fourth group (MR) did not receive any surgery. GG rats were fed by infusion of artificial milk directly into the stomach. GO rats were fed by intraoral infusion of artificial milk. GM and MR rats were reared by their mother with free access to mother's milk, water and rat chow. Rats from all groups were similar in body weight and length by P21. On P21 rats in all groups were intraorally presented with 0.5 M sucrose solution and the brains were extracted and processed for c-Fos immunohistochemistry. Taste-elicited c-Fos expression in both the gustatory waist area, and the external lateral subnucleus of the PBN in rats in the GG group was significantly more robust than in the other three groups. These findings suggest a substantial alteration in orosensory-evoked neuronal response in this nucleus, due to sensory or motor deprivation during a critical developmental stage.

Keywords

Neonatal rat; Gastrostomy; Taste; Development; Parabrachial nucleus; Sensory deprivation; Artificial rearing; Fos Immunohistochemistry

1. Introduction

The development of normal feeding in human infants is dependent on experience with sensory reception of oral stimuli (Ayano et al., 2000). Often, infants either born prematurely or

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possessing congenital diseases need temporary nutritional support via tube feeding. Frequently, these infants display feeding impairments and motor speech delays (Bier et al., 1993; Jennische and Sedin, 1998,1999; Hawdon et al., 2000; Dodrill et al., 2004). It is possible in these cases that impaired oral feeding is due to hypersensitivity in the oropharyngeal region. Weaning is a critical period for the development of normal feeding (Illingworth and Lister, 1964), and it is likely that orosensory experience during this period is crucial. However, there exist few basic studies investigating the significance of sensory reception in the development of feeding behavior.

Mammals possess a functional sense of taste at birth, although the gustatory system continues to develop into adulthood (for reviews see Mistretta, 1991; Barlow, 2000; Mistretta and Hill, 2003; Krimm 2007). There is copious evidence that development in the taste central nervous system (CNS) of rodents may be altered prenatally by environmental manipulation, such as low-sodium diet fed to the dam (Krimm and Hill, 1997; Mangold and Hill, 2007). Studies also show that central gustatory development can be altered by manipulation of feeding in neonatal animals. For example, Lasiter and Diaz (1992) demonstrated that rats receiving artificial rearing (AR) via an intragastric cannula from postnatal days 4 to 14 (P4 – P14) possess reduced terminal field volumes in the nucleus of the solitary tract (NST) as compared with mother-reared controls (Lasiter and Diaz, 1992). Further studies using AR rats demonstrated that this altered neuroanatomical development was specifically due to lack of gustatory stimulation (Lasiter, 1995). On the other hand, taste exposure during the suckling period was found to be unnecessary for the development of normal taste behavioral responses in rats (Bernstein et al., 1986).

The purpose of the current study was to examine how differences in feeding state during the critical weaning period affect gustatory-evoked neural activity in the taste CNS of AR rats. This was accomplished by comparing taste responses in rats fed during the critical weaning period (P9 – P20) via either a gastric fistula or an intraoral cannula. Once the rats reached P21, taste-evoked activity was measured in the parabrachial nucleus (PBN). Studies examining c-Fos immunoreactivity (Fos-IR) in this region following intraoral stimulation with various tastants suggest the existence of a “taste map” in the PBN (Yamamoto et al., 1994; Yamamoto, 2006). Distinct patterns of Fos-IR across PBN subnuclei are evoked by stimuli classified as hedonically positive, such as sweeteners; a different pattern is evoked by aversive stimuli such as quinine. We hypothesized that rats fed intragastrically, without oral stimulation, would possess diminished or altered Fos-IR in the PBN in response to sweet taste stimulation. Sweet-tasting stimuli evoke appetitive or consumatory behavior in rats, and evoke responses distinct groups of gustatory neurons in the CNS (for review see Yamamoto, 2003). Sweeteners also elicit including suckling, a consumatory behavior, in neonatal rats (Kozlov et al., 2006).

2. Methods

The study was approved by the Animal Care and Use Committee standards of Showa University (authorization No. 19006), and animals were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996.

