Muscarinic ACh Receptors On Spider Peripheral Mechanosensilla

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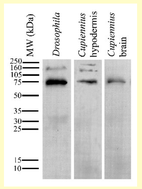
Introduction

Spider peripheral mechanosensory neurons receive extensive efferent innervation. In the tropical wandering spider, *Cupiennius salei,* most of the efferent neurons are GABAergic and the mechanosensory neurons respond to agonists of ionotropic and metabotropic GABA receptors (Fabian-Fine et al. 2003; Panek et al. 2002; 2003). Some of the sensory neurons show acetylcholine esterase (AChE) activity. In addition, immunoreactivity to choline acetyltransferase (ChAT), the enzyme that catalyzes acetylcholine synthesis, has been found in the mechanosensory neurons and in some of the efferent fibers (Fabian-Fine et al. 2003), suggesting a cholinergic innervation of the sensory neurons. In arthropods, muscarinic acetylcholine receptors (mAChRs) have been shown to play an important role in the modulation of synaptic transmission by increasing or reducing the excitability of neurons postsynaptic to mechanosensory neurons (Trimmer 1995). These effects could be caused by mAChRs on the sensory neurons or on neurons presynaptic to the sensory neurons.

Here, we investigated the distribution and function of mAChRs on peripheral mechanosensilla of *Cupiennius salei* using Western blot analysis, immunocytochemistry and extracellular electrophysiological recordings from tactile trichobothria hairs on the spider tibia.

Western blot analysis

Western blot analysis was used to test the specificity of a monoclonal antibody (M35) (Argene Biosoft 10-217) against mAChRs in spider brain and peripheral nervous tissues. The peripheral tissue was obtained from the hypodermis in the spider femur and patella. The tissue was frozen with liquid nitrogen and then ground. After SDS-page separation and transfer to nitrocellulose or PVDF membranes, the samples were incubated in the primary antibody in blocking solution (1:1,000). Peroxidase conjugated goat anti-mouse secondary antibody (1:1,000) was obtained from Jackson laboratories. Immunoreactive protein bands were visualized using an ECL plus chemiluminescent kit (Amersham) according to the manufacturer's instructions.



M35 is an antibody that recognizes all five subtypes of vertebrate mAChR (André et al. 1987) and has been shown to bind to invertebrate mAChR (Wegener et al. 1996). In *Cupiennius,* a specific band was seen at approximately 75 kDa in both brain and peripheral nervous tissues. This molecular weight is typical for muscarinic receptors. A similar band was observed in tissue extracts from *Drosophila* heads.

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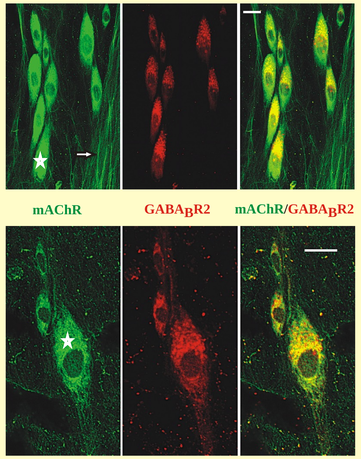
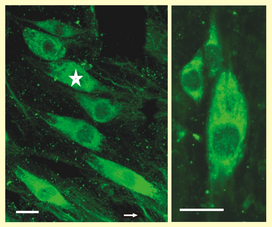
Immunostaining

Immunostaining was performed to see if the M35 antibody would label mAChRs in spider mechanosensilla. Pieces of tissue from the patella of the spider were used for whole mount experiments. Several groups of tactile hairs and one of the lyriform slit organs (VS-3) were present in these samples. The tissue was fixed in 4% paraformaldehyde, followed by incubation in the primary M35 antibody in blocking solution (1:200) overnight at 4°C. The secondary antibody was Alexafluor 488 conjugated goat anti-mouse (1:100; Molecular Probes A-11029). To see if the M35 antibody labeling coincided with the previously shown labeling with metabotropic GABA receptors, we performed double labeling experiments with the M35 and an antibody against $GABA_BR2$ receptors (1:1,000 Chemicon AB5394). A CY-3 conjugated goat anti-guinea pig antibody (1:1,000; Jackson Labs 106-165-006) was used as a secondary antibody. Control preparations where the primary antibody was replaced with mouse ascites fluid or blocking solution showed no staining. The samples were observed using either epifluorescent optics or by laser scanning confocal microscopy (LSM510, Zeiss) with an argon-krypton laser for Alexafluor 488 and a helium-neon laser for CY-3.



Confocal images of double stainings with M35 and $GABA_BR2$ in a VS-3 lyriform mechanosensory organ (top) and a tactile sensillum (bottom). The somata of the mechanosensory neurons showed strong staining with the M35 antibody (asterisk) and many of the nerve axons in the main leg nerve were also labeled with this antibody (arrow). The anti-$GABA_BR2$ staining was clustered in the distal parts of the mechanosensory neurons and no labeling was present in the nerve axons. All of the sensory neurons were stained similarly (scale bars 20 µm in all figures).

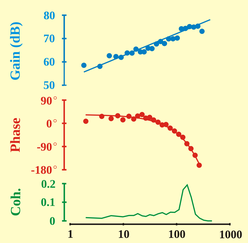


Epifluorescent images of a VS-3 organ (left) and a tactile sensillum (right) showing labeling with the M35 antibody. The staining was seen in the cell bodies (asterisk) and in the axons (arrow) (scale bars 20 µm).

Electrophysiology

Electrophysiological recordings were performed to test the effects of an agonist of muscarinic receptors, oxotremorine (Sigma O-100), on the spider peripheral mechanosensory neurons. Action potentials were recorded extracellularly using an autotomized leg fixed to a Styrofoam support with beeswax (Right). Spider saline was perfused through the leg using a hypodermic needle fixed in the femur. The system was lightly pressurized by a small aquarium pump (Elite 799). An opening on the distal part of the tibia allowed the saline to flow out. 1 mM oxotremorine was applied directly in the saline flow just before it entered the leg, thus reaching the target tissues typically within a few seconds. Two silver wire electrodes (0.25 mm diameter) were pushed into the femur and the patella. This arrangement allowed consistent recordings from the small trichobothria sensilla (~700 µm long) situated at the proximal end of the tibia. A stimulator consisting of a tungsten wire (0.25 mm diameter) mounted on a loudspeaker was attached to one of these hairs with petroleum jelly. Stimulator position was detected by a matched infrared LED-photodiode pair, which also provided second-order servo position control. The hair was stimulated by pseudorandom noise displacement with a maximum amplitude of approximately 50 µm. Action potentials and the stimulator position were sampled by 16-bit analog-to-digital convertors and recorded by a personal computer using custom-written software.

Frequency response function



To test if the mAChR agonists affected the dynamic behavior or information capacity of the spider sensory neurons we performed a series of experiments using frequency response analysis. After stimulating a sensillum with pseudorandom noise displacement and recording the action potentials, we calculated the gain, $G(f)$, and the coherence function, $\gamma^2(f)$, by direct spectral estimation, using position as input and action potential response as output. Gain was fitted by the power law relationship:

$$G(f) = Af^{k}$$

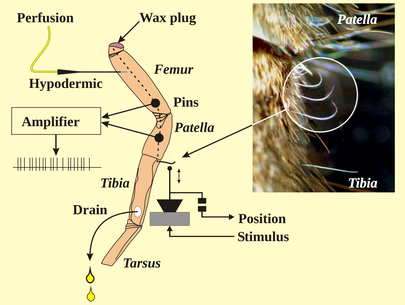
Where f is frequency and A and k are fitted parameters describing the sensitivity of the neuron and the rate of adaptation, respectively.

The coherence function, $\gamma^2(f)$, provides a measure of the linear correlation between the input and output signals as a function of frequency. It also allowed us to estimate the linear information capacity R, using the Shannon formula (Shannon 1949):

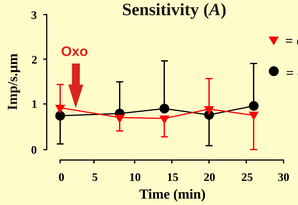
$$R = \int \log_2 \left(\frac{1}{1-\gamma^2(f)} \right) df$$

Information capacity (in bits/second) is the maximum achievable rate of information transmission through a communication system.

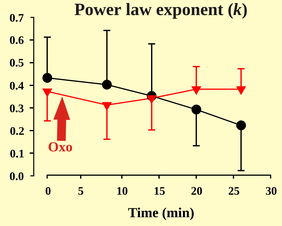
Experimental Setup



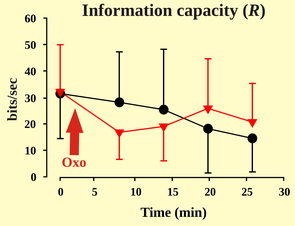
Sensitivity (*A*)



Power law exponent (*k*)

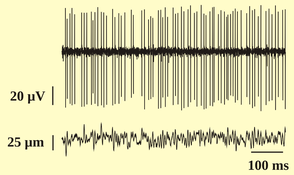


Information capacity (*R*)



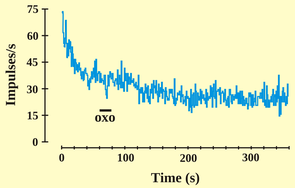
The control lines (●) were recordings where no drugs were applied, but frequency responses were measured from six minute recording segments five times during the 26 min period. The first recording (time = 0) in the oxotremorine (▼) line was prior to drug application. Then 1 ml of 1 mM oxotremorine was applied and four recordings of six minutes duration were performed. The preparation was perfused continuously with spider saline during the experiments. Gain and information capacity were estimated from the last five minutes of each recording. The sensitivity of the neuron (A) remained constant during the control recording and was also unaffected by oxotremorine. However, the exponent (k) describing the rate of adaptation of the neuron, decreased slightly and reversibly during the first 10 min after oxotremorine application while it decreased more slowly in the control recordings. Information capacity (R) also decreased reversibly in response to oxotremorine application. A slower decrease was seen in the control recordings. Each graph shows means and standard deviations from seven recordings (n = 7).

Typical recording



Typical recording showing pseudorandom noise stimulus (bottom) and the resulting action potentials (top) recorded from a trichobothrium.

Firing Frequency



Firing frequency was generally unaffected by oxotremorine. Starting at 60 sec, 1 ml of 1 mM oxotremorine was applied. The sharp decrease in frequency seen at the beginning of the recording was commonly observed. For this reason the first minute of each recording was discarded when calculating the frequency response.

Summary and conclusions

- Western blot analysis using M35 antibody suggested that mAChRs were present in the spider brain and peripheral nervous tissue.

- Immunocytochemistry showed strong labeling with the M35 antibody in all of the sensory neurons of the spider leg. The main leg nerve was also strongly stained. These results suggest that mAChRs are present in the sensory neurons and probably also in the efferent neurons and/or the glial cells

- Action potential firing frequency was not significantly affected by oxotremorine treatment.

- Small and reversible changes in the power law exponent (k) and information capacity (R) were seen in the frequency response recordings. A slow decrease in both these parameters was also seen in neurons that were not treated with oxotremorine, suggesting that a slow decrease in the cell's response occurs normally with time.

-These results support previous findings that spider sensory neurons receive cholinergic innervation. While the presence of mAChRs in the sensory neurons seems clear, we found only small effects of ACh agonist on cell function here. It is likely that mAChR receptors have other functions in these sensilla that are not directly related to regulation of neuronal excitability.

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