Immunocytological and electrophysiological study of acetylcholine and octopamine receptors on peripheral mechanosensory neurons of the spider *Cupiennius salei*

by

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Abstract

Peripheral mechanosensory neurons of the spider (*Cupiennius salei)* receive extensive efferent innervation. Previous work has shown that many of these efferent neurons are immunoreactive to GABA, while ionotropic and metabotropic GABA receptors are found on the mechanosensory neurons themselves. The ACh synthesizing enzyme choline acetyltransferase has also been found in the sensory neurons and in some efferent nerve fibers, suggesting a cholinergic innervation of the mechanosensory neurons. Octopamine immunoreactive neurons have previously been found in the spider CNS making it possible that the sensory neurons are also modulated by octopamine. I investigated the distribution and function of acetylcholine and octopamine receptors on mechanosensilla in the spider leg. Fluorescent antagonists and immunocytochemistry revealed that muscarinic acetylcholine receptors (mAChRs) were present in all the mechanosensory neurons in the spider leg, but intra- and extracellular electrophysiological recordings performed on two types of mechanosensory organs gave no clear responses to muscarinic agonists. However, frequency response analysis revealed that muscarinic agonists abolished a small decay in the rate of firing and information capacity that occurred over time in control recordings, suggesting that the muscarinic system could attenuate long-term adaptation of the mechanosensory neurons. It is also possible that mAChRs have other functions unrelated to neuronal excitability. Immunocytochemistry against octopamine receptors (OARs) indicated that these receptors are present in all mechanosensory neurons, concentrated in the proximal parts of the somata and axon hillock. Double labeling experiments with an antibody against synapsin suggested that OARs are associated with presynaptic vesicles. Octopamine immunolabeling demonstrated the presence of at least one octopamine immunoreactive efferent fiber in close proximity to the sensory neurons. Using electrophysiology, octopamine and its precursor tyramine greatly increased the firing rate of mechanically stimulated tactile hairs. This effect was inhibited by the OAR blocker phentolamine. Frequency response analysis revealed an increase in sensitivity and information capacity in response to octopamine. Finally, experiments with a cAMP analog and a PKA inhibitor indicated that OARs are positively coupled to adenylyl cyclase and act via a PKA mediated pathway.

List of Abbreviations and Symbols Used

A	sensitivity
ACh	acetylcholine
AChE	acetylcholine esterase
AP/s	action potentials per second
bits/AP	bits per action potential
bits/s	bits per second
8-Br-cAMP	8-bromoadenosine-3',5'-cyclic monophosphate
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
ChAT	choline acetyl transferase
Cl^-	chloride ion
CNS	central nervous system
CO_2	carbon dioxide
DABCO	1,4-diazabicyclo(2,2,2)octane
DAG	diacylglycerol
DUM	dorsal unpaired median neuron
EC_{50}	half-maximal effective concentration
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FMRFamide	Phenylalanine-Methionine-Arginine-Phenylalanine-amide
g	gravitational acceleration
GABA	γ-aminobutyric acid
Gi, Gq, Gs	G-proteins
HEK293	human embryonic kidney cells
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
H_2O	water
HPLC	high pressure liquid chromatography
I_{Na}	voltage-activated sodium current
IP_3	inositol 1,4,5-trisphosphate
k	fractional exponent
K^+	potassium ion
KCl	potassium chloride
kDa	kilodalton
KH_2PO_4	potassium phosphate monobasic
LED	light emitting diode
M_1-M_5	muscarinic receptor types
M1-M4	transmembrane domains
mAChRs	muscarinic acetylcholine receptors
$MgCl_2$	magnesium chloride
mRNA	messenger ribonucleic acid
MRO	muscle receptor organ

MW	molecular weight
n	Hill coefficient
Na^+	sodium ion
nAChRs	nicotinic acetylcholine receptors
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate dibasic
OAMB	octopamine receptor in mushroom bodies
OARs	octopamine receptors
OHC	outer hair cell
PBS	phosphate-buffered saline
pH	negative logarithm of the hydrogen ion concentration
PKA	protein kinase A
R	information capacity
Rp-cAMPS	adenosine-3',5'-cyclic monophosphorothioate
SDS	sodium dodecyl sulfate
VS-3	vorderseite-3 (front side-3)
VUM	ventral unpaired median neuron
~	approximately

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1. Introduction

Mechanosensory information is crucial to many physiological functions of vertebrates and invertebrates but much remains to be learned about the transduction and encoding of mechanical signals, their integration by central nervous systems and the central control of peripheral mechanoreceptors by neural or humoral pathways. Most mechanoreceptors have complex morphology that limits experimental access to their sensory neurons. For example, vertebrate skin mechanoreceptors and proprioceptors have their cell bodies in the dorsal root ganglia and their output synapses in the spinal cord, while their sensory endings in the integument, musculature and viscera are distant from the cell bodies and surrounded by complex accessory structures. These features restrict both anatomical and physiological research. In arthropods the structures are simpler, with the somata of the mechanosensory neurons close to the sensory structures, and in some preparations accessible to intracellular recordings (reviewed by McIver 1986; French 1988; Keil et al. 1997; Chapman 1998; French and Torkkeli 2001).