2.1. Subjects

A total of 28 male Sprague-Dawley neonate rats (Sankyo laboratory, Tokyo, Japan), weighing between 5.0 – 7.0 g at the start of the experiments were used. 28 of these were reared with their mother in a colony room for 8 days after birth (P1 – P8), and then randomly assigned to one of four experimental groups (see below). These were reared with their mother until P21. All rats were housed in an animal colony room with a 12 h light/12 h dark cycle (lights on at 0800 h, off at 2000 h) and constant temperature (25 ± 1 °C) and humidity (50 – 70%). Seven additional

rats were excluded from the final number due to decannulation, leakage of artificial milk from the stomach, or excretion problems during the course of the experiments.

2.2 Surgery and groups

2.2.1 Gastrostomy surgery—The animals were arbitrarily divided into the GG (gastrostomy + intragastric infusion), GO (gastrostomy + intraoral infusion), GM (gastrostomy + mother rearing) and MR (mother rearing) groups consisting of 7 rats each (Fig. 1). On day 9 (P9) rats in the GG, GO and GM groups were anesthetized with diethyl ether and polyethylene tubing gastric cannula (PE10, Becton Dickinson and Co., NJ, USA) was implanted in the forestomach as described previously (Ooka et al., 2008). After surgery, the GG and GO animals were returned to the colony and individually housed in plastic home cages to prevent decannulation. Animals in the GG group were reared by intragastric infusion of sterilized artificial milk (Bean Stalk Snow Co., Ltd, Tokyo, Japan). The milk was first infused into the stomach 6 – 12 hours after surgery. The total volume of artificial milk infused on P9 was 2 – 3 ml. From P10 – 21, the volume of artificial milk for one day was calculated by the following formula: Postnatal day $\times$ 0.65 ml (modified from Messer et al., 1969). Volume for one infusion was 1 – 2 ml. Infusion started at 0800 h and was given every 2 hours at a rate of 0.5ml/min. Animals in the GO group were fed in a similar fashion as those in the GG group except that they were infused intraorally with artificial milk. Both groups were maintained under this feeding schedule until P21. After surgery, the GM rats were returned to their mother and allowed free access to dam milk, water and rat chow (Nosan Co., Kanagawa, Japan) until P21. Animals in the MR group did not receive gastrostomy surgery, and were reared by their mother with free access to dam milk, water and rat chow throughout the experiments.

2.2.2 Intraoral cannula implantation—We developed an original method for intraoral cannula implantation due to the fact that the conventional method, where the cannula is anchored to the skull with screws (e.g. Yamamoto and Sawa, 2000; Tokita et al., 2007), is too invasive to neonate rats. On P16 rats in all the groups were anesthetized with diethyl ether. A snorkel-like intraoral cannula, made with a 24G stainless steel needle (Terumo Co., Tokyo, Japan) and polyethylene tubing (PE50, Becton Dickinson and Co., NJ, USA), was fixed on the right cheek with dental adhesive resin cement (Super-Bond C&B, Sunmedical Co., Shiga, Japan) and inserted from outside the mouth. The tip of the cannula was smoothed with dental drill engine to prevent damage to the oral cavity and placed between the cheek and gum next to the first maxillary molar (Fig. 2).

2.3. Measurement of body weight and length

The body weight of each animal was measured daily at 0900 h during the period between P8 and P21 to monitor body growth. On P21, the body length, defined as the length from the tip of the nose to the tip of the tail, was also measured.

2.4. Taste stimulation

On P21, following 5 hours of food deprivation, rats were intraorally infused with 6 ml of 0.5 M sucrose solution at a rate of 1 ml/min for 6 minutes using an infusion pump (PHD Infusion 2000, Harvard apparatus, MA, USA). Taste stimuli were prepared from reagent-grade chemicals dissolved in distilled water, and were presented at room temperature.

2.5. Perfusion and immunohistochemical staining

Three hours following the onset of oral taste stimulation with sucrose, rats were anesthetized with an overdose of diethyl ether and perfused transcardially with 250 ml of 0.02 M phosphate-buffered saline (PBS), followed by 500 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and postfixed for 24 hours in the same fixative, and

cryoprotected in a 30% sucrose solution in 0.1 M PB at 4 °C for at least 24 hours. Coronal sections were cut at 50 µm thick using a freezing microtome (Yamato Kohki Industrial Co., Ltd, Saitama, Japan). The floating sections were processed for Fos-IR detection using the labeled streptavidin (LSAB) method (Dako, Glostrup, Denmark). First, the sections were incubated with PBS containing 0.02% hydrogen peroxide to inactivate endogenous peroxidase. After being rinsed with PBS three times, the sections were incubated with rabbit polyclonal anti-c-fos antibody (1:5000, #sc-a52, Santa Cruz Biotechnology, CA, USA) overnight at room temperature. The sections were rinsed (PBS) and then incubated in goat anti-rabbit IgG (1:1000, Dako) for 120 min, and peroxidase-conjugated streptavidin (1:1000, Dako) for 60 min at room temperature. All antibodies were diluted with PBS containing 1% normal goat serum and 0.5% Triton X-100. For the peroxidase reaction, 3, 3'-diaminobenzidine with nickel ammonium sulfate intensification was used to visualize immunoreactivity. These sections were mounted on aminosilane-coated slides (Matsunami Glass Ind., Ltd, Osaka, Japan) and coverslipped.

2.6. Microscopic analysis of sections

Fos-IR in the waist area (defined as the central medial and ventral lateral subnuclei), dorsolateral (dls) and external lateral subnuclei (els) in the PBN was imaged and analyzed using a microscope (BZ-9000, Keyence corporation, Osaka, Japan). Three serial sections per subregion with most apparent FLI were chosen as representative ones. Neuron counts were based on these sections and the average number of Fos-IR cells was calculated. After imaging of Fos-IR in all sections, slides were incubated with xylene for 24 hours and coverslips were removed, and then sections were counter-stained with cresyl violet. Cytoarchitectonic identification of PBN subnuclei was made with the use of these Nissl-stained sections, and according to previous studies in the rat, hamster and mouse (Fulwiler and Saper, 1984; Halsell and Frank, 1991; Hashimoto et al., 2009).

2.7. Data analysis

Data were analyzed using a general linear model (repeated measures or one-way ANOVA). Post-hoc comparisons were made using Scheffe's test where appropriate. Statistical analysis software was used for all analyses (SPSS14.0, SPSS Japan, Tokyo, Japan). The criterion for significance was set a priori at $p < 0.05$.

3. Results

3.1. Physical assessments

All the groups showed a similar weight gain curve during the P8 – 21 period (Fig. 3A). A repeated measures (day) ANOVA with a factor for group revealed significant main effects of group ($F_{3,24} = 3.29$; $p < 0.05$), day ($F_{13,312} = 1308.70$; $p < 0.01$) and a group $\times$ day interaction ($F_{39,312} = 7.44$; $p < 0.01$). Generally, rats from the GG group possessed a somewhat lower body weight than the other groups from about P14 – P19. However, there was no significant difference in the mean body weight between the groups by P21 (Scheffe's test). Fig. 3B shows the mean body length on P21. Although a one-way ANOVA indicated an overall effect of group ($F_{3,24} = 3.15$; $p < 0.05$), post-hoc Scheffe's test revealed no significant difference between the groups.

3.2. Histological identification of PBN subnuclei

Based on the Nissl-stained brain sections of 5 P21 rats the location and rostrocaudal extent of each subregion were identified. In spite of differences in the overall size of the brain, cytoarchitectonic identification of PBN subnuclei was possible according to previous reports using adult rat, hamster and mouse (Fulwiler and Saper, 1984; Halsell and Frank, 1991;

Hashimoto et al., 2009). The distance between the caudal end of the cms and the contact of the inferior colliculus to the pons was approximately 400 – 450 μm in P21 rats (in adult rats, this distance is reported to be approximately 750 μm , i.e. Yamamoto and Sawa, 2000). Our analysis was based on data obtained from this 400 – 450 μm region. The cms and vls were located from approximately 400 to 0 μm caudal to the contact. The els was located from 220 to 0 μm caudal to the contact. The dls was located from 250 to 0 μm caudal to the contact.

3.3. Taste-elicited Fos-IR in the PBN

Fig. 4A–E shows photomicrographs of representative waist area sections for each of the 4 experimental groups. Oral sucrose stimulation elicited robust Fos-IR in the GG group. In the other three groups, especially in the GM and MR groups Fos-IR was weak.

