Comparison of social interaction and neural activation in the main olfactory bulb and the accessory olfactory bulb between Microtus mandarinus and Microtus fortis

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Abstract To gain insight into the function of AOB and MOB during different social interaction and in different vole species, the behaviors and neural activation of the olfactory bulbs in social interactions of mandarin voles Microtus mandarinus and reed voles Microtus fortis were compared in the present research. Mandarin voles spent significantly more time attacking and sniffing their opponents and sniffing sand dust than reed voles. During same-sex encounters, mandarin voles attacked their opponents for a significantly longer time and sniffed its opponent for shorter time compared with male-female interactions. However, no significant behavioral differences were found during encounters of two individual reed voles regardless of gender composition of the pair. Using c-Fos as an indicator of neural activation, we observed that neural activation was significantly higher in almost all sub-regions of the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB) of mandarin voles compared with reed voles. Numbers of c-Fos-IR neurons in almost all sub-regions of the AOB and the MOB during male-female interactions were also higher than those in interactions of the same sex. Anterior-posterior ratios of Fos-IR neurons in the AOB, MOB and the AOB-OBG in male-female interaction were significantly higher than those in interaction of the same sex. The AOBMR of male mandarin voles and reed voles were larger than those of females in male-female interactions. Behavioral patterns are consistent with cellular activity patterns. Consistent level of neural activation in MOB and AOB suggests important roles of both the main olfactory bulb and the accessory olfactory bulb in social interaction in two species. Current Zoology 55(4): 279 – 287 (2009).

Key words Mandarin vole □ Microtus mandarinus □ Reed vole □ Microtus fortis □ Main olfactory bulb □ Accessory olfactory bulb □ Cellar activity □ Social interaction

The microtine rodents are ideal for comparative study of the neurobiological bases of social behaviors because many of the species show profound differences in reproductive biology and social organization. Young and Wang (2004) Aragona et al. (2006). Mandarin voles Microtus mandarinus and reed voles Microtus fortis are two species of microtine rodents. Previous studies of mate choice and other related characteristics found that mandarin voles are socially monogamous (Tai et al., 2001) and family members of mandarin voles live in one burrow system (Tai and Wang, 2001) suggesting that they communicate and interact more frequently. On the other hand, reed voles have larger litter sizes (Bo et al., 2006) and invest less in parental care suggesting they may have a polygynous mating system. Previous research also found that mandarin voles remember and recognize individual odor better than reed voles (Tai et al., 2005). We suggest that mandarin voles and reed voles show significant differences in the social behavior that are found in other species of voles with different social organization (Pierce and Dewsbury, 1991; Trivers, 1972). In addition, field investigation found that reed voles are solitary except during the reproductive period mandarin voles live in communal groups with extend family (Wu et al., 1996). We predict that mandarin voles interact socially more often than reed voles.

The main olfactory and vomeronasal systems are two primary and important chemosensory systems for mediation of pheromonal influences on socio-sexual functions in rodents (Kumar et al., 1999). Pheromones are closely associated with a wide variety of socio-sexual functions including puberty onset, estrous cycling, aggression, copulation behavior and recognition of reproductive state, gender, and strain (Doving and Trotter, 1998). Gill et al. (1998) and Vandenbergh (1994). Mating activities increase the number of Fos-IR neurons in the granule layer of the AOB and other related brain regions of both male and female musk shrews Crocidura coerulea.

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It was also found that the response in the ventral medial nucleus of the hypothalamus was dimorphic where mating significantly increased Fos-ir in female and not in male. In both sexes only the granule layer of the AOB displayed an increase in Fos-ir after exposure to chemosensory cues alone. Gill et al. 1998. Cushing et al. 2003 reported that heterosexual pairing induced significant Fos-ir increase in the hypothalamus to which pheromonal inputs were conveyed. In the traditional view AOB play major roles in social interaction that mainly depend on the pheromone. On the other hand the main and accessory olfactory systems converge downstream at several levels including the cortical-medial amygdala Matthieu et al. 2009. The respective roles played by the main and the accessory olfactory systems in the control of social behavior such as mate recognition and sexual behavior are at present still controversial and is not clear-cut Matthieu et al. 2009.

