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Neuronal activation associated with paternal and aversive interactions toward pups in the Mongolian gerbils (*Meriones unguiculatus*)

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ABSTRACT

Approach/avoid model is used to analyze the neural regulation of maternal behavior in the laboratory rat. This model proposes that the medial preoptic area (mPOA) and bed nucleus of stria terminalis (BNST) are brain regions involved in facilitating mechanisms. By contrast, anterior hypothalamic nucleus (AHN), ventromedial hypothalamic nucleus (VMH), and periaqueductal gray participate in the inhibiting mechanisms of neural regulation of maternal behavior. We hypothesized that there are also facilitating and inhibiting mechanisms in the neural regulation of paternal behavior. Here, we determined which neural areas are activated during paternal and aversive interactions with pups in the Mongolian gerbils (*Meriones unguiculatus*). By testing paternal behavior, we selected 40 males aggressive toward pups and 20 paternal males. These males were organized into six groups of 10 animals in each group: aggressive males that interacted with pups (AGG-pups) or candy (AGG-candy), paternal males that interacted with pups (PAT-pups) or candy (PAT-candy), and males with testosterone (T)-induced paternal behavior that interacted with pups (IPAT-pups) or candy (IPAT-candy). After interacting with pups or candy, the brains were extracted and analyzed for immunoreactivity (ir) with *c-fos*. Males that interacted with pups had significantly higher *c-fos*-ir in the mPOA/BNST than males that interacted with candy. Males that displayed aggression had significantly higher *c-fos*-ir in the AHN, VMH, and periaqueductal gray than aggressive males that interacted with candy. These results suggest that in the neural regulation of paternal behavior in the Mongolian gerbil underlie positive and negative mechanisms as occurs in maternal behavior.

1. Introduction

The neuroendocrine basis of maternal behavior in mammals has been extensively studied using the laboratory rat as the main model (Factor et al., 1993; Fleming et al., 1980; Hansen et al., 1991; Koch and Ehret, 1991; Numan and Insel, 2003). Maternal behavior occurs when the tendency to approach and interact with stimuli from pups is greater than the tendency to avoid these stimuli. The approach or avoidance of pups depends on the physiological state of the female. Virgin female rats avoid pups, but when they are treated with progesterone and estradiol, which simulate the hormonal changes that occur at the end of pregnancy, the avoidance behavior changes to maternal behavior (Rosenblatt and Mayer, 1995; Dulac et al., 2014). This duality of approach/avoidance involves several regions of the brain: a facilitating mechanism that descends from the medial preoptic area (mPOA) through the bed nucleus of stria terminalis (BNST) to the midbrain and an inhibitory mechanism that descends from the middle hypothalamus, anterior hypothalamic nucleus (AHN), and ventromedial hypothalamus nucleus (VMH) to the midbrain (periaqueductal gray) (Numan and Insel, 2003; Numan, 2014; Lonstein et al., 2015; Bales and Saltzman, 2016). Both neural regions involved in the facilitation and inhibition of maternal behavior have multiple connections with other regions such as the medial amygdala (MeA), which receives projections from the olfactory bulb (OB) (Numan and Insel, 2003; Numan, 2014; Dulac et al., 2014; Bales and Saltzman, 2016).

Knowledge of the neural mechanisms that regulate maternal

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behavior has been obtained by using electrolytic lesions, exocytotoxic lesions, magnetic resonance imaging, and neuronal activation markers such as the products of early expression genes including the *fos* family, which are used for mapping the functional anatomy of the neuroendocrine system (Baum and Everitt, 1992; Coolen et al., 1996; Hoffman et al., 1993; Koch and Ehret, 1991; Lee et al., 2000; Lonstein and Stern, 1998; Newman, 1999; Numan and Insel, 2003; Numan, 2014).

Some studies have shown that several regions that are part of the neural circuit of maternal behavior also play an important role in regulating paternal behavior. In the California mouse, electrolyte lesions in the mPOA, nucleus accumbens, and MeA cause a decrease in the time invested in retrieving, crouching, and grooming (Lee and Brown, 2007). In prairie voles, interactions with pups were found to activate regions such as the mPOA, BNST, lateral septum, and MeA by using *c-fos* as a marker of neuronal activity (Kirkpatrick et al., 1994a, 1994b).

As females, most virgin males of biparental species tend to attack pups and possibly even kill them. However, when these males are fathers, they show a paternal behavior (Elwood, 1977, 1980; Gubernick et al., 1994; Vella et al., 2005). The transition from infanticidal male to paternal male has a high adaptive value because the survival of offspring is vital for the continued existence of any species (Hrdy, 1979). Virgin males aggressive toward pups of the Mongolian gerbil and Mexican volcano mouse (*Neotomodon alstoni*) show paternal behavior when they are primed with testosterone (T) (Luis et al., 2010; Martínez et al., 2015; Luis et al., 2017). This duality in the behavior of males toward pups is similar to that observed in females. Based on this, we hypothesized that there are facilitating and inhibiting mechanisms in the neural regulation of paternal behavior and that these mechanisms underlie neural regions that are activated according to the response of the male toward pups. The aim of this study was to provide evidence of the existence of inhibiting and facilitating mechanisms in the neural regulation of paternal behavior by using Mongolian gerbil (*Meriones unguiculatus*) as the model. Neural circuit of paternal behavior has not been established, although brain areas such as mPOA, BNST, OB, MeA, lateral *septum*, and nucleus *accumbens* are involved in the regulation of paternal behavior (Lee and Brown, 2007; Kirkpatrick et al., 1994a, 1994b; Lonstein et al., 2015). The Mongolian gerbil is a monogamous species; the male significantly participates in the care of pups (Elwood, 1975). In the Mongolian gerbil, social factors such as copulation, cohabitation with a pregnant female, and the presence of pups may activate neuroendocrine changes that facilitate paternal behavior (Brown, 1993; Brown et al., 1995; Reburn and Wynne-Edwards, 1999). This transition is associated with a significant increase in T (Martínez et al., unpublished data).

