Distribution of Like-muscarinic Acetylcholine Receptor M2 in the Brain of Three Castes of Polyrhachis vicina

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Abstract: The cholinergic system plays an important role in the central nervous system of insects and is closely related to the complex behavior of insects. The immunohistochemical technique was performed to detect the expression of like-muscarinic acetylcholine receptor M2 in the brain of three castes of Polyrhachis vicina. A positive expression of like-muscarinic acetylcholine receptor M2 was observed in the mushroom body, central body and antennal lobes of the ant brain; but there is great diversity in their location and intensity among worker, queen and male ants. It is speculated that like-muscarinic acetylcholine receptor M2 plays a critical role in the central nervous system, in terms of projecting visual information and olfactory information into the protocerebrum and integrating many inputs.

Key words: Polyrhachis vicina; Insect brain; Cholinergic system; Like-muscarinic acetylcholine receptor M2; Central nervous system

The central nervous system (CNS) of insects contains relatively large amounts of acetylcholine (Ach), which is thought to serve as a neurotransmitter (Pitman, 1985). There are two main types of cholinergic receptors – muscarinic and nicotinic acetylcholine receptors. Muscarinic receptors belong to a large family of membrane-bound receptors that are coupled to G-proteins. In mammals, muscarinic receptors are further classified into M1–M5 subtypes (Hosey, 1992). Coupled to stimulatory G-proteins, M1 muscarinic acetylcholine receptors have a stimulatory effect on neurotransmission when bound by an agonist (Ach). M3 and M5 receptor subtypes also have a stimulatory effect on the target tissue, whereas the M2 and M4 subtypes are inhibitory (Hulme et al, 1990; Parker et al, 1991; Smrcka et al, 1991).

Muscarinic acetylcholine receptor is one of the most significant receptors in the insect nervous system and also widely distributed throughout the neurons of the CNS of insects such as, drosophila, silkworms,
cockroaches and grasshoppers (Shapiro et al, 1989; Orr et al, 1991; Blake et al, 1993; Bai & Sattelle, 1994; Aizono et al, 1997; Heinrich et al, 2001; Wenzel et al, 2002). Insect muscarinic acetylcholine receptors all are coupled to stimulatory G-proteins, and induce adenylate cyclase activity. In drosophila, mAchR is structurally and functionally most similar to the mammalian m1, m3, and m5 subtypes. The drosophila mAchR binds monoclonal antibodies of the rat brain m1 receptor (Shapiro et al, 1989). In the central nervous system, all kinds of hormones, neurotransmitters, neuropeptides and chemokines bind to their receptors which are coupled to multiple G proteins to modulate many different signal transduction pathways. mAchR is concerned with this network of signal transduction pathways, which play a significant role in feeling, movement, memory, learning and other cognitive processes. But the M2 receptor in insects (inhibitory receptor) has not been studied previously.

*Polyrhachis vicina* is a typical eusocial insect exhibiting complex behavior, making it a good biological model for anatomical and immunohistochemical studies because of its concentrated nervous system and well-defined brain structure. *Polyrhachis vicina* is also an important medical insect and has a high economic value. In this paper, the immunohistochemical technique was performed to detect the expression of like-muscarinic acetylcholine receptor M2 in the brain of *P. vicina* in order to study the structure and function of insect acetylcholine receptor and accumulate data about insect neurophysiology.

1 Material and methods

1.1 Experimental animals

*Polyrhachis vicina* ants were purchased from the Handan Hualong King of Ant Force Development Co, Ltd, Hebei Province, China. The ants were maintained in the laboratory in plastic boxes (40cm×40cm) containing a floor made of plaster with a nest inside (13cm×8cm). A glass roof separated the nest from the foraging area. Colonies were kept at 25°C, 70% RH and a 12/12 LD photoperiod, and were provided with fresh honey three times a week.

1.2 Tissue preparation

Thirty worker ants, thirty virgin queen ants, thirty queen ants and thirty male ants were selected from colonies. The heads together with pronotums were quickly isolated from the bodies and fixed in 4% paraformaldehyde at 4°C for 8 hours. After thoroughly rinsing in 0.1 mol/L PBS, the samples were dehydrated through ethanol in ascending concentrations, and embedded in paraffin wax. The pronotums were removed during the embedding process. The specimens were then sectioned with a rotatory microtome. Serial sections (8 µm) were cut in the horizontal planes, and mounted on Poly-L-Lysine-coated glass slides, which were used directly in staining experiments.

1.3 Immunohistochemistry for muscarinic acetylcholine receptor M2

The immunohistochemical assay of the expression of like-muscarinic acetylcholine receptor M2 in brain sections was performed by the StreptAvidin-Biotin Complex (SABC) immunohistochemical technique (SABC kit; Boster Biological Technology Ltd, Wuhan, China). After deparaffinization and rehydration, the histological sections were peroxidase-blocked with 3% hydrogen peroxide for 15 min, heated in a microwave oven to retrieve the antigen twice for 10 min each, incubated with 5% BSA for 20 min, then incubated with a rabbit anti-muscarinic acetylcholine receptor M2 polyclonal antibody (Sigma, Predivided by Boster, China; diluted at 1∶400 in 0.1 mol/L PBS) overnight at 4°C. The next day, after washing with PBS, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (Boster Biological Technology Ltd, Wuhan, China) for 30 min washed with PBS, and incubated with SABC (Boster Biological Technology Co Ltd, Wuhan, China) for 30 min. Subsequently, the sections were covered with a chromogenic agent solution of 3, 3′-diaminobenzidine and H2O2 (DAB, Boster Biological Technology Co Ltd, Wuhan, China) for 3-5 min, rinsed with distilled water. Finally, they were dehydrated with alcohol, cleared in xylene and mounted with neutral balsam.

The following controls were used: no-primary antiserum control, which involved running the whole immunohistochemical procedure excluding the primary antiserum. The results were observed and photographed under an Olympus microscope.

1.4 Image collection and data processing

Five positive sections from each caste were selected and every section was placed in HPIA S2100 high definition and color picture analyzing system. The intensity of positive staining was calculated by the gray value of the images. The mean gray value of different castes were automatically collected (all data was collected in 40×object lens) in the range 0-255 (the value 0 is most intense, the value 255 is least intense; the lower
value, the more intense). The data was then input into SPSS 13.0 software and analyzed using one-way ANOVA with Dunnett’s multiple comparison tests where appropriate.

2 Results

By observing the brain like-muscarinic acetylcholine receptor M2 immunoreactivity sections of three castes, we described the different localization of like-muscarinic acetylcholine receptor M2 immunoreactivity in the brain of three castes (*P. vicina*).

2.1 Distribution of like-muscarinic acetylcholine receptor M2 immunoreactivity in the brain of worker ant

Protocerebrum Like-M2 receptor positive reaction existed in connective fibers between the lateral calyx and medial calyx of the mushroom body (Fig. 1a) and the terminal of α-lobes (Fig. 1b). Other parts of the mushroom body could not be observed. Like-muscarinic acetylcholine receptor M2 immunoreactivity was not located in Kenyon cells around the calyx. There was no distribution of like-muscarinic acetylcholine receptor M2 immunoreactivity in the optic lobes. Apart from the MBs, the central body was one of the most strongly labeled regions in the brain of worker ants, such as the upper central body (Fig. 1c) and inner antenno-cerebral tracts beside the central body (Fig. 1d).

Deutocerebrum Antennal lobes exhibited like-muscarinic acetylcholine receptor M2 immunoreactivity (Fig. 2a). Middle antenno-cerebral tract showed a diffuse and homogeneous meshwork of like-muscarinic acetylcholine receptor M2 immunoreactive fibres (Fig. 2b). Dorsal lobes and tritocerebrum exhibited negative like-muscarinic acetylcholine receptor M2 immunoreactivity.

2.2 Distribution of like-muscarinic acetylcholine receptor M2 immunoreactivity in the brain of virgin queen ant and queen ant

2.2.1 Virgin queen ant Protocerebrum Kenyon cells around the calyx and connective fiber between the lateral calyx and medial calyx of the mushroom body did not exhibit like-muscarinic acetylcholine receptor M2 immunoreactivity. But there were some weaker like-muscarinic acetylcholine receptor M2 immunoreactivities within the terminal of α-lobes (Fig. 7a). The medulla-protocerebrum tract was labeled (Fig. 8a). Antenno-cerebral tracts consisted of the inner antenno-cerebral tract, the middle antenno-cerebral tract and the outer antenno-cerebral tract. The inner and outer antenno-cerebral tract showed like-muscarinic acetylcholine receptor M2 immunoreactivity (Fig. 7b) (Fig. 8b).

Deutocerebrum Antennal lobes exhibited some weak like-muscarinic acetylcholine receptor M2 immunoreactivities (Fig. 9a). Tritocerebrum was not labeled.

2.2.2 Queen ant Protocerebrum Kenyon cells around the calyx and connective fiber between the lateral calyx and medial calyx of the mushroom body did not exhibit like-muscarinic acetylcholine receptor M2 immunoreactivity. But there were some weaker like-muscarinic acetylcholine receptor M2 immunoreactivities within the terminal of α-lobes (Fig. 11a). Other parts of the mushroom body could not be observed. The lower central body exhibited obvious like-muscarinic acetylcholine receptor M2 immunoreactivity (Fig. 11b). In addition, the fibre tract above the pharynx which connected two antennal lobes, showed the like-muscarinic acetylcholine receptor M2 immunoreactivity (Fig. 11c). Deutocerebrum and tritocerebrum were negative parts.

Control: sections were unlabeled when treated with PBS (Fig. 12) instead of primary antibodies.

2.3 Distribution of like-muscarinic acetylcholine receptor M2 immunoreactivity in the brain of male ant

Protocerebrum The dense like-muscarinic acetylcholine receptor M2 immunoreactive staining was observed in the terminal of α-lobes (Fig. 11a). Other parts of the mushroom body could not be observed. The lower central body exhibited obvious like-muscarinic acetylcholine receptor M2 immunoreactivity (Fig. 11b). In addition, the fibre tract above the pharynx which connected two antennal lobes, showed the like-muscarinic acetylcholine receptor M2 immunoreactivity (Fig. 11c). Deutocerebrum and tritocerebrum were negative parts.

Control: sections were unlabeled when treated with PBS (Fig. 12) instead of primary antibodies.

2.4 Image analysis and statistical result

The mean gray values of different castes were automatically collected. The data above was put into SPSS 13.0 software, and analyzed using one-way ANOVA with Dunnett’s multiple comparison tests. Each group with a different letter superscript (a, b, c) indicates that the means are at 5% levels of significance; while those with the same letter, are not significantly different (Tab.1).

3 Discussion

This study gives the first account of the representation of like-muscarinic acetylcholine receptor M2 neuronal systems in the brain of insects. The results showed that like-muscarinic acetylcholine receptor M2 is widely distributed in the brain of *Polyrhachis vicina*. However, like-muscarinic acetylcholine receptor M2
Figs. 1-12: 1. Worker ant’s brain section showing like-M2 receptor positive connective fiber between lateral calyx and medial calyx. (a), α-lobes (b), upper central body (c), inner antenno-cerebral tract (d); 2. worker ant’s brain section showing like-M2 receptor positive antennal lobe (a), middle antenno-cerebral tract (b); 3. worker ant’s brain section showing control response of PBS replacing M2 receptor antibody; 4. virgin queen ant’s brain section showing like-M2 receptor positive Kenyon cells (a), connective fiber between lateral calyx medial calyx (b); 5. virgin queen ant’s brain section showing like-M2 receptor positive α-lobes (a), lobula-protocerebrum tract (b), inner antenno-cerebral tract (c); 6. virgin queen ant’s brain section showing control response of PBS replacing M2 receptor antibody; 7. queen ant’s brain section showing like-M2 receptor positive α-lobes (a), inner antenno-cerebral tract (b); 8. queen ant’s medulla-protocerebrum tract showing like-M2 receptor positive (a), outer antenno-cerebral tract (b); 9. queen ant’s brain section showing like-M2 receptor positive antennal lobe (a); 10. queen ant’s brain section showing control response of PBS replacing M2 receptor antibody; 11. male ant’s brain section showing like-M2 receptor positive α-lobes (a), lower central body (b), uppharyngeal fiber tract (c); 12. male ant’s brain section showing control response of PBS replacing M2 receptor antibody.
innervation, showed distinct differences in the different castes of \textit{P. vicina} (Tab. 1), suggesting that like-muscarinic acetylcholine receptor M2 had a different effect on special behaviors of different castes.

The mushroom bodies are paired and higher-order neuropils in the insect brain are involved in complex functions such as learning and memory, sensory integration, context recognition and olfactory processing. The mushroom body contains many Kenyon cells, and consists of a calyx, pedunculus and two lobes, one medial (alpha lobe) and one vertical (beta lobe). The calyx houses dendritic branches of Kenyon cells and the pedunculus and lobes contain the axons and terminals of these neurons respectively. The cells of the calyces, and part of the alpha lobe, receive sensory input of all modalities. Kenyon cells’ two synapses from olfactory receptor bundles of long thin fibers might be involved in temporal integration of sensory signals (Xin, 1987; Sjöholm, 2006). Different castes of \textit{P. vicina} ant all showed like-muscarinic acetylcholine receptor M2 immunoreactivity in the mushroom body, especially, the terminal of \textalpha-lobes (Tab. 1). In mammals, the M2 muscarinic acetylcholine receptor gene is expressed at high levels in the basal forebrain, striatum and the septohippocampal pathway (Levey et al, 1991). Studies on the function of the basal forebrain have focused on cholinergic neurons that project to cortical and limbic structures critical for various cognitive abilities. Recent experiments suggest that these neurons serve a modulatory function in cognition, by optimizing cortical information processing and influencing attention. LTP is formed in the hippocampus and corpus striatum. The hippocampus contributes to memory processes and particularly to spatial memory (Rouse & Levey, 1996; Rose, 1997).

The central complex is a topographically ordered neuropil structure in the center of the insect brain. It consists of three major subdivisions, the upper and lower divisions of the central body and the protocerebral bridge (Xin, 1987). To further characterize the role of this brain structure, biologists have recorded the responses of identified neurons of the central complex to visual stimuli. Biologists also report that particular types of central complex interneurons are sensitive to polarized light (Müller et al, 1997; Martin et al, 1999). Considering the receptive fields of the neurons and the biological significance of polarized light in insects, the central complex might serve a function in sky compass-mediated spatial navigation of the animals (Vitzthum et al, 2002). Worker ants and male ants (\textit{P. vicina}) exhibited the obvious like-muscarinic acetylcholine receptor M2 immunoreactivity in the central complex. This could indicate that like-muscarinic acetylcholine receptor M2 played a special role in optic integration and limb cooperation. In mammals, we know little about inferior olivary function, but its very intimate association with the cerebellum and its accepting visual information input suggests it is involved in motor coordination and most likely motor “learning”. The inferior olivary nucleus was found to express muscarinic acetylcholine receptor M2 (Miyoshi et al, 1989).

The antennal lobes are another prominent pair of structures located in the front of the brain. These neurons receive input from the chemosensors in the antennae and are responsible for the preprocessing of olfactory and chemosensory information. The lobes are globular structures. The glomeruli form the functional units of the olfactory code. Each of these units can be uniquely identified by its sensitivity to a specific chemical or set of chemicals. The two lobes are connected to each other across the midline by a bundle of nervous tissue called the ‘suprasesophageal commissure’. Another bundle, called the ‘olfactorio-globularis tract’, connects the antennal lobes with the mushroom bodies (Xin, 1987; Hansson & Anton, 2000). Worker ants are engaged in more activities, and communicate with other ants and their environment. Like-muscarinic acetylcholine receptors M2 are abundant in the antennal lobes of worker ants. Olfactorio-globularis tracts of worker ants and queen ants showed like-muscarinic acetylcholine receptor M2 immunoreactivity (Tab. 1). In addition, people found that olfactory tubercle enriched m4-receptors similar to m2-receptors. Muscarinic acetylcholine receptor M2 also is present in the temporal lobe which accepts auditory input (Levey, 1996).

In summary, the results demonstrated that like-muscarinic acetylcholine receptors M2 are distributed widely in the central nervous system of \textit{P. vicina}. We

\begin{tabular}{|c|c|c|}
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\textbf{Caste} & \textbf{Protocerebrum} & \textbf{Deutocerebrum} \\
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Worker ant & 176.4673±4.20685 \textsuperscript{a} & 176.9000±4.43895 \textsuperscript{a} \\
Queen ant & 189.2333±3.99272 \textsuperscript{b} & 208.6000±3.74303 \textsuperscript{b} \\
Male ant & 182.7667±3.71412 \textsuperscript{c} & - \textsuperscript{c} \\
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\end{tabular}

\textit{n}=30: Sample size. Means with different superscripts are statistically different (\textit{P}<0.05, One-Way ANOVA, Two-tailed, LSD).
have proved that Apis mellifera carnica also expressed this analogue. Compared with former studies, we know Sf9 insect cells have successfully expressed mammal muscarinic acetylcholine receptors M2 (Mosser et al, 2002); D2 dopamine receptor is the inhibitory G protein-coupled receptor, which has been proved to exist in the honey bee, A. mellifera (Beggs et al, 2005). Now, muscarinic acetylcholine receptors M2 in mammals are emerging as a hot spot for research, which play an important role in working memory, declarative memory, sustained visual attention and psychomotor speed (Levey et al, 1991; Mrzljak et al, 1993; Jones et al, 2004; Ellis et al, 2006). The muscarinic acetylcholine receptor M2 gene is also a risk factor for the correlated clinical characteristics of alcoholism and depression (Wang et al, 2004). Like-muscarinic acetylcholine receptors M2 could have significant effects on the social insects’ complex behavior. However, further studies on demonstrating that like-muscarinic acetylcholine receptor M2 exists in social insects at a molecular level and exploring of its function in pharmacology are necessary.

References:


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