Recent studies report that there is a functional dichotomy within the vomeronasal system Kumar et al. 1999 Tai et al. 2006. The vomeronasal organ VNO contains two subdivisions of vomeronasal receptor neurons VRNs. These two populations of VNO neurons are anatomically segregated within the VNO into apical and basal zones. The apically located VRNs coexpress Gi and putative pheromone receptor V1R and project to several glomeruli in the rostral part of the AOB Belluscio et al. 1999 thereby the basally located VRNs express Go putative pheromone receptor V2R and project to the caudal part of the AOB. Subpopulations of accessory olfactory bulb neurons were activated distinctly by different chemo-sensory stimulation Dudley and Mos 1999 Kumar et al. 1999. These subdivisions of the vomeronasal receptors that project into distinct zones of the AOB have been found in a wide variety of species Keverne 1999. Different cellular activity patterns in the AOB were also found while mandarin voles were exposed to substrate from the cage on an individual of different sex Tai et al. 2006. However it is not clear whether cellular activities patterns of AOB in different social interaction and different vole species are different.

A recent study also revealed that prairie M. ochrogaster and meadow voles M. pennsylvanicus which have different mating systems show different Fos-immunoreactive Fos-ir expression in several brain areas including the medial preoptic area ventromedial hypothalamusamygdala and prefrontal cortex differently following social isolation coupled with the elevated plus maze EPM test Stowe et al. 2005. Although the anatomy and cyto-architecture of vomeronasal organ and AOB have been found to vary within different vole species Tai et al. 2004 it is not clear that whether different species of voles with different social organization show different social interaction and induce different levels of neural activation in the AOB and MOB. Therefore one objective of present research was to characterize the variation in social behaviors between two species of voles. Another objective was to determine if there is a dichotomy of activation pattern in AOB during different social interactions in microtine rodents and whether there are differences in cellular activation of MOB and AOB in two species of voles.

c-Fos the protein product of the immediate early gene c-fos is a useful marker for identifying initial neural responses to a variety of stimuli including social interaction Cushing et al. 2003 Bressler and Baum 1996. To gain insight into the function of AOB and MOB during different social interaction and in different vole species response properties of MOB and AOB were examined for the first time using induction of c-Fos.

1 Materials and Methods
1.1 Animals

Mandarin voles and reed voles used in the experiments came from an out-bred colony reared at the College of Life Sciences Shaanxi Normal University in Xi'an China. The colony of mandarin voles was established in 1997 with wild captured animals from Lingbao City Henan Province. The reed vole colony was established in 2000 and captured from Qingtongxia City Ningxia autonomous region. The animals were maintained in clear plastic cages 40 x 28 x 50 cm supplemented every year. The breeding colonies were retained under a photoperiod of 12L:12D and temperature of 18–20 °C. Hardwood shavings and cotton batting were provided as substrate and bedding. Rabbit chow Lab Anim. Center Xi'an Medical University carrot and malt were available ad libitum. All the voles used in the experiments were about 90 days old. Female voles were sexually naive because sexual experience may affect the Fos expression during different social interactions Fewell and Meredith 2002. In addition all females were in an anestrous phase that was identified by vaginal smear in which leukocytes predominated. Three to four days prior to behavioral testing subjects were individually housed in clear cages with wood shaving and were completely undisturbed during separation. All animals were treated according to guidelines approved by Animal Care and Use Committee of Shaanxi Normal University.

1.2 Behavioral testing

The experiments were conducted in a plastic box 80 x 40 x 40 cm which was divided into two equal chambers 40 x 40 x 40 cm by a baffle plate. One vole was placed in each of the two chambers for 30 min prior to the start of the test to adapt to environment. Then the baffle plate was taken away carefully. Each test lasted for 2 hours with interactions being continuously observed and recorded for the first 30 min. Any unrelated stimuli such as noise light or handle was eliminated to minimize their
effects of the experiment. Behaviors such as attacking, sniffing body, licking within 1 cm of the object’s face, body or genital region and sniffing sawdust were recorded. To compare the differences in social behavior and neuron response pattern of olfactory bulb between different genders and species, the following intra-specific interactions for mandarin and reed voles were observed: 1 male with strange male; 2 male and strange female; and 3 female with strange female. As a control, two voles were put into each of the 2 compartments for 30 min, then one vole and theaffle plate were removed. The control vole remained in the box for two hours. To prevent injury from aggressive interactions any fights lasting more than 10 seconds were interrupted by clicking the side of the observed cage. After the experiment the voles were deeply anesthetized with pentobarbital and transcardially perfused with 0.1 M phosphate buffer and 4% paraformaldehyde pH 7.4.