2. Materials and methods

2.1. Animals

In this study, we used virgin male Mongolian gerbils of age 180–210 days old. Gerbils were weaned between the age of 25 and 28 days. The animals were obtained from a breeding colony kept at the Facultad de Estudios Superiores Iztacala, UNAM. The colony was maintained under an inverted photoperiod of 12:12 h (light–dark cycle; onset of light at 18:00 h) at an ambient temperature between 17 and 21 °C. Gerbils were fed with pellets of Lab Chow 5001 (Nutrimentos Purina, México) and tap water *ad libitum*. At the beginning of the study, three or four gerbils of the same sex were housed in a polycarbonate cage (37 × 27 × 15 cm) with sawdust bedding. We used virgin male Mongolian gerbils to avoid the effect of sexual experience. Virgin males of this rodent may show an aggressive or paternal behavior toward foreign pups of the same species. The criteria for aggressive male behavior included sniffing and attacking pups and moving them violently. In addition, pups may be bitten if they are not withdrawn. Paternal males sniff and touch pups with the nose and groom and crouch beside them. Through screen tests of paternal behavior, 40 males aggressive toward pups and 20 paternal males were selected; in this study, we name males aversive to those males aggressive toward the pups because virgin aversive female rats also attack the pups (Peters et al., 1991). During these tests, each male was placed in a polycarbonate cage (37 × 27 × 15 cm) with clean sawdust bedding. After 10 min, two 1- to 3-day-old pups were introduced into the cage. The pups were withdrawn quickly if they were attacked (Elwood, 1991; Martínez et al., 2015). When the males displayed paternal behavior, the observation period was for 10 min. These selected gerbils were organized into six groups of 10 animals in each group. In groups 1 and 2, we included gerbils aversive toward pups; the males of group 1 interacted with pups (AGG-pups) and those of group 2 interacted with candy (AGG-candy). The gummy bear was used as control stimulus for its softness and size, thereby trying to make it similar to the pups of the Mongolian gerbil. Candy has also been utilized as the control stimulus in male–pup interactions in prairie voles (Kirkpatrick et al., 1994a, 1994b). Groups 3 and 4 were integrated with paternal males. In group 3, males interacted with pups (PAT-pups), whereas males of group 4 interacted with candy (PAT-candy). In groups 5 and 6, we included males that were initially aversive toward pups, but those males were primed with T to induce paternal behavior (Martínez et al., 2015). The males of group 5 interacted with pups (IPAT-pups), and those of group 6 interacted with candy (IPAT-candy). Males with T-induced paternal behavior were included to compare the neural activation of these males with that of spontaneously paternal males. Before interactions with the pups or candy, aggressive and paternal males remained in individual cages for 24 h, and they were not disturbed to isolate them of stimuli that could cause neural activation. Tests of paternal behavior or interactions with candy were performed on the morning of the following day. The T concentrations were quantified to analyze the correlation of T levels with neural activity.

All experiments were performed in accordance with the ethical guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023) and the ethical guidelines and technical specifications of the Mexican Official Norm for the Production, Care and Use of Laboratory Animals (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists, 2016).

2.1.1. Induction of paternal behavior

Aggressive gerbils were primed with a T solution (1 mg T/10 ml of sesame oil, Sigma-Aldrich, St. Louis, MO, USA) (Siegel and Rosenblatt, 1975). Each gerbil was injected subcutaneously in the dorsal region with 0.1 ml of this solution after asepsis of the zone with benzalkonium chloride.

2.1.2. Tests of paternal behavior

At 24 h after isolation, behavioral tests were performed following the method described above for the paternal behavior screening. Two pups were used in each test session for a total of 240 pups. The test was ended when pups were attacked. Six pups were slightly bitten, and their wounds were healed with application of gentian violet (1%) because this antiseptic has no odor. Then they were returned to their parents. The exposure period was 90 min for paternal males to interact with the pups or candy and for aggressive males to interact with candy. In the test with paternal males, the pups were changed two times during the exposure period. These interactions were videotaped with a high-definition infrared camera (IR Bullet camera, 2.1 megapixels). Latencies of the onset of paternal behavior (the time elapsed since the pups were introduced until the male made contact with one of them) and the time invested in crouching and grooming were recorded. Observations were made between 11 and 14 h.

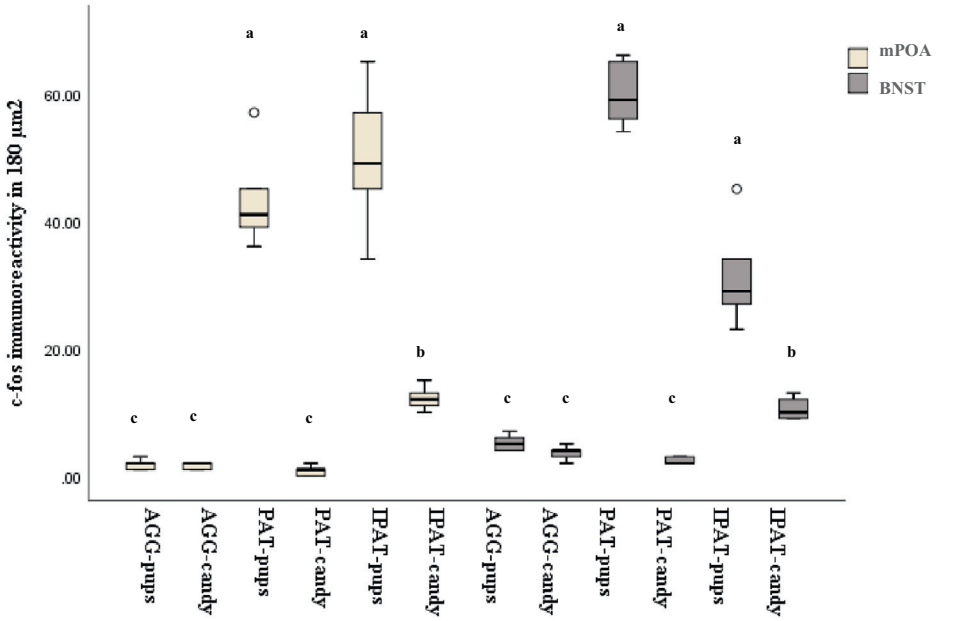

Fig. 1. Paternal males that interacted with pups (PAT-pups and IPAT-pups) have a significantly higher number of *c-fos*-ir cells in mPOA/BNST than paternal males that interacted with candy (PAT-candy and IPAT-candy) and aggressive males (AGG-pups and AGG-candy). Data are presented as the median. Circles indicate outliers, and letters indicate significant differences.

2.2. *c-fos* immunohistochemistry

Five gerbils from each group were deeply anesthetized with a dose of 10 mg/kg xylazine and 90 mg/kg ketamine for 70 min after the interactions because the product of the expression of *c-fos* reaches its maximum between 60 and 90 min following exposure to a stimulus (Morgan et al., 1987; Hoffman et al., 1993). Then, they were intracardially perfused with physiological saline (0.9%), followed by a 2% paraformaldehyde solution in sodium phosphate buffer (0.1 M PB; pH 7.6). The brain was then removed and postfixed for 18 h in the same fixative solution. Subsequently, this tissue was processed and cut into 30 µm thick coronal sections with a cryostat. The neural areas were located by comparison with the stereotaxic atlas of the Mongolian gerbil (Loskota et al., 1974). The OB (+1.7 mm from bregma, image 300), mPOA (−0.1 mm, image 520, the same section), MeA and AHN (both −0.8 mm, image 590, the same section), VMH (−1.3 mm, image 640, the same section), and periaqueductal gray (−3.5 mm, image 860, the same section). Once sections of the different neural areas were obtained, the samples were placed on gelatinized slides (Nutrient Gelatin, 70151-500G-F; Sigma-Aldrich, CA, USA). Each of the following steps was followed by rinsing in phosphate-buffered saline (PBS) for 5 min: (1) 10 min of incubation in 3% H_2O_2 in PBS, (2) 20 min of incubation in 5% normal goat serum (Vectastain ABC kit, PK-4000; Vector Laboratories) in PBS, and (3) 16 h of incubation at 25 °C with 1:50 dilution of a rabbit c-*fos* antibody epitope located in the N-terminus of human origin (sc-52; Santa Cruz Biotechnology, Inc.) in PBS. After two 5 min rinses in PBS, the sections were incubated with biotinylated goat antirabbit antibody in PBS for 90 min and rinsed two times in PBS (Vectastain ABC kit, PK-6102; Vector Laboratories). The sections were then incubated with an avidin–biotin complex (Vectastain ABC kit, PK-6100; Vector Laboratories) for 30 min, followed by two additional rinses with PBS. Finally, binding was visualized by using 3,3′-diaminobenzidine as the chromogen (DAB Peroxidase Substrate, SK-4100; Vector Laboratories). The sections were dehydrated and then cover-slipped. Negative controls were obtained by omitting the primary antibody incubation step. All neural tissues were processed with the same assay. *c-fos* antibody has been utilized in other rodents such as *Microtus ochrogaster* (Kirkpatrick et al., 1994a, 1994b).

2.2.1. *Image analysis*

The number of cells that showed *c-fos* immunoreactivity (ir) was quantified in microphotographs with an area of 180 µm^2. Microphotographs were taken with a Motic camera (10 megapixels) attached to a Leica microscope. Three sections of the same area for each animal were bilaterally quantified for *c-fos*-ir. After the data obtained from each animal were added, the mean and standard deviation were calculated for each group.

2.2.2. *Hormone assay*

Immediately after the parental behavior test, blood samples (250 µl) were collected from the retro-orbital sinus of all the gerbils in each group. Five males in each group were anesthetized with a mild dose (5 mg/kg xylazine and 60 mg/kg ketamine) for obtaining blood samples, and the remaining five males were anesthetized with a heavy dose for obtaining blood samples before perfusion. These samples were collected in heparinized capillary tubes. Each sample was taken for 1 min between 11 and 14 h. Plasma was separated by centrifugation and stored at −70 °C. Hormonal analysis was conducted by ELISA. The T level was measured with a DRG commercial kit (DRG Diagnostics, Marburg, Germany) at a sensitivity of 0.083 ng/ml. The intra-assay and inter-assay coefficients of variation were 3.9% and 4.6%, respectively. The T assay was validated, and it demonstrated a correlation between the dilutions of serum from Mongolian gerbils and the standard curve. The recovery rate for T was 95.0% (r = 1.0). The plate was read in a plate reader (model Multiskan Ascent V1.25, with a filter of 450-nm wavelength; Thermo Electron Corporation).

2.3. *Statistical analysis*

2.3.1. *c-fos immunoreactivity data*

The number of *c-fos* immunoreactivity (ir) cells in the neural areas of males of the AGG-pups, AGG-candy, PAT-pups, PAT-candy, IPAT-pups, and IPAT-candy groups was analyzed with a nonparametric Kruskal–Wallis test because of the non-normal distribution of the data (Anderson–Darling test, $P < 0.05$). Bonferroni correction for multiple testing was applied for pairwise comparisons.

2.3.2. *Testosterone quantification data*

The T levels between the AGG-pups, AGG-candy, PAT-pups, PAT-candy, IPAT-pups, and IPAT-candy groups were also analyzed with the Kruskal–Wallis test. Bonferroni correction for multiple testing was applied for pairwise comparisons.

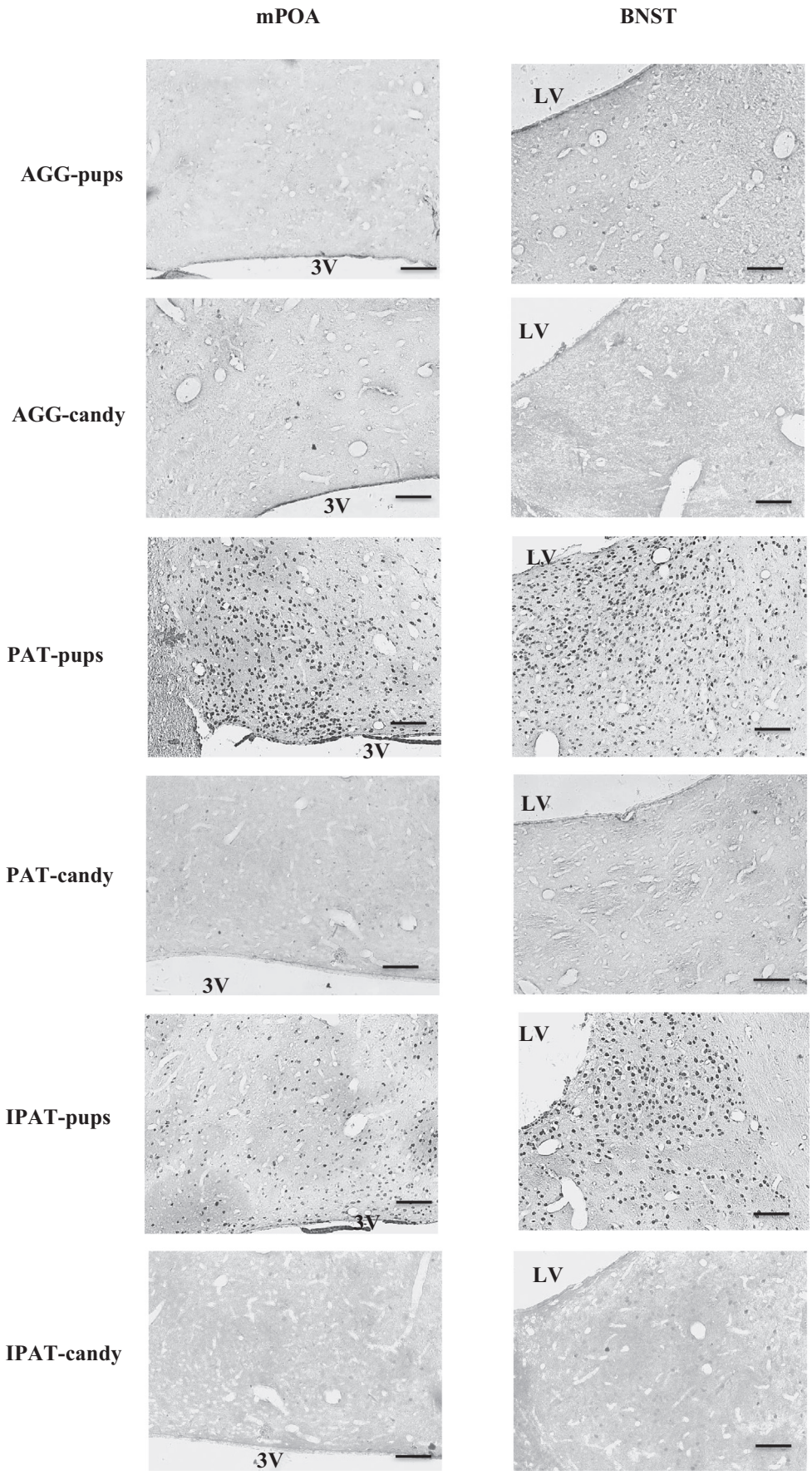

Fig. 2. Representative photomicrographs showing *c-fos*-ir cells in the mPOA/BNST of the males of different groups. 3V = third ventricle, LV = lateral ventricle. Coronal sections, scale bars = 100 μm.

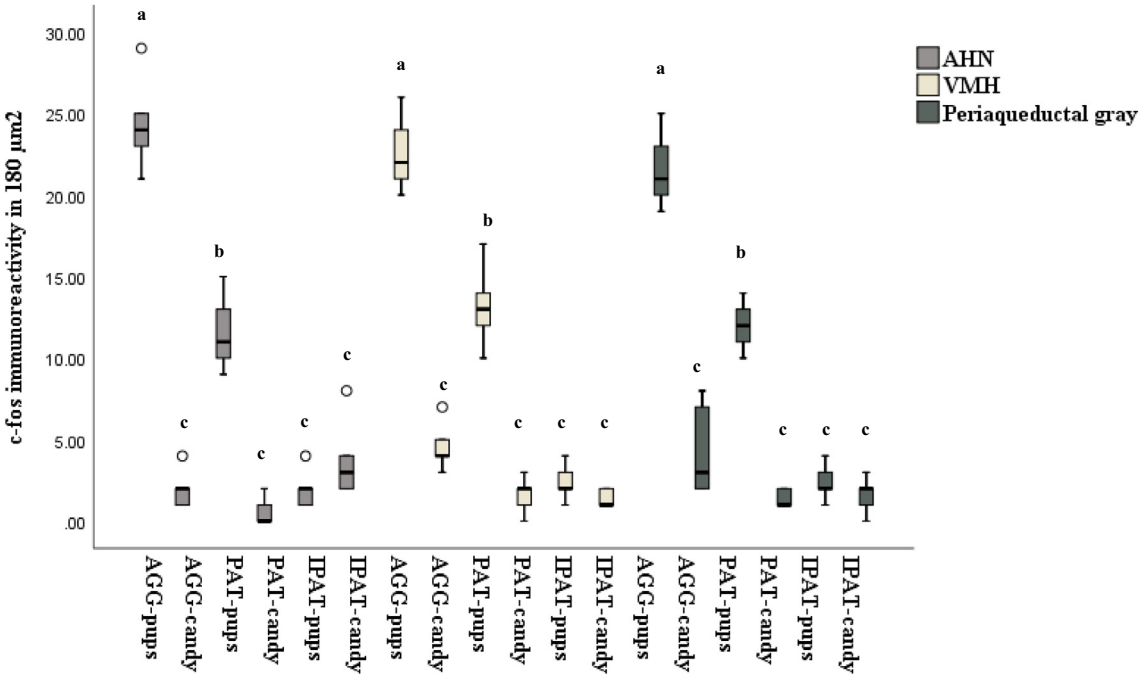

Fig. 3. Number *c-fos*-ir cells in aggressive males that interacted with pups (AGG-pups) had significantly higher c-*fos*-ir in AHN, VMH, and periaqueductal gray than aggressive males that interacted with candy (AGG-candy) and paternal males (PAT-pups, PAT-candy, IPAT-pups, and IPAT-candy). Data are presented as the median. Circles indicate outliers, and letters indicate significant differences.

2.3.3. Behavioral data

The latency of the onset of paternal behavior between the PAT-pups and IPAT-pups groups was compared with the nonparametric Mann–Whitney *U* test. The time spent by these males in grooming and sniffing was compared with the same test.

Finally, T levels were correlated with time invested in huddling and grooming by using Spearman correlation analysis. This analysis was also used to correlate the T concentrations with the number of *c-fos*-ir cells in the mPOA and BNST.

Statistical analyses were performed using SPSS version 21.0 (IBM SPSS, Armonk, NY).

3. Results

3.1. c-fos-immunoreactivity

The number of *c-fos*-ir cells varied significantly between the AGG-pups, AGG-candy, PAT-pups, PAT-candy, IPAT-pups, and IPAT-candy groups in the mPOA ($H = 27.17$, $df = 5$, $P < 0.05$, Figs. 1 and 2) and BNST ($H = 25.54$, $df = 5$, $P < 0.05$, Figs. 1 and 2). *Post hoc* analyses revealed that males of the PAT-pups group had significantly higher number of *c-fos*-ir cells in the mPOA and BNST than males in the PAT-candy, IPAT-pups, IPAT-candy, AGG-pups, and AGG-candy groups. Males in the IPAT-pups group had higher number of *c-fos*-ir cells in these areas than males of the PAT-candy, IPAT-candy, AGG-pups, and AGG-candy groups (Bonferroni adjusted $P = 0.003$).

The number of *c-fos*-ir cells in the AGG-pups, AGG-candy, PAT-pups, PAT-candy, IPAT-pups, and IPAT-candy groups showed significant difference in the AHN ($H = 23.95$, $df = 5$, $P < 0.05$, Figs. 3 and 4), VMH ($H = 24.97$, $df = 5$, $P < 0.05$, Figs. 3 and 4), and periaqueductal gray ($H = 22.78$, $df = 5$, $P < 0.05$, Figs. 3 and 4). Pairwise comparison revealed that males of the AGG-pups group had significantly higher number of *c-fos*-ir cells in the AHN, VMH, and periaqueductal gray than males in the AGG-candy, PAT-pups, PAT-candy, IPAT-pups, and IPAT-candy groups (Bonferroni adjusted $P = 0.003$).

The number of *c-fos*-ir cells in the OB and MeA in the AGG-pups, AGG-candy, PAT-pups, PAT-candy, IPAT-pups, and IPAT-candy groups was significantly different ($H = 25.49$; $H = 27.23$, $df = 5$, $P < 0.05$, respectively, Figs. 5 and 6). Pairwise comparison revealed that males of the PAT-pups group had significantly more *c-fos*-ir cells in the OB and MeA than males of the AGG-pups, AGG-candy, PAT-candy, IPAT-pups, and IPAT-candy groups. Males of the IPAT-pups group had a greater number of *c-fos*-ir cells in these areas than males of the AGG-pups, AGG-candy, PAT-candy, and IPAT-candy groups. Similarly, males of the AGG-pup group had a greater number of *c-fos*-ir cells in OB and MeA than males of the AGG-candy group (Bonferroni adjusted $P = 0.003$).

3.1.1. Testosterone levels

The concentrations of T in plasma from the AGG-pups, AGG-candy, PAT-pups, PAT-candy, IPAT-pups, and IPAT-candy groups showed significant difference ($H = 56.03$, $df = 5$, $P < 0.05$, Fig. 7). Pairwise comparison revealed that males of the IPAT-pups group had significantly higher T concentrations in plasma than males of the AGG-pups, AGG-candy, PAT-pups, PAT-candy, and IPAT-candy groups. Males of the PAT-pups group had higher concentrations of T in plasma than males of the AGG-pups, AGG-candy, and PAT-candy groups. Unexpectedly, males of the AGG-pups group had lower T concentrations than males of the AGG-candy group (Bonferroni adjusted $P = 0.003$).

3.1.2. Paternal behavior

The latency of the onset of paternal behavior between the PAT-pups and IPAT-pups groups was significantly different, with a longer latency in the onset of paternal behavior in the PAT-pups group ($U = 64.5$, $P < 0.01$, Fig. 8). The time invested in crouching over pups was significantly longer in males of the IPAT-pups group than in males of the PAT-pups group ($U = 55.0$, $P < 0.01$, Fig. 8). Similarly, males of the IPAT-pups group invested more time in grooming than males of the PAT-pups group ($U = 57.0$, $P < 0.01$, Fig. 8).

We did not observe significant correlations between the T levels in plasma and the number of c-fos-ir cells in the mPOA ($r = -0.87$, $P > 0.05$) and BNST ($r = 0.38$, $P > 0.05$) in males of the PAT-pups group. We found no correlations between T levels and the number of *c-fos*-ir cells in the mPOA ($r = -0.00$, $P > 0.05$) and BNST ($r = 0.15$, $P > 0.05$) in males of the IPAT-pups group. No significant correlations

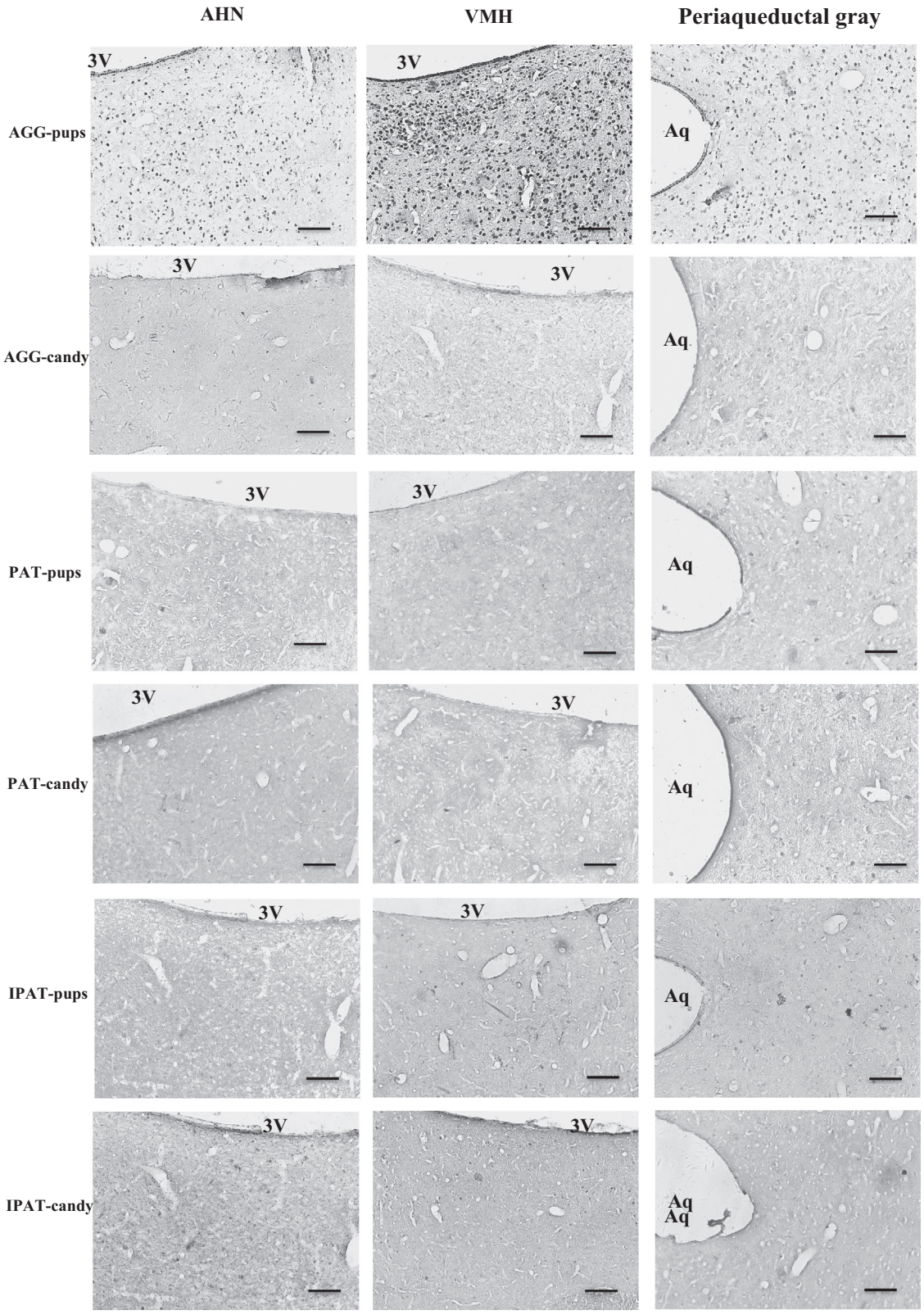

Fig. 4. Representative photomicrographs showing *c-fos*-ir cells in the AHN, VMH, and periaqueductal gray of the males of different groups. 3V = third ventricle, Aq = aqueduct. Coronal sections, scale bars = 100 μm.

were observed between the time invested in huddling and grooming and the number of c-*fos*-ir cells in the mPOA ($r = 0.53$, $P > 0.05$; $r = 0.01$, $P > 0.05$, respectively) and BNST ($r = 0.74$, $P > 0.05$; $r = 0.00$, $P > 0.05$, respectively).

4. Discussion

Mongolian gerbil males that interacted (approached) with pups had significantly higher number of *c-fos*-ir cells in mPOA and BNST than

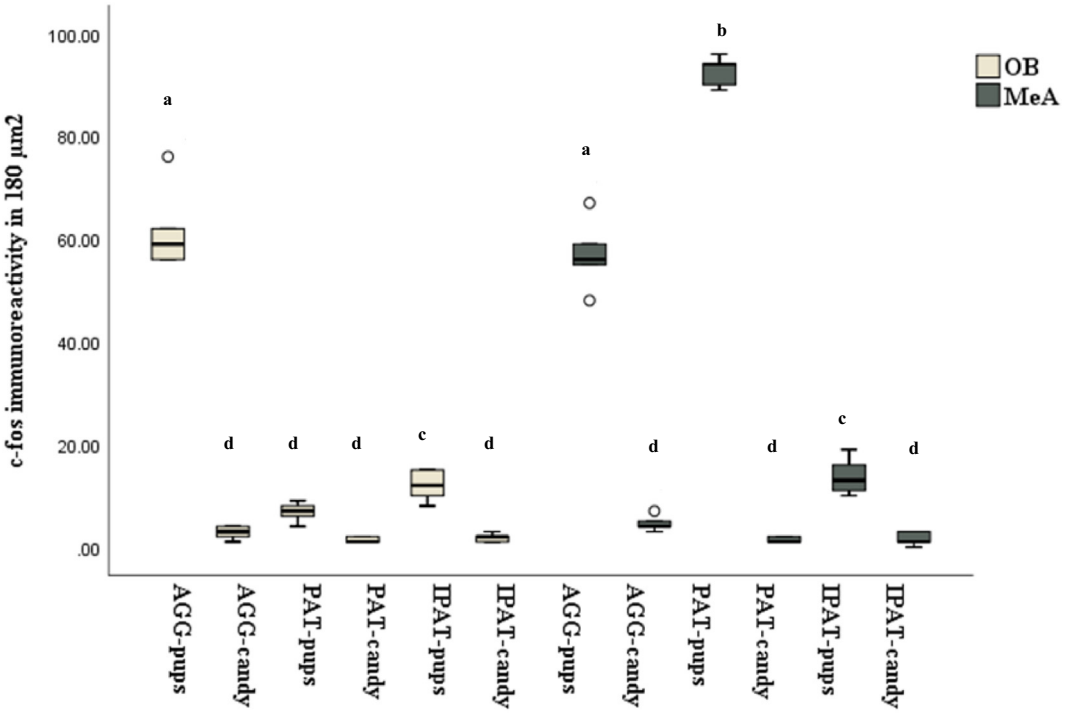

Fig. 5. Both paternal and aggressive interactions with pups had greater *c-fos*-ir cells in OB and MeA than those that interacted with candy. Data are presented as the median. Circles indicate outliers, and letters indicate significant differences.

males that interacted with candy. These results showed that these neural regions are strongly activated when Mongolian gerbil males display paternal behavior, which indicates that the mPOA and BNST regions are involved in the neural regulation of paternal behavior in this rodent. In the laboratory rat, these neural areas are central to the neural circuit that specifically regulates maternal behavior (Numan, 2007, 2014; Lonstein et al., 2015). In prairie voles, virgin males that interact paternally with pups of the same species have higher activation in the mPOA and BNST than males that interact with candy (Kirkpatrick et al., 1994a, 1994b). In virgin California mouse males, electrolytic lesions in the mPOA cause a decrease in the time spent in licking pups and huddling pups (Lee and Brown, 2002, 2007).

Mongolian gerbil males that displayed an aggressive behavior toward pups had a significantly higher number of *c-fos*-ir cells in the AHN, VMH, and periaqueductal gray than aggressive males that interacted with candy. These results suggest for the first time that these neural regions are involved in the regulation of aversive interactions in males. The AHN, VMH, and periaqueductal gray are components of the neural circuit that mediates aversive and defensive behaviors in female laboratory rats (Canteras, 2002; Numan and Insel, 2003; Sheehan et al., 2000). Exocytotoxic lesions of the AHN and VMH stimulate the display of maternal care in nulliparous rats treated with estradiol and progesterone (Bridges et al., 1999). It has also been observed that electrostimulation of VMH inhibits the firing of neurons in the mPOA, which is a region that is involved in the display of parental care (Mayer, 1981; Lonstein et al., 2015). Behavioral evidence indicates that projections from the AHN to the periaqueductal gray play an important role in defensive behavior as well as fear and avoidance responses (Numan and Insel, 2003; Sheehan et al., 2000).

The OB and MeA were activated in both paternal and aversive males possibly because these neural regions are part of the positive and negative mechanisms that regulate paternal behavior in the Mongolian gerbil. As already mentioned, in the female rat, these two neural areas have multiple connections with the facilitators and inhibitors of the mPOA/BNST and AHN/VMH regions, respectively, of the neural circuit of the maternal behavior (Numan and Insel, 2003; Numan, 2014; Brunton and Russell, 2015; Bales and Saltzman, 2016). The OB is an important component of the olfactory system in rodents; in virgin female rats, the anosmia eliminates fear and withdrawal responses toward pups (Fleming and Rosenblatt, 1974; Fleming et al., 1979; Brunton and Russell, 2015). However, the physiological changes in females postpartum make the odors from pups attractive (Kinsley and Bridges, 1990). Similarly, virgin females with MeA lesions no longer avoid pups, and it is possible that this neural structure mediates avoidance responses toward pups (Fleming et al., 1980; Numan et al., 1993). However, this avoidance mechanism would have to be depressed at parturition for maternal behavior to occur (Numan, 2007). On the basis of these results, we suggest that these neural areas can participate in the positive and negative mechanisms that regulate paternal behavior in this rodent. Studies in California mouse and prairie voles showed that lesions in olfactory areas interrupt both paternal and maternal behavior (Lee and Brown, 2007; Kirkpatrick et al., 1994a, 1994b; Williams et al., 1992). The MeA has been related to neural regulation of other social behaviors such as mating and aggression in the laboratory rat and golden hamster (*Mesocrisetus auratus*) (Fleming et al., 1980; Koolhaas et al., 1990; Lehman and Winans, 1982). In prairie voles, paternal interactions activate the MeA (Kirkpatrick et al., 1994a, 1994b). Thus, these neural regions participate in multiple social behaviors. Therefore, they are activated during displays of these behaviors (Numan and Insel, 2003).

These results also showed that paternal or aversive interactions with pups are strong stimuli for neural activation because aggressive and paternal males that interacted with pups had significantly higher number of *c-fos*-ir cells than the males that interacted with candy. In prairie voles and California mouse, the number of *c-fos*-ir cells is greater in males exposed to pups than in males that interacted with candy or marble in brain areas such as the mPOA, BNST, OB, and MeA (Kirkpatrick et al., 1994a, 1994b; Horrell et al., 2017). According to Kirkpatrick et al. (1994a, 1994b), this increase is because pups are a source of multiple stimuli that cause neuronal activation in males during these interactions.

In this study, we observed that *c-fos*-ir cells in the mPOA/BNST in

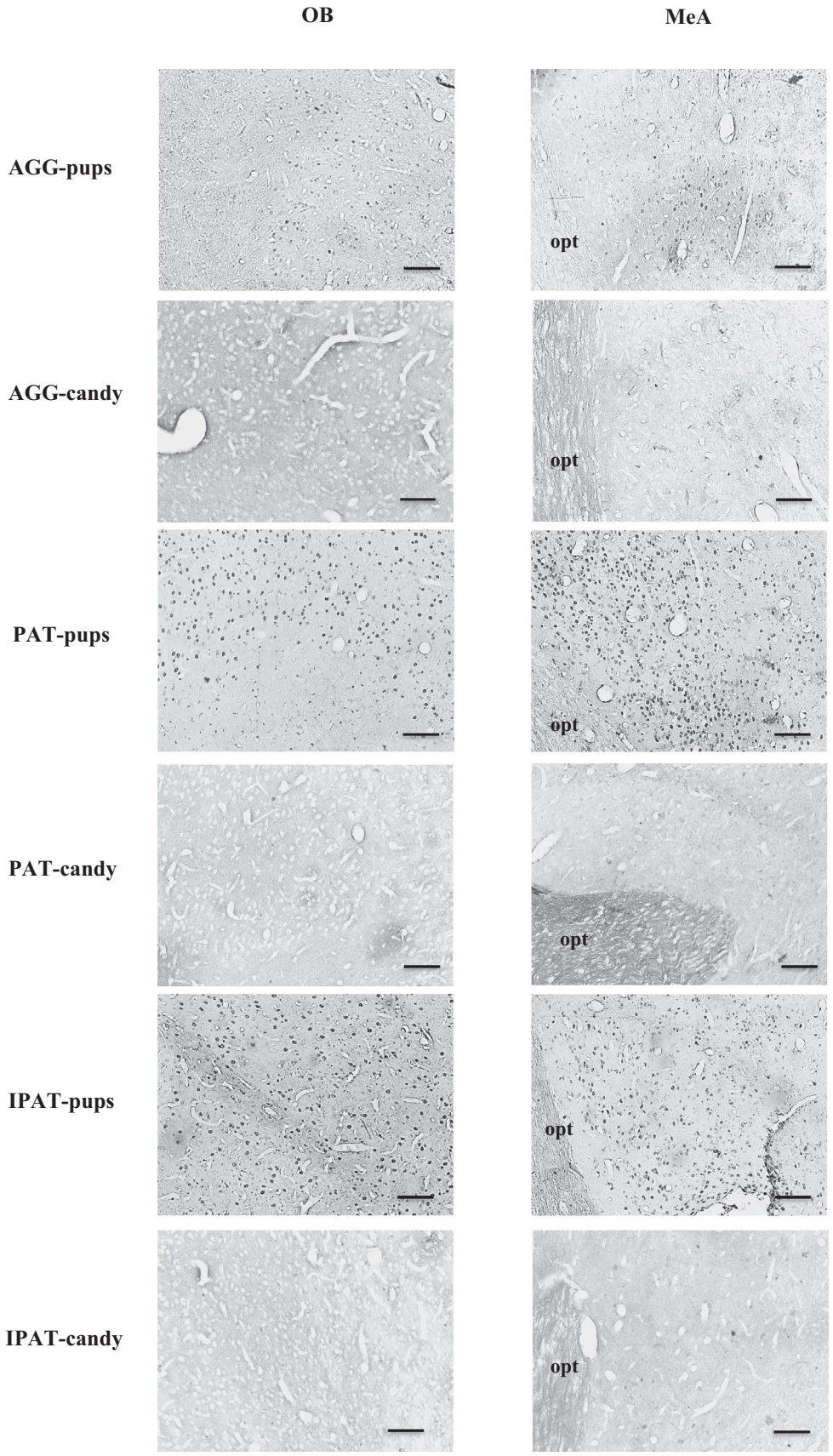

Fig. 6. Representative photomicrographs showing c-fos-ir cells in the OB and MeA. opt = optic tract. Coronal sections, scale bars = 100 μm.

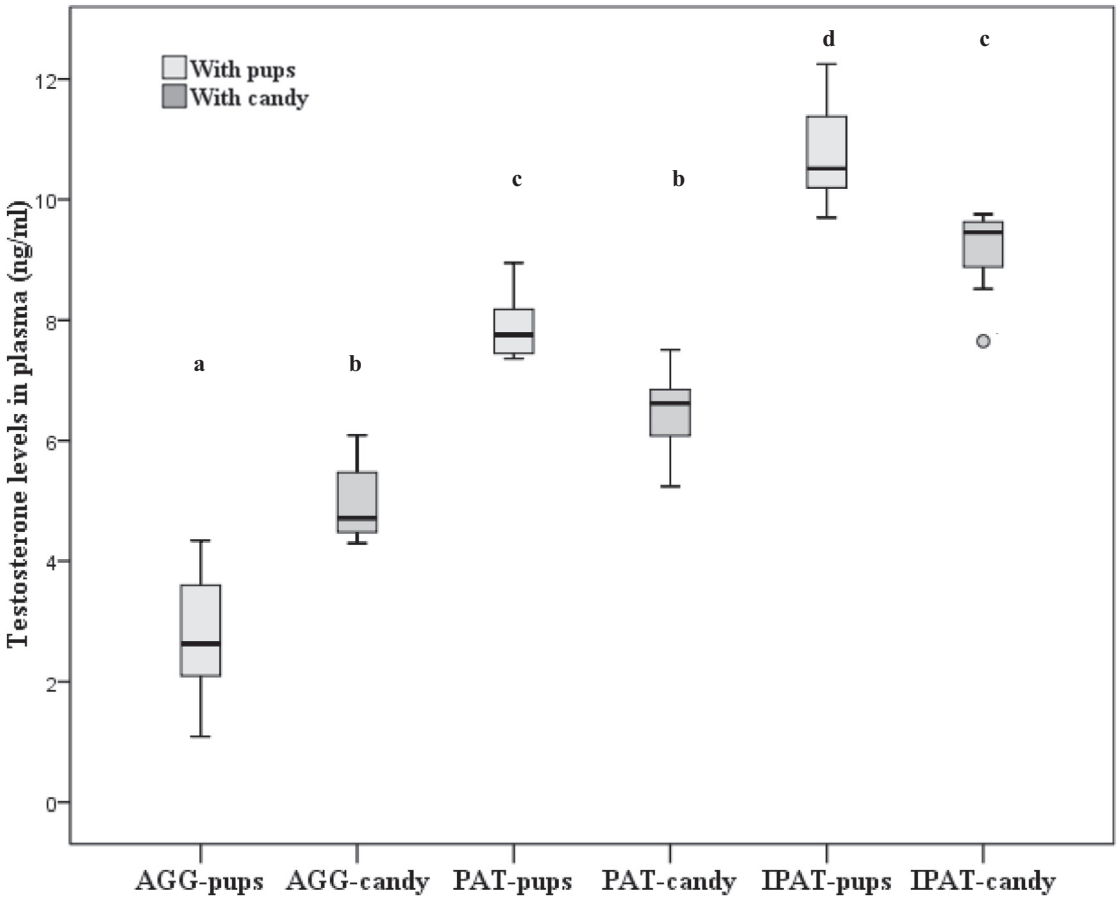

Fig. 7. Paternal males that interacted with pups (IPAT-pups and PAT-pups) had significantly higher T levels than paternal males that interacted with candy (IPAT-candy and PAT-candy) and aggressive males (AGG-pups and AGG-candy). Data are presented as the median. Numbers indicate outliers, and letters indicate significant differences.

males of the AGG-pups group were absent or very low in number. The same was observed in the AHN, VMH, and periaqueductal gray regions in males of the IPAT-pups and PAT-pups groups. These results reinforce that these neural areas are involved in the regulation of paternal and aversive interactions, respectively.