1. 1. Arthropod mechanoreceptors

Arthropods have two types of mechanosensilla: Type I or cuticular sensilla are associated with the exoskeleton and Type II mechanosensilla are not linked to the cuticle but function as proprioceptors or internal stretch receptors (McIver 1986; French 1988). Type I mechanosensilla are innervated by bipolar sensory neurons that have their somata in the periphery close to the sensory ending. Among type I receptors three groups can be

distinguished (McIver 1986, French 1988): 1) Hair sensilla are found on the exoskeleton of most arthropods and can be of various shapes and sizes. In some cases hair sensilla are innervated by both mechano- and chemosensory neurons. The hair is located inside a socket allowing movement. The mechanosensory neuron is stimulated by compression of its dendritic ending, which is closely attached to the base of the hair (Chapman 1998, French and Torkkeli 2001). In most insect hair sensilla, a single neuron innervates each hair while in spiders and crustaceans, two or three sensory neurons are associated with each sensillum (French and Torkkeli 2001).

2) In insects, campaniform sensilla detect stress in the cuticle. Their cuticular part is dome shaped and these sensilla are commonly found in critical strain areas of the exoskeleton (McIver 1986; French 1988). The sensory dendrites run through a canal in the cuticle and enter a dome near the surface of the exoskeleton so that stress in the cuticle compresses the dendritic tip. In arachnids, the role of cuticular stress detectors is fulfilled by slit sensilla (see below).

3) Chordotonal organs are vibration receptors without clear external parts, but they are attached to the cuticle with specialized attachment cells. They are composed of a single unit or many units called scolopidia, each innervated by one or more neurons that are surrounded by a scolopale cell (Chapman 1998; Field and Matheson 1998). Chordotonal organs serve many roles from detecting joint movements to hearing (Field and Matheson 1998; Eberl 1999).

Type II mechanoreceptors are stretch receptors often associated with muscle fibers or connective tissues and consist of multipolar neurons with free nerve endings usually found deeper within the body (McIver 1986; French 1988; Chapman 1998).

1. 2. The spider *Cupiennius salei*

Cupiennius salei (Keys. 1877) is a Central American hunting spider found from Mexico to Honduras (Barth et al. 1988a) (Fig. 1A). It is the largest of the nine known *Cupiennius* species, with a body length reaching 4 cm and a leg span of 12 to 15 cm in females, the males being slightly smaller. *C. salei* does not build webs but it is closely associated with particular species of plants where it hides during the day and on which it hunts and courts at night (Barth et al. 1988a; reviewed by Barth 2002). The dwelling plants, such as banana trees (*Musa sapientium*) usually possess large and strong leaves that offer a protective space for the spider (Barth et al. 1988a). *C. salei* leaves its retreat after sunset and waits nearby until complete darkness, then goes further on the leaf and lies waiting for a potential prey such as a cockroach, earwig, moth or small frog (Barth 2002). For a successful capture the spider must rely on vibratory clues created by the moving prey and transmitted through the substrate, in this case the leaf of the host plant, or through the air (Barth et al. 1988b; Barth 2002). During courtship, potential mates emit vibrations as a means of communication by scratching and drumming on the host plant using their pedipalps (Barth 2002). These vibrations from prey and mates have been described in detail and shown to have broad-band spectra with high frequency peaks, between 400 Hz and 900 Hz, for running prey like cockroaches. Courtship signals have lower frequencies that peak around 75 to 115 Hz for males and 20 to 50 Hz for females (Barth et al. 1988b). Evidently, spiders possess numerous mechanoreceptors to perceive these signals.

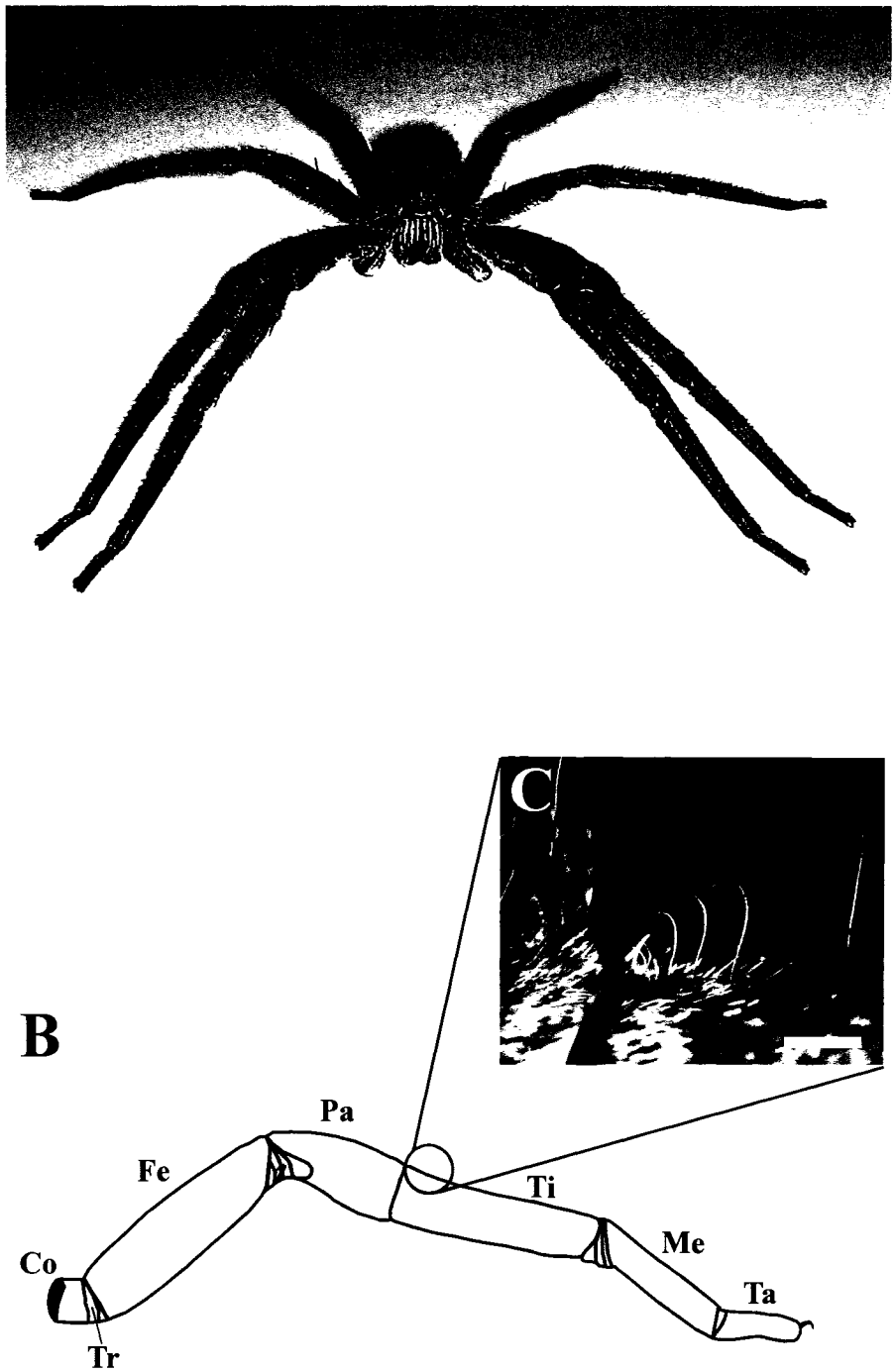

Figure 1:

The spider *Cupiennius salei*.
A: Photograph of a male *C. salei* (courtesy of Ulli Höger). *B*: Schematic drawing of the spider leg with the different parts indicated. co = coxa; tr = trochanter; fe = femur; pa = patella; ti = tibia; me = metatarsus; ta = tarsus. *C*: Photograph of three trichobothria sensilla on the proximal part of the tibia, showing their typical bent apex and gradation in length. Scale bar: 500 μm

1. 3. Spider trichobothria sensilla

One type of spider mechanoreceptors are the trichobothria (reviewed by Barth 2004). They are Type 1 hair mechanoreceptors located on the spider legs. The walking legs are subdivided into several segments, starting with the coxa closest to the body, followed by the trochanter, femur, patella, tibia, metatarsus and tarsus most distally (Fig. 1B). Approximately 100 trichobothria are found on the tarsus, metatarsus and tibia of each leg, most often dorsally. They are often assembled in groups from a few to 24 hairs and each group is distinguished by a gradation in the length of the hairs (Fig. 1C). For example, the lengths of trichobothria on the proximal part of the tibia range from 100 to 1,200 μm. The basal diameter of the hair shaft varies from 10 to 15 μm in long sensilla to 5 to 7 μm in short ones, and both types taper toