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RESEARCH ARTICLE

Effects of neonatal rearing by different types of foster mother on the distribution of corticotropin-releasing factor neurons in the central amygdaloid nucleus in rats

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Abstract

The mother–child relationship of newborns plays an essential role in the development of the central nervous system, and an inadequate relationship, such as mother–child separation, can cause deficits of mental function in adulthood. However, insufficient research has examined the effects of foster mothers. We assigned some neonatal rats to one of two foster mothers: one that was lactating and feeding her first litter (FL group) and one that had one previous experience of childbirth and feeding but no current litter (FE group). Other pups were raised by their own mother (OM group) or subjected to maternal separation (MS group). Pups were placed with the foster mother (FL and FE groups) or separated from their mother (MS group) for 3 h/day on postnatal days 1–20. At age 6 weeks, each group was divided into two subgroups, one with 30 min of acute restraint stress loading (FL-R, FE-R, OM-R, and MS-R) and one without it (FL, FE, OM, and MS). Then, we compared the density of corticotropin-releasing factor-immunoreactive (CRF-ir) neurons in the central amygdaloid nucleus (CeA). The density of CRF-ir neurons in the CeA was significantly lower in the FL-R and MS-R subgroups than in the FL and MS subgroups, respectively. The results suggest that differences in care received during the neonatal period affect maturation of CRF neurons in the CeA and may have negative effects on the synthesis and release of CRF.

Keywords Corticotropin-releasing factor neuron · Central amygdaloid nucleus · Foster · Mother–child relationship

Introduction

The neonatal mother–infant relationship plays an integral role in the development of the central nervous system. In rats, separating pups from their mothers was shown to increase anxiety and depression-related behaviors in adulthood (Lambás-Señas et al. 2009) and cause behavioral and learning impairments (Mirescu et al. 2004; Kwak et al. 2008; Mogi et al. 2011), probably due to changes in brain structure. The findings of Malter Cohen et al. (2013) and Coplan et al. (2014) from animal studies revealed that maternal separation causes structural changes in the amygdala, the region that plays a central role in memory, integration, and retrieval of fear experiences and mediates various defensive responses (Jolkkonen et al. 2002; Maren 2005; Balleine and Killcross 2006; Tsvetkov et al. 2015).

Corticotropin-releasing factor (CRF) in the paraventricular hypothalamic nucleus (PVN) is an important regulator of the hypothalamic–pituitary–adrenal (HPA) axis, which acts as a defense system against stress. Dysregulation of the HPA axis is associated with depression and neuro-immunodeficiency (Li et al. 2015; Raineki et al. 2016; Jiang et al. 2019). Environmental stress in early childhood, such as mother–infant separation, is thought to alter CRF in the PVN, affecting the stress response of the HPA axis (Plotsky and Meaney 1993). Also, early rearing conditions alter the central CRF system (Plotsky et al. 2005), which may affect the expression of endocrine responses to stress in adulthood (Desbonnet et al. 2008; O'Malley et al. 2011).

CRF is found in a wide range of the brain regions besides the PVN, including the amygdaloid nucleus (Weiss et al. 2011; Diamantopoulou et al. 2013; Li et al. 2015). The central amygdaloid nucleus (CeA) receives signals from

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the basolateral amygdalar complex, which associates conditioned and unconditioned fear-related stimuli (Pitts et al. 2009). Previous studies showed that CeA lesions attenuate both stress-induced anxiety behavior and activation of the HPA axis (Ventura-Silva et al. 2013). Conversely, continuous expression of CRF in the CeA is associated with dysregulation of the HPA axis, leading to increased anxiety behavior and impairment of reproductive physiology and behavior (Keen-Rhinehart et al. 2009). Maternal separation also was shown to reduce CRF receptor type 2 (Weiss et al. 2011) and increase CRF-like immunoreactivity and CRF mRNA levels (Plotsky et al. 2005) in the CeA.

From the above, we hypothesized that the mother–child and foster mother–child relationship in the neonatal period may affect the formation and maturation of neurons that show immunoreactivity to CRF (CRF neurons) in the CeA. Therefore, we designed new foster mother models with foster mothers that were lactating and feeding their first litter (FL) and others that had one previous experience of childbirth and nursing but did not currently have their own litter (FE). We compared the change in density of CRF neurons in the CeA in rats raised during the entire lactation period by their own mother (OM group) with that in rats that, for 3 h/day, were placed with one of these foster mothers (FL and FE groups) or separated from their own mother (MS group). Last, in each group, we compared the density of CRF neurons in male rats with and without 30 min of acute restraint stress (ARS) loading.

Materials and methods

All animal experiments were approved by the Saga Medical School Animal Care and Use Committee, and all experiments complied with all applicable laws, regulations, and standards in Japan.

In total, 12 virgin female and 6 male Sprague–Dawley rats aged 9–10 weeks were purchased for reproduction from Japan SLC, Inc (Shizuoka Japan) to obtain offspring (the ARS loading experiment required a total of 24 male offspring; see below). No criteria were set for including or excluding animals. Rats were housed in groups of three rats of the same sex in a standard rat cage in a humidity- and temperature-controlled vivarium (humidity, 55%; temperature, 22 °C) with a 12-h light/dark cycle (lights on at 8:00 PM); standard rodent chow and water were available ad libitum. Bedding materials were changed twice a week. After an acclimation period of at least 6 days from arrival, one male (11–17 weeks old) was mated with one or three female(s) (9–16 weeks old) over a 7-day period; each pregnant female was housed alone during the last 2 weeks of gestation. The day of delivery was designated as postnatal day (PND) 0. All but one of the litters contained 12 to 16 pups of both sexes, and one litter contained 7 pups of both sexes.

On PND 1, the neonatal rats in each litter were divided into three or four groups without culling and without being removed from the litter (four or five pups were assigned to each group, and each group had approximately the same number of males and females; no randomization was used for allocation to groups, no controlling for confounders was required, because all rats underwent the respective intervention at the same time). Then, each of the groups was assigned to one of four conditions for PND 1–20, i.e., the day before weaning. The following three conditions were applied for 3 h/day from 8:00 AM to 11:00 AM: placement with a foster mother that was feeding her own first litter (FL group), placement with a foster mother that was not feeding a litter but had one previous experience of birthing and nursing (FE group), and placement alone in a new cage for maternal separation (MS group). In the fourth condition, neonatal rats were left with their own mother (OM group). We separated rats in the FL, FE, and MS groups for 3 h a day until PND 20, because this approach has been used in several previous studies (Ren et al. 2007; Malter Cohen et al. 2013; Danielewicz and Hess 2014; Yasuda et al. 2016; Paternain et al. 2016). Individual animals in each group were marked by numbering them in different colors applying a picric acid solution to their back or the base of the tail.

In the FL, FE, and MS groups, the procedure was as follows: first, the mother was transferred to a new cage, and each group of pups from the litter was moved to the respective cage of the FL, FE, or MS group. Then, the mother was returned to her own cage where the pups of the OM group remained, if applicable. The FL, FE, and MS cages were placed in a separate room from the real mother so that the pups would not be affected by the sounds and odors of the mother. At the end of the 3-h period, the neonatal rats in the FL, FE, and MS groups were returned to own mothers. The foster mother of the FL group received only foster pups during the neonatal handling period and otherwise reared her own pups. In the litter with only seven pups, all were assigned to the OM group. After weaning, animals were housed in groups of three and bred normally. No blinding procedures were used.

Six weeks after birth, on the day the animals were to be euthanized, three males of the FL, FE, MS, and OM groups were exposed to ARS loading (FL-R, FE-R, MS-R, and OM-R subgroups, respectively). For ARS loading, animals were lightly anesthetized with isoflurane and put into an acrylic resin animal holder with multiple breathing holes (38 mm in diameter, 152 mm in length; AS ONE, Osaka, Japan) for 30 min. Afterward, rats were housed for 90 min in a cage with access to food and water ad libitum. In each group, three other males were not exposed to ARS loading controls (FL, FE, OM, and MS subgroups).

After the ARS loading experiment, all study animals (130–275 g body weight) were anesthetized by intraperitoneal injection of a mixture consisting of 0.15 mg/kg medetomidine hydrochloride, 2 mg/kg midazolam, and 2.5 mg/kg butorphanol tartrate and fixed by transaortic perfusion of physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The brains were removed, divided into three pieces (by cutting just rostral to the optic chiasm and caudal to the mammillary body) and re-fixed by immersion in the same fixative for 2 h or more. After immersion in 10% and 20% sucrose in phosphate-buffered saline (PBS) for 2 h at room temperature and overnight at 4 °C, frontal sections were serially cut at a thickness of 40 µm throughout the entire amygdala with a freezing microtome (Yamato Kohki Industrial Co, Saitama, Japan) and placed in PBS. Then, every sixth section (cut at 240-µm intervals) was used for CRF immunohistochemistry with the avidin biotin complex method. First, each section was incubated with 2% normal donkey serum for 1 h to block nonspecific reactions, then with rabbit antibody to CRF (Peninsula Laboratories International, San Carlos, CA; T-4037; diluted 1:6,400) for 1 h at room temperature and overnight at 4 °C. On the next day, sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA; diluted 1:200) and avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA; diluted 1:200) for 1 h each. Between each step, sections were rinsed with PBS three times for 5 min each. Then, the sections were treated with 0.02% diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan) in 0.05 M Tris buffered saline (TBS, pH 7.6) containing 0.005% H_2O_2 and rinsed with TBS and PBS. After that, the sections were mounted on gelatin-coated glass slides and viewed and photographed with an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP71 digital camera (Olympus, Tokyo, Japan). To demarcate the amygdala, the sections adjacent to those stained for CRF were stained with hematoxylin (Fig. 1). Image processing software (Adobe Photoshop) was used to make minor adjustments to the brightness and contrast of the digital microscopy images.

The density of CRF-immunoreactive (CRF-ir) neurons in the CeA was calculated by dividing the total number of CRF-ir neurons counted bilaterally in every sixth section by the total CeA area where CRF fibers or terminals were distributed in each section. In one animal in the FE-R subgroup, cells were counted on only one side, because the amygdala regions were damaged during tissue preparation. Accordingly, the data of the FE males with ARS were obtained from counting neurons and fibers/terminals in five sections from each of three animals. Cells were counted and areas measured with a Java image processing program from the public domain (ImageJ v. 1.49e from National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

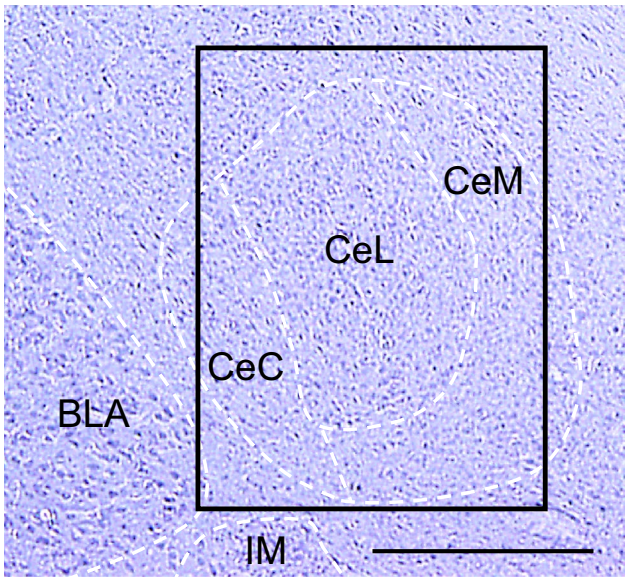

Fig. 1 HYPERLINK "sps:id::fig1|locator::gr1|mediaobject::0" Photomicrogram showing hematoxylin staining of the central amygdaloid nucleus and its vicinity, corresponding to Bregma −2.12 mm of "The Rat Brain in Stereotaxic Coordinates" (Paxinos and Watson). The section is adjacent to that shown in Fig. 2h. Subnuclei are demarcated by white broken lines. The approximate area in the solid black box is shown in Fig. 2. *BLA* basolateral amygdaloid nucleus, anterior part; *CeC* central amygdaloid nucleus, capsular part; *CeL* central amygdaloid nucleus, lateral division; *CeM* central amygdaloid nucleus, medial division; *IM* intercalated amygdaloid nucleus, main part. Bar = 500 µm

Statistical analysis

All animals were included in the analysis. Data are expressed as mean ± standard error of the mean (*SEM*). We analyzed results by two-way analysis of variance (ANOVA) with the factors fostering, ARS treatment, and fostering × ARS treatment and then performed a post hoc test. In addition, we compared each of the fostering groups by one-way ANOVA and analyzed differences among means with Tukey–Kramer multiple comparison tests. The restraint (with ARS) and non-restraint (without ARS) subgroups were compared by two-sided Student's *t* test. Because each of the fostering groups was an unrelated and independent group, we compared the restraint (with ARS) and non-restraint (without ARS) conditions within each of the fostering groups also by two-sided Student's *t* test. A *p* value of less than 0.05 was considered statistically significant. All analyses were performed with JMP15 (SAS Institute Japan, Tokyo, Japan).

Results

CRF immunoreactivity in the CeA was almost completely limited to the central part of the lateral subdivision of the CeA and seen only to a small extent in the medial subdivision of the CeA (Fig. 2). Ovoid or multipolar CRF-ir neurons were observed in the CeA in all subgroups, even though they were not treated with colchicine (a drug that is used to stop axonal flow and accumulate synthesized neurochemical substances in the cell body).

Two-way ANOVA showed a significant difference following ARS treatment ($F_{1,39}=8.48$, $p=0.006$). We did not find any significant differences for the factors fostering ($F_{3,39}=1.92$, $p=0.142$) or fostering × ARS treatment ($F_{3,39}=1.26$, $p=0.302$).

Compared with the mean ± *SEM* density of CRF-ir neurons (cell number/mm^2) in the CeA in the OM subgroup, the density tended to be higher in the FL subgroup and lower in the FE and MS subgroups without ARS (OM, 73.0 ± 9.6; FL, 89.5 ± 11.2; FE, 62.2 ± 8.0; and MS, 66.4 ± 6.5; Fig. 3). The comparison of each of the fostering groups by one-way ANOVA did not show any significant differences ($F_{3,20}=1.78$, $p=0.184$), and Tukey–Kramer multiple comparison tests found no difference between the means. After ARS loading, the mean ± *SEM* density of CRF-ir neurons (cell number/mm^2) in the CeA tended to be lower in the FL-R, FE-R, and MS-R subgroups than in the OM-R subgroup (OM-R, 64.3 ± 8.1; FL-R, 56.6 ± 8.1; FE-R, 58.0 ± 6.6; and MS-R, 44.7 ± 4.9; Fig. 3). The comparison of each of the fostering groups by one-way ANOVA did not show any significant differences ($F_{3,19}=1.38$, $p=0.280$), and Tukey–Kramer multiple comparison tests found no difference between the means. No difference in the occurrence and distribution of CRF fibers and terminals in the CeA was observed between each subgroup, indicating that the areas used for counting CRF-ir neurons were almost the same in every subgroup.

The mean density of CRF-ir neurons (cell number/mm^2) in the CeA of the four subgroups with ARS was 55.8 ± 3.65, which was significantly lower ($p=0.007$) than the density in the four subgroups without ARS (72.8 ± 4.73). The same was found when comparing each individual subgroup with ARS with the corresponding subgroup without ARS, whereby the difference was significant only between the MS-R and MS subgroups ($p=0.023$) and between the FL-R and FL subgroups ($p=0.039$) (Fig. 3).

Discussion

To our knowledge, this study was the first to use the foster mother models FE and FL. The present study evaluated the influence of various types of neonatal rearing on the density of CRF-ir neurons in the CeA in rats. The generally used separation model consists of 3 h/day of maternal separation until postnatal day 14 (Lippmann et al. 2007), but we continued to separate neonatal rats from their mothers until PND 20, because several previous studies that used separation until PND 21 showed additional changes, such as in behavior, neural activity, or neurochemical substances (Ren et al. 2007; Malter Cohen et al. 2013; Danielewicz and Hess 2014; Yasuda et al. 2016; Paternain et al. 2016). The main finding of the study was that the density of CRF-ir neurons in the CeA of the FL-R and MS-R subgroups was significantly lower than that in the CeA of the respective subgroup (i.e., FL and MS).

An earlier study found that, in response to a stimulus, CRF mRNA is created in 60 min and CRF is synthesized in 20–30 min in CRF neurons in the rat PVN (Watts 2005). Although the study examined in the PVN, it is conceivable that a similar process occurs in CRF neurons in the CeA. A non-maternal separation study by Sterrenburg et al. (2012) showed that the number of CRF-ir neurons in the CeA was not different in male rats 1 h after a 1 h-restraint stress and non-stressed male rats but that the number of CRF mRNA-containing CeA neurons was increased after the same stress. Another study found that the CRF mRNA level in the CeA increased after acute stress (electric foot shock) in male rats subjected to maternal separation (24 h, on PND 9) but not in non-separated animals (Barna et al, 2003). The present study showed that the density of CRF-ir neurons in the CeA 90 min after 30 min' ARS in the OM-R subgroup was not different from that in the non-stressed OM subgroup. This finding is consistent with that of Sterrenburg et al. (2012), although the present study did not evaluate CRF mRNA expression.

We found no significant difference in the density of CRF-ir neurons also in the FE groups with and without ARS, suggesting that these handling conditions may have represented stress-free environments for the neonates. A study in monkeys found that cohabitation with a stuffed surrogate during childhood resulted in normal development (Reite et al. 1978). The foster mothers with previous experience of parenting (FE group) may have accepted foster pups earlier; previous studies reported that even virgin rats can be induced to express maternal behavior by repeated exposure to pups for 5–7 days (Rosenblatt, 1967). Although the FE foster mother in the present study could not suckle the pups, she probably behaved as a surrogate mother and thereby may have provided a stress-free environment; consequently, the synthesis and release of CRF were not negatively affected.

Maternal separation is known to represent a model of chronic stress during the neonatal period (Coplan et al. 2014; Malter Cohen et al 2013; O'Malley et al. 2011). In the present study, ARS decreased the density of CRF-ir neurons in the CeA of the MS-R subgroup compared with the MS

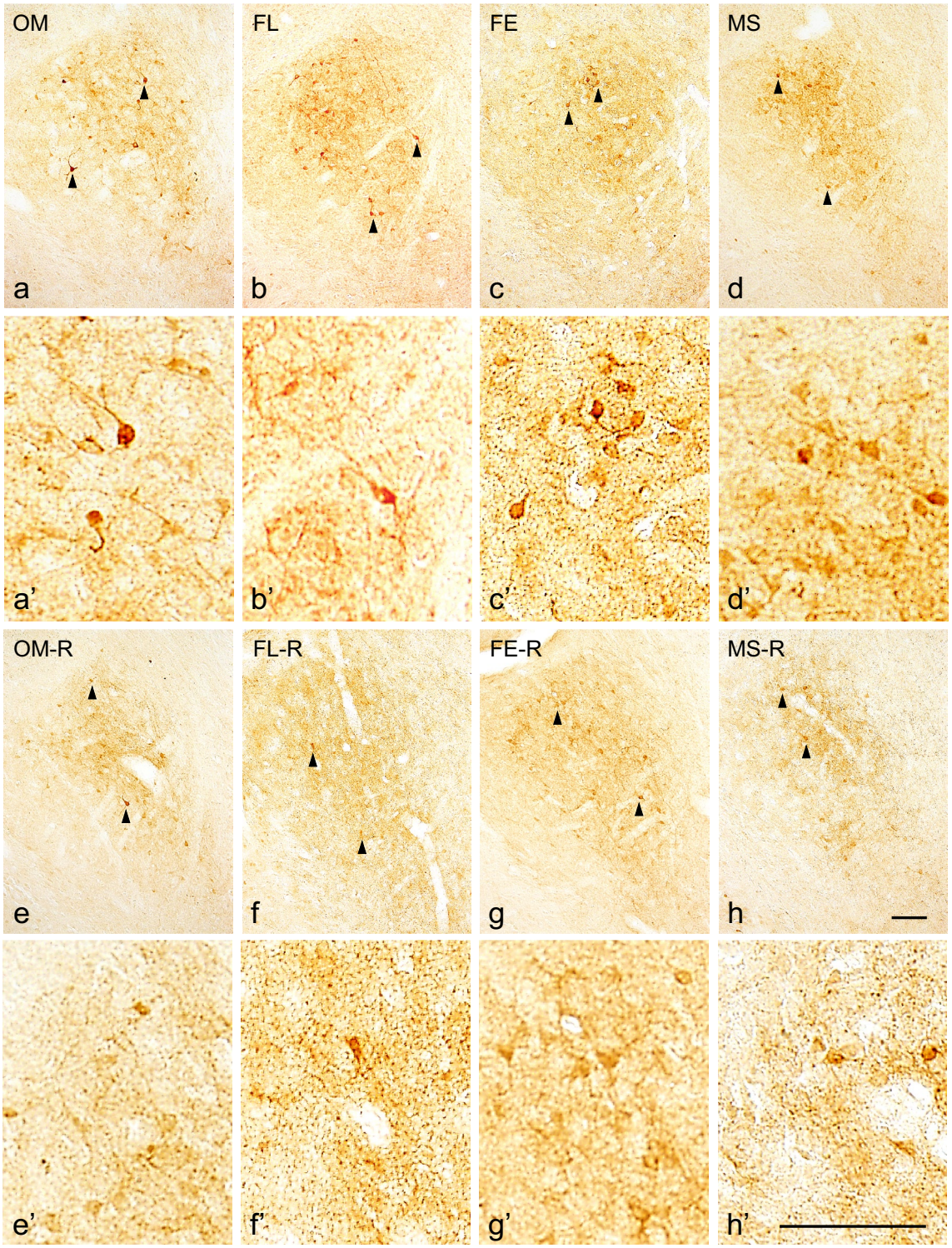

Fig. 2 Photomicrograms showing corticotropin-releasing factor immunoreactivity in the central amygdaloid nucleus of 6-week-old rats. Note that immunoreactive cell bodies (some of them are indicated by arrowheads) are intermingled with nerve fibers or terminals in the central amygdaloid nucleus, especially in its lateral division. The region around the cell indicated by the upper arrowhead is magnified in **a'** to **h'**. Note that corticotropin-releasing factor-immunoreactive cell bodies and fibers are visible. Sections are from rats left with their own mother (OM; **a**), subjected to one of three handling conditions for 3 h/day on postnatal days 1–20 (placed with a foster mother rearing her first pups [FL; **b**], placed with a foster mother that had experienced childbirth and nursing once [FE; **c**], separated from their mother [MS; **d**]), or subjected to one of these handling conditions and acute restraint stress (OM-R, FL-R, FE-R, and MS-R; **e**–**h**). Bars = 200 µm. Note that the bar in **h** also applies to **a**–**g** and the bar in **h'** also applies to **a'**–**g'**

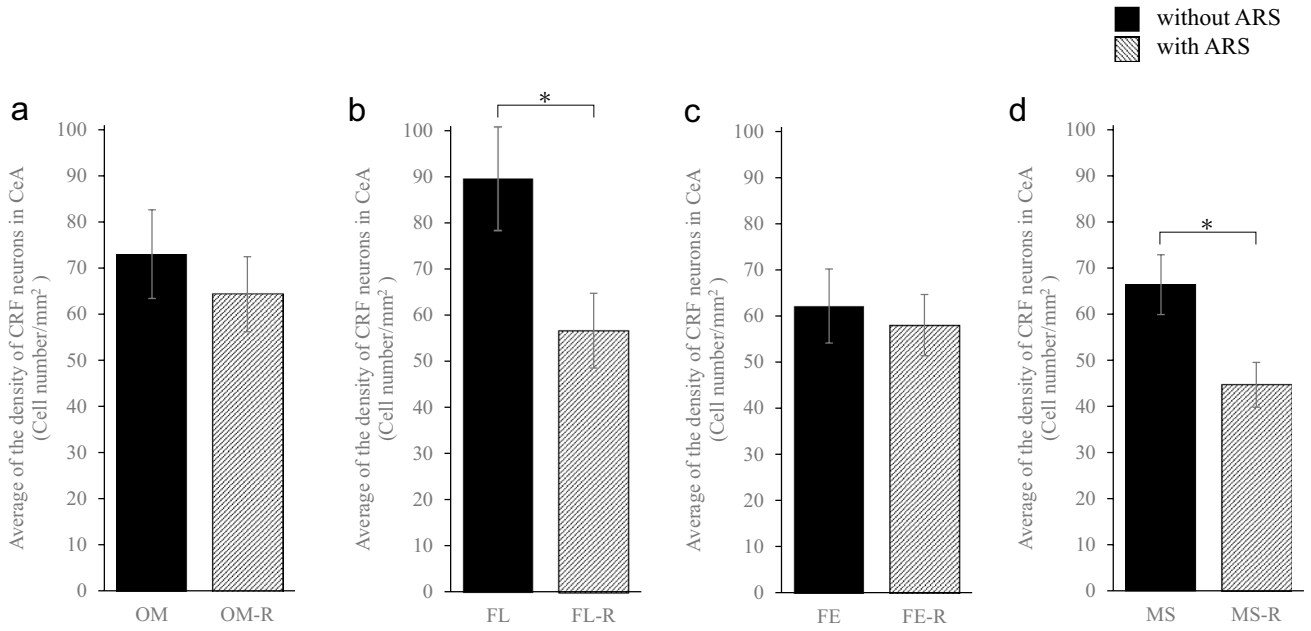

Fig. 3 Density of corticotropin-releasing factor-immunoreactive neurons in the central amygdaloid nucleus of rats left with their own mother (OM, **a**), rats placed with a foster mother rearing her first rats (FL, **b**), rats placed with a foster mother that had experienced childbirth and nursing once (FE, **c**), and rats separated from their mother (MS, **d**) with (OM-R, FL-R, FE-R, and MS-R; hatched bars) and without (solid bars) acute restraint stress. Bars represent mean ± standard error of the mean. *$p < 0.05$. *CeA* central amygdaloid nucleus; *CRF* corticotropin-releasing factor; other abbreviations as for Fig. 2

subgroup. A similar study by Desbonnet et al. (2008) showed that the number of CRF-ir neurons in the CeA 90 min after acute stress (forced swimming) decreased in rats with neonatal maternal separation but not in rats without maternal separation. As suggested by Desbonnet et al. (2008), the decrease in the density of CRF-ir neurons in the CeA after neonatal maternal separation may be due to delayed synthesis of CRF resulting from an impaired ability of CRF synthesis in some CeA neurons rather than to diminution or disappearance of CRF neurons themselves.

The density of CRF-ir neurons in the CeA was also lower in the FL-R subgroup than in the FL subgroup. Lactating rodent mothers have been shown to recognize their offspring and distinguish between their own and other pups (Ostermeyer and Elwood 1983). Pups in the FL group may have experienced a certain psychological burden from spending time with the lactating foster mother. Accordingly, the present findings suggest that experiencing placement with a lactating foster mother in the neonatal period causes chronic stress and affects the functional maturation of CRF-ir neurons in the CeA.

Unexpectedly, we did not find a significant difference in the density of CRF-ir neurons between each neonatal handling group (FL, FE, and MS) and the OM group. Previous maternal separation studies that compared early (PND 1–14 or PND 1–6) and late (PND 14–21 or PND 16–21) neonatal handling revealed behavioral and biochemical changes (in plasma corticotropin level, c-Fos activity, and body weight) in the former period but not in the latter period (Horii-Hayashi et al. 2013; Matsumoto et al. 2006). We suggest that the longer neonatal handling (i.e., PND 1–20) causes a partial disturbance of maturation of CRF-ir neurons in the CeA, but it does not affect immunohistochemical detection obviously when acute stress is not loaded.

In the present study, we did not perform behavioral observations on fostering-related mother–pup relations, but such observations may be useful for precisely monitoring the rearing environment. Further studies are required, including studies on other types of neonatal handling, such as the use of virgin foster mothers or maternal separation with a feeding bottle.

In conclusion, we suggest that the maturation of CRF-ir neurons in the CeA is partially impaired by rearing by a certain type of foster mothers, which influences the synthesis and release of CRF in neurons. This hypothesis is supported by the finding that the density of CRF-ir neurons in the CeA in adolescent rats was decreased after ARS in rats that were placed with a foster mother feeding her own first litter, a finding that was similar to that in rats exposed to maternal separation.

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Author contributions Both authors contributed to the study conception and design and performed material preparation and data collection and analysis. The first draft of the manuscript was written by MN-N, and HK commented on all versions of the manuscript. Both authors read and approved the final manuscript.

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Data availability The data that support the findings of this study will be made available upon reasonable request to the corresponding author.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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