Male Mongolian gerbil that exhibited paternal behavior had significantly higher concentrations of T in plasma than males that displayed aversion toward pups. The concentration of T was significantly higher in males of the IPAT-pup group than in males of the PAT-pups group. Furthermore, males of the IPAT-pups group had a significantly higher number of *c-fos*-ir cells than males of the PAT-pups group in the mPOA/BNST. However, no correlation was found between the T concentrations and the number of *c-fos*-ir cells in these neural areas. Investigations in our laboratory have shown that paternal males have significantly higher T levels than aggressive males (Martínez et al., unpublished data). According to Numan et al. (2006), the occurrence of an approach toward pups (paternal behavior) depends on the physiological state of the animal. This difference in T concentration may be the hormonal basis in response to pups.

Males of the IPAT-pups and PAT-pups groups huddled, groomed, and sniffed the pups. The time spent by males of the IPAT-pups group was significantly higher than that of males of the PAT-pups group. Furthermore, males of the IPAT-pups group also had a significantly higher number of *c-fos*-ir cells in the mPOA/BNST than males of the PAT-pups group. These results suggest that high T concentrations enhance paternal behavior and increase neural activity in these brain regions that regulate paternal interactions. However, in males of the IPAT-pups group, no significant correlations were observed between T concentration and time invested in huddling and grooming and the number of *c-fos-ir* cells in the mPOA/BNST. This could be because the males of IPAT-pups group had T concentrations in plasma above of the physiological level of those observed in virgin spontaneously paternal males.

Males of the AGG-pups group had a significantly lower T concentration than males of the AGG-candy group; this result is surprising because social interactions such as aggression between conspecific males, copulatory behavior, and paternal behavior have been associated with an increase in the T level (Gleason et al., 2009; Martínez et al., 2015). However, in this study, a decrease in T levels is reported when aversive males interacted with foreign pups of the same species. We believe that the presence of pups could cause stress in these males, which consequently affected the synthesis of T. Some studies have mentioned that stress affects the biosynthesis of luteinizing hormone, thus causing a decrease in T concentrations (Gray et al., 1978; Dong et al., 2004).

These results suggest that the duality of the response of males toward pups in the Mongolian gerbil underlie positive and negative neural mechanisms, in which the mPOA/BNST are part of the positive mechanism of paternal behavior and the AHN/VMH and periaqueductal gray are part of the negative mechanism of neural circuit paternal behavior.

Future studies in other species with paternal care should support these results.

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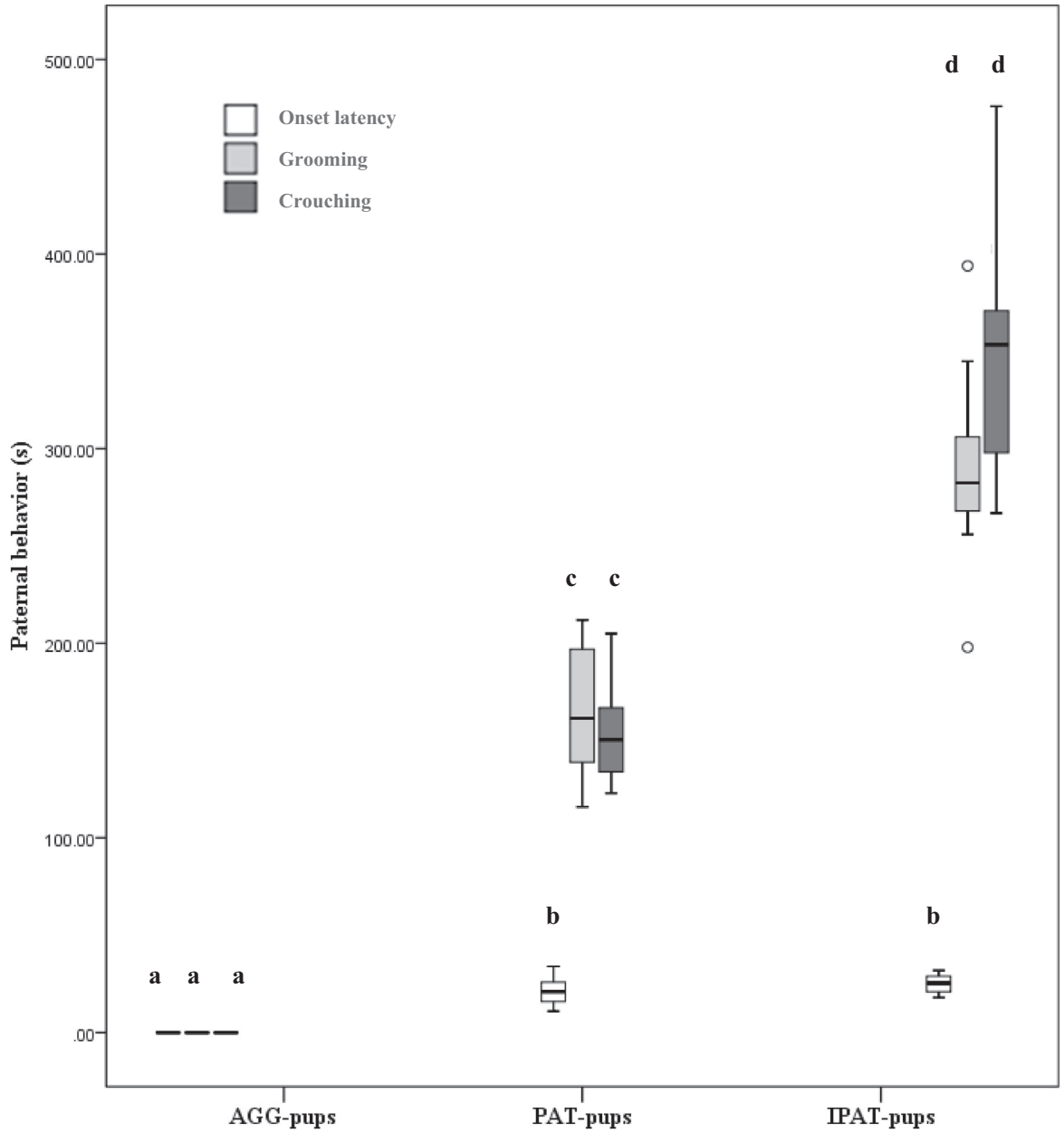

Fig. 8. Males of the IPAT-pups group had an onset latency of paternal behavior shorter than males of the PAT-pups group. The males of the IPAT-pups group also spent significantly more time ($P < 0.05$) in crouching and grooming than males of the PAT-pups group. Data are presented as the median. Numbers indicate outliers, and letters indicate significant differences.

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