Fig. 5A–E shows photomicrographs of representative PBN sections for each of the 4 experimental groups. Although oral stimulation with 0.5 M sucrose elicited comparable Fos-IR in the dls in each group (open arrowheads), Fos-IR in the els (closed arrowheads) in the GG group was strikingly more robust in terms of cell numbers than in the other groups.

Fig. 6 displays the mean number of Fos-IR in the waist area (A), dls (B) and els (C) in each of the 4 experimental groups. A one-way ANOVA indicated a significant main effect of group in the waist area ($F_{3,24} = 34.83$; $p < 0.01$) and in the els ($F_{3,24} = 67.76$; $p < 0.01$) but not in the dls ($F_{3,24} = 2.32$; $p = 0.1$). Fos-IR in both waist area and els in the GG group was significantly higher than in the other three groups (Scheffe's test, $p < 0.01$). Furthermore, in the waist area the GO group showed significantly more Fos-IR than the MR group (Scheffe's test, $p < 0.05$).

4. Discussion

In this study, we found striking differences in sucrose-elicited c-Fos expression in the PBN of juvenile rats, which were dependent on postnatal experience with oral feeding. Only GG (gastric feeding) rats did not have experience with oral stimulation from postnatal day 9 – 21, either through normal suckling or via intra-oral feeding. Despite bypassing the normal feeding mechanism, GG rats were similar to the other groups in body weight and size by P21. When given an intraoral infusion of sucrose solution on P21, GG rats displayed a significantly greater amount of c-Fos expression in both the gustatory waist region and the els of the PBN as compared to the other groups. This effect was perhaps most striking in the els. Here, Fos-IR counts were, on average, 4-fold higher in GG rats.

Like other mammals, rats are born with a taste system that is functional to the point that 1-day old pups are capable of behavioral responses to taste stimuli (Johanson and Shapiro, 1986; Mistretta, 1991; Barlow, 2000; Mistretta and Hill, 2003). However, the taste system, especially the central taste nuclei, continues to develop throughout the postnatal period until adulthood. The PBN appears to develop later than the peripheral taste system or NST: According to Lasiter and Kachele (Lasiter and Kachele, 1988, 1989), a period of development and maturation of PBN taste neurons, including increased dendritic morphology and synaptogenesis, occurs between roughly P16 and P40. Hill found increased neural activity to taste stimulation in PBN neurons after P14; these increases persisted to at least P50 and are reflective of increases in morphological development (Hill, 1987). Previous studies show that reducing or eliminating orosensory input during postnatal development, either through peripheral damage or gastric feeding, disrupts morphological development in the NST, indicating a critical period (Lasiter and Kachele, 1990; Lasiter, 1991; Lasiter and Diaz, 1992). In the current study, GG rats were deprived of orosensory input during part (P9 – P21) of what appears to be a critical period for PBN development. These rats did not possess a diminished neural response to sucrose, but

rather a significantly increased response in several PBN regions as assessed by c-Fos expression.

The significance of this altered taste response pattern in GG rats is not obvious, but insight may be gleaned from studies examining c-Fos expression in response to taste and ingestion of sapid stimuli in adult rats. Functional neuroanatomical approaches implicate the waist region as the primary gustatory relay area (Norgren and Pfaffmann, 1975; Halsell and Travers, 1997; Karimnamazi et al., 2002), and intraoral sucrose, as well as NaCl, elicits c-Fos expression in this area in intact rats (e.g. Yamamoto and Sawa, 2000; Tokita et al., 2007). Electrophysiological studies also show that sucrose-best neurons are preferentially located, although intermingled with other best neurons responsive to other basic tastes, in the waist area of the PBN (Shimura et al., 2002; Tokita et al., 2004; Geran and Travers, 2009). Among the four groups of rats tested, GG rats possessed a more robust response to sucrose in this region, as evidenced by higher Fos-IR counts.

It is intriguing that the GO mice were intermediate in response in the waist region (Fig. 6A), as these animals received less optimal orosensory stimulation (intraoral injection) than rats in the GM and MR groups, which suckled. A possible explanation for increased c-Fos expression in the GG group is due to the novelty of the specific taste stimulus (sucrose), or the novelty of orosensory stimulation in general. Mother's milk contains various sugars (e.g. Anseloni et al., 2005), and previous studies support an increase in c-Fos expression in taste CNS regions following taste experience in novel vs. experienced rats (Montag-Sallaz et al., 1999; Koh et al., 2003). However, this effect was far more pronounced in the forebrain. In the Koh et al. study, differences in c-Fos expression were not significantly apparent in the PBN or NST (Koh et al., 2003).

In this study, sucrose-elicited Fos-IR was elevated in the GG group in the els, but not the dls, relative to the other groups. Previous studies indicate that ingestion of palatable stimuli elicits Fos-IR strongly in the dls of adult rats, whereas unpalatable stimuli elicit c-Fos expression in the els (Yamamoto et al., 1994; Yamamoto, 2006). Both of these subnuclei are associated with visceral processing, but along with the waist region, both receive at least some visceral and gustatory input (Halsell and Travers, 1997; Karimnamazi et al., 2002). Yamamoto suggested that the distribution of taste-evoked Fos-IR neurons in the dls is concerned with ingestive behavior, or with positive hedonic processing (Yamamoto, 2006). Whatever the function of this region may be, our results (Fig. 6B) did not support modulation via orosensory deprivation.

The els has been proposed to be a site for the representation of negative hedonics associated with taste or ingestion (e.g. Yamamoto, 2006). Thus, intraoral sucrose may be perceived as negative by mice, a consequence of sensory deprivation during development. However, purely visceral stimuli, such as intraperitoneal injection of LiCl or bradykinin, also evoke robust Fos-IR and electrophysiological responses in this region (Bernard et al., 1994; Yamamoto et al., 1994). Interestingly, intragastric delivery of sucrose was found to produce far more robust c-Fos expression in the els as compared to intraoral delivery (Yamamoto and Sawa, 2000). This elevation was similar in magnitude to that seen in the present study with orosensory-deprived rats. This similarity suggests that intraoral sucrose in GG rats provokes a gastric-like response, indicative of at least an altered if not diminished taste response. A gastric response may also be provoked in the waist region, resulting in stronger c-Fos expression. Studies indicate there is visceral input to this area (Karimnamazi et al., 2002) and some PBN neurons can be activated by both oral and visceral stimulation (e.g. Baird et al., 2001).

Another possibility is that increased Fos-IR in the PBN is due to deprivation related to oromotor activity. In this study, animals in the GG group were deprived of not only taste input, but also mechanical stimulation in the oral cavity. Previous studies have reported that somatosensory

stimulation of the mucosa in the oral cavity (Hermanson and Blomqvist, 1997) and teeth (Yamashiro et al., 1997; Hiroshima et al., 2001) also results in c-Fos expression in the PBN, especially in the external subnuclei. Taste neurons from the tongue and palate can be activated by mechanical stimulation, and the afferent inputs of taste and mechanical modalities converge on the els (Ogawa et al., 1982; Halsell and Travers; 1997). Some NST neurons relay mechanical stimulation to the PBN (Ogawa and Hayama, 1984; Ogawa et al., 1984). In a rat model, Westneat and Hall describe that nipple attachment relies on alternating contraction of the masseter with the genioglossus and digastric muscles (Westneat and Hall, 1992). Physiological studies using rats or humans indicate that changes in intraoral pressure result from tongue movement (Smith et al., 1985; Westneat and Hall, 1992). However, mice fed intraorally did not significantly differ in either waist or els Fos-IR from mice that suckled (Fig. 6A,C). Wayne found that AR from P3 – P14 produces changes in fatigue resistance in the genioglossus muscles of rats (Moore et al., 2007). Kinirons et al. demonstrated that AR during the same period produced long term muscle phenotype changes in a tongue retractor muscle, the styloglossus, indicating that normal suckling behavior is necessary for the normal postnatal development of the tongue (Kinirons et al., 2003). In light of these developmental alterations in rats deprived of oral feeding or suckling, it is possible that intraoral administration of sucrose may be followed by oromotor dysfunction, especially in swallowing. This dysfunction may in turn lead to stress-induced Fos-IR in the PBN.

In the present study, we did not assess non-chemical effects of taste stimulation on Fos-IR, i.e. the baseline level of c-Fos expression, using water as an intraoral stimulus. In some studies, intraoral water stimulation leads to moderate Fos-IR in the nucleus of the solitary tract (Harrer and Travers, 1996; King et al., 1999). However, Yamamoto and Sawa (2000) showed only negligible Fos-IR following intraoral and intragastric water stimulation in both nucleus of the solitary tract and PBN. This discrepancy can be explained by differences in the duration of stimulus presentation. Studies showing water-induced Fos-IR in the gustatory brainstem used much longer fluid presentations, on the order of 7–7.5 ml in 25–30 minutes (Harrer and Travers, 1996; King et al., 1999). In contrast, little water-evoked FOS-IR was found when 7.5 ml of water was delivered in 7.5 min (Yamamoto and Sawa; 2000). This finding implies that water but not taste stimulation requires a longer time to induce detectable Fos-IR in brainstem gustatory nuclei, or that there is a timing difference in terms of Fos-IR between the NST and PBN. Considering the short duration of stimulus presentation in the present study (6ml for 6 min), it is likely that different levels of Fos-IR across groups are due to altered parabrachial neuronal sensitivities to taste quality rather than post-ingestive or mechanical properties of taste stimuli presented in the oral cavity.

In this study, we show for the first time that depriving an animal of taste or somatic stimulation during postnatal development leads to altered neuronal activity in the PBN following orosensory stimulation. However, the mechanism underlying this alteration has not yet been uncovered. Furthermore, it should be noted that while the use of c-Fos immunocytochemistry allows visualization of large amounts of activated neurons at once, it indicates increased activity, and not decreases in activity or inhibition. Future studies using electrophysiological techniques are needed to dissociate the effect of gustatory factors from oromotor factors on PBN development. Further study of these mechanisms should prove crucial for the establishment of evidence-based medicine feeding therapy for infants who require nutritional support with tube feeding.

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Figure 1.

Schematic drawing of the procedures and timelines for each of the 4 experimental groups. GG, gastrostomy + intragastric infusion; GO, gastrostomy + intraoral infusion; GM, gastrostomy + mother rearing; MR, mother rearing.

Figure 2. Schematic diagram of the intraoral cannula used in the present study. The taste solution was presented through the cannula, which was fixed on the right cheek with dental adhesive resin cement. The stimulus was introduced into the oral cavity (vestibule) at about the first maxillary molar, similar to a conventional skull-fixed intraoral cannula.

Figure 3.

Body weight (g) and size (length; mm) of rats in each group. *A*: Body growth as indicated by weight gain during P8 – P21 period. Post-hoc analysis revealed no significant difference on P21. *B*: There were no significant group differences in the mean ($\pm$ S.E.) body length on P21.

Figure 4.

Representative photomicrographs of the waist (gustatory) area located approximately 250 μm caudal to the contact of the inferior colliculus to the pons from individuals in each group. *A*: Nissl-stained section illustrating the location of the central medial (cms) and ventral lateral subnuclei (vls) relative to the brachium conjunctivum (BC). *B*: GG group; *C*: GO group; *D*: GM group; *E*: MR group. Scale bars = 200 μm .

Figure 5.

Representative photomicrographs of the dorsal lateral (dls) and external lateral subnuclei (els) located approximately 50 μm caudal to the contact of the inferior colliculus to the pons from individuals in each group. A: Nissl-stained section illustrating the location of the dorsal lateral (dls) and external lateral subnuclei (els) relative to the brachium conjunctivum (BC). B: GG group; C: GO group; D: GM group; E: MR group. Scale bars = 200 μm .

Figure 6.

Mean ($\pm$ S.E.) number of Fos-IR+ nuclei in the waist area (A), dorsal lateral (B), and external lateral subnuclei (C) in each of the 4 experimental groups. Fos-IR in the waist area and external lateral subnucleus in the GG group was significantly higher than in the other three groups. In the waist area Fos-IR of the GO group was also significantly higher than the MR group. * $p < 0.05$. ** $p < 0.01$.