1.3 Tissue-processing procedure

After perfusion the brains were removed and placed in 4% paraformaldehyde for 12 hr then 24 hr in 30% sucrose and then sectioned immediately. The immunohistochemical and counting procedures followed those of Zhang and Mei 2000. Briefly, sagittal sections 40 μm thick were cut on a cryostat and floating sections were processed using a primary antibody and the avidin-biotin complex ABC® Boster Company method. The primary antibody kindly provided by Santa Cruz Biotechnology® 1:1500 USA® was a rabbit polyclonal directed against the specific peptide corresponding to human c-fos amino acid residues 3–16 of N-terminal region. The secondary antibody was biotinylated goat anti-rabbit ® Boster Company®. DAB was used for visualization of c-Fos immunoreactivity® Fos-ir® and the counting of stained nuclei was performed using an Olympus microscope. Slides were coded and randomly selected for microscopic analysis so that the group designation of subjects was unknown during analysis. The numbers of cells within the each brain region that show Fos-ir labeling were quantified by eye with the aid of a reticle placed in one ocular lens of microscope 20 × 10® Lunstein and De Vries® 2000. Sections were chosen by their correspondence to the reference atlas plate and not by the levels or intensity of Fos-ir labeling. For each animal the number of Fos-ir neurons in the glomerular region of the olfactory bulb, nucleus accumbens, and nucleus basalis was counted from sections of two OBs and two MObS two left and two right. The sections chosen for counting were from middle portion along sagittal plane and were those in which the anterior-posterior axis of the OB was longest. All Fos-ir neurons in the anterior and posterior parts of the OB were counted. Since the Fos-ir neurons in the MOB seemed to be evenly distributed throughout mitral and lateral granule layer and there is a significant difference between lateral and middle granule layer an area standard area is 200 × 100 μm² just dorsal to the three layers and middle granule were chosen arbitrarily for Fos-ir neurons quantification. The mean ratio of the number of stained cells in the anterior portion to the number of stained cells located in the posterior portion was calculated. Generally the mean ratio of the small number of Fos-ir neurons in PG they were not included in this analysis. Chosen sections were photographed with a Nikon® Tokyo® Japan® camera attached to an Olympus microscope.

1.4 Statistics

All data are presented as means ± SE and p < 0.05 was used for significance testing. SPSS 13.0 was used for data analysis. The social interaction behaviors and numbers of Fos-ir neurons in different regions of olfactory bulbs were analyzed using a three-way analysis of variance ANOVA® with fixed factors such as sex® species and pair. Post-hoc tests using Scheffe’s method assuming equal variance was used for multiple comparisons among different groups. Correlations among Fos-ir neurons of different olfactory layers of AOB and MOB were performed with Pearson’s correlation coefficient. Correlation is significant at the 0.01 level 2-tailed.

2 Results

2.1 Behavioral analysis of social interactions

Three-way ANOVA analysis showed an effect of species on duration of attacking, sniffing body and substrate behaviors where mandarin voles spent significantly more time attacking F0.05.013 = 385.4 P < 0.001 and sniffing its opponent F0.05.0112 = 223.6 P < 0.001 and sniffing sawdust F0.05.0112 = 377.9 P < 0.001 Fig.1. There was significant interaction between species and pair observed for attacking F0.05.0112 = 177.6 P < 0.001. Same sex encounters of mandarin voles resulted in significantly longer time attacking the opponent F0.05.0134 = 117.8 P < 0.0001 and and less time sniffing the opponent F0.05.0134 = 331.84 P < 0.0001 compared to male-female encounters. However, no significant difference was found in reed vole between different encounters. No interaction between sex and species was observed on attacking behavior F0.05.0112 = 0.2 P = 0.619 and sniffing substrate F0.05.0112 = 0.05 P = 0.818. No interaction among sex® species and pairs was observed for attacking F0.05.0112 = 0.181 P = 0.671 sniffing body F0.05.0112 = 2.5 P = 0.111 and sniffing substrate F0.05.0112 = 1.54 P = 0.198. Multiple comparisons showed that voles in male-female encounters spent the most time sniffing the body in both species P < 0.001 Fig.1.

2.2 Fos-ir neurons in MOB and AOB during different encounters

Three way ANOVA analysis revealed that the number of Fos-ir neurons in almost all sub-regions of the AOBs and MOBs of mandarin voles were significantly higher
than those in reed vole $P < 0.001$ Fig. 2 3 4. In mandarin vole numbers of Fos-ir neurons in all subregions were significantly higher relative to those of controls in all encounters $P < 0.001$. However in reed voles these differences were only found in subregions of the AOBs in male-female encounters and in MOBGM in both same sex and male-female encounters. Furthermore numbers of Fos-ir neurons in almost all subregions of AOB and MOB during male-female interactions were also higher than those in encounters of same sex $P < 0.0001$. More over significant interactions between species and pairs were observed in almost in all subregions except MOBL $F_{10\,94} = 1.78 \ P = 0.175$ and MOBGRM $F_{10\,94} = 1.056 \ P = 0.352$. Furthermore a significant interaction between sex and pair was observed on AOBMR AOBGM and MOBGM $P < 0.001$. There was no interactions among species sex and pairs for Fos-ir neurons in all subregions of AOB and MOB $P > 0.05$ Fig. 2 3 4.

It was found that numbers of Fos-ir neurons in three layers of MOB were correlated with those in AOB in different social interactions Pearson correlation test $P < 0.001$ two tailed test for instance MOBM-AOBMA $r = 0.783 \ P < 0.001$ MOBGM-AOBGA $r = 0.767 \ P < 0.001$.

Anterior-posterior ratios in AOBM AOBMR and AOBGM AOBGR were also calculated under different social interactions for each species. It is found that AOBGR and AOBMR in male-female encounter were significantly higher than those in encounters of same sex for AOBGR $F_{10\,94} = 24.359 \ P < 0.001$ for AOBMR $F_{10\,94} = 20.117 \ P < 0.001$. Multiple comparisons also revealed that AOBMR of male mandarin voles and reed voles were larger than female in male-female encounters for mandarin vole $P = 0.028$ for reed vole $P = 0.008$. There is interaction between sex and pairs of different sex for AOBMR $F_{10\,94} = 12.347 \ P < 0.001$. Interaction among species sex and pairs of different sex was not observed for AOBMR $F_{10\,94} = 0.389$ ns and AOBGM $F_{10\,94} = 0.315$ ns.

3 Discussion

3.1 Interspecific difference in behavior patterns and cellular activities

The present research has shown that mandarin voles spent significantly more time in social behaviors than reed voles. These results were expected given the social organization of mandarin voles Tai et al. 2001 as has been found also in the highly social prairie voles Getz et al. 1981. The reed voles on the other hand like meadow voles are promiscuous and display low levels of social interaction Lim et al. 2004 Madison 1980. These results add further evidence that socially monogamous voles exhibit more subtle behaviors and stronger sociality than polygamous voles Pierce and DeWsbury 1991 Tai et al. 2000 Tai et al. 2001.

The present research also found that mandarin voles produced significantly more Fos-ir neurons than reed voles in all layers of MOB and AOB in different social interactions. This is consistent with our prediction that socially monogamous and polygamous voles would exhibit variation in the level of activation of MOB and AOB during social interactions likely resulting from interspecific variation in social behaviors. Several researchers have also reported that different patterns of behavior and neural activation are related with mating and agonistic behavior sexual and social experience Gushing et al. 2003 Kollack-Walker and Newman 1995 Wang et al. 1997. Based on these results we may infer that
different neuron activities of AOB and MOB may be associated with different social organizations in two vole species. But recently, Stowe et al. (2005) found that male prairie voles had more Fos-ir cells in every brain area examined than did male meadow voles, showing inter-specific differences in brain responses to the EPM associated non-social stimuli. They explain that there may be species differences in the binding affinity of the antibody. However, locomotor activities of prairie voles were significantly higher than meadow voles (Stowe et al. 2005) which suggests an alternative explanation for our findings that differences between two species may be due to innate behavioral differences associated with mating strategy/life history.

3.2 Social interaction can induce similar increases of cellular activities in both MOB and AOB

The present study found that social interactions resulted in significant cellular activations in almost all layers of MOB and AOB with the exception of the glomerular layer of AOB. Similar results were also found in mice (Kumar et al. 1999), Dudley and Moss (1999), hamsters (Fiber and Swann 1996), and rats (Dudley et al. 1992). The results showed that there is correlation in the cellular activation of the MOB and AOB in different behavioral contexts. Exposure to female hamster vaginal secretions induced significant increases in neuronal Fos-IR in both the MOB and the AOB (Fiber and Swann 1996). Also, Kollack-Walker and Newman (1995) found
that pheromones detected by the MOE main olfactory epithelium and initially processed by the MOB are able to augment neuronal activity in the MA medial amygdala and activate hypothalamic neurons as well. Such effects depend on the sex of the subject and pheromonal stimulus presented. From results described above we can conclude that both main olfactory bulb and accessory olfactory bulb play important role in social interactions of different sexes and different species.

3.3 Cellular activities and behavioral patterns of different sex

Regardless of species animals were more aggressive and spent less time investigating same-sex conspecifics compared with heterosexual encounters. Furthermore female mandarin voles spent significantly more time attacking and less time sniffing compared to males. Heterosexual encounters evoked significantly more cellular activation in almost all layers of the MOB and the AOB and glomerular layer of the AOB compared with encounters of the same sex. In male-female interactions males had significantly more Fos-ir neurons in the AOBGA MOBGR and significantly less Fos-ir neurons in
AOBMP compared with females. These results suggest that cellular activation of the MOB and the AOB is consistent with behavioral observations in different social interactions as was found for prairie vole[58] Wang et al. [9] 1997 [9] and Syrian hamsters Kollack-Walker and Newman [13] 1995 [13]. In musk shrews mating can significantly increase Fos-ir in the ventral nucleus of hypothalamus of females but not in males Gill et al. [21] 1998 [21]. Cushing et al. [56] 2003 [56] found that in monogamous prairie voles heterosexual cohabitation stimulates a similar pattern of immediate early gene activation in males and females with increased c-Fos IR in the medial amygdaloid nucleus MeA[54] the bed nucleus of the stria terminalis[55] BST[56] the ventromedial hypothalamic nucleus VMN[57] and the medial preoptic nucleus MPO[58]. This is consistent with neuron response patterns of cohabitation in polygamous species. In rats and other species activation of the MeA[54] BST[56] and MPOA and the VMN[57] especially in females is assumed to be part of the sexual response Pfau and Hech[97] 1997 [97]. Cohabitation with a novel unfamiliar male resulted in a significant increase in c-Fos IR in the CeA of male. These results may be consistent with our studies that male and female mandarin vole or reed voles showed different behavioral pattern in different social interactions.

3.4 Cellular activities dichotomy in AOB of mandarin vole and reed vole

Mandarin voles and reed voles exhibit cellular activity dichotomy in the AOB. The result is consistent with previous results that the chemoarchitectural dichotomy of the peripheral vomeronasal system serves to segregate pheromone signals based on their behavioral context Dudley and Moss [1999] Kumar et al. [1999] Tai et al. [2006]. All AP ratios in different groups are larger than 1 indicating more Fos-ir neurons distributed in the rostral portion than caudal portion regardless of different social interactions. Voles in male-female interactions had higher AP ratios than interactions of the same sex. In male-female interactions AP ratios of stained mitral cells in males were higher than those in
females. This cellular activity pattern of AOB was also consistent with our previous research results while mandarin voles were exposed to substrate from different sex.Tai et al. 2006]. Some researchers reported that exposure of male mice to urine collected from females at different stages of the estrous cycle also results in preferential activation of the rostral AOB with higher numbers of activated cells observed during stages of the cycle associated with sexual heat. Dudley and Moss 1999. Previous reports found that male and female excrete different sex- and hormone-dependent constituent in urine Schende et al. 1986. A hormone-dependent component in female urine is likely to be responsible for the VNO-dependent release of luteinizing hormone observed in male mice Clancy et al. 1984. On the other hand, testosterone-dependent compounds in male mouse urine have been shown to promote aggressive behavior in males Muggford and Nowell 1970 Novotny et al. 1985. Indeed the VLH ventral nucleus of hypothalamus which is activated in females after exposure to male pheromones has been implicated in the steroid control of feminine sexual behavior in female rodents Rubin and Barfield 1980. Female urine can inhibit males’ aggressive behavior. This suggests that a hormone-dependent component of female urine may be received by apical VNO cells and evoke activation of cells in the rostral portion of the AOB. On the other hand, apical VNO cells may inhibit basally situated cell groups and further inhibit cellular activation in caudal portion of AOB. These hypotheses need to be elucidated with further research.

In conclusion, mandarin vole showed significantly higher social interaction behavior and higher numbers of Fos-ir neurons in almost all layers of olfactory bulb compared with reed voles. Mandarin voles in male-female encounters exhibited more sniffing body behavior and less aggressive behavior higher anterior-posterior ratios of Fos-ir neurons compared with individuals in encounters of same sex. Behavioral patterns are consistent with cellular activity patterns. A consistent level of activation of MOB with AOB suggests important roles of both MOB and AOB in the social interactions in two species. Mandarin voles and reed voles exhibit obvious cellular activity dichotomy in AOB. These results may help us understand the neurobiological basis of social interaction behavior and different mechanisms of different behaviors in different vole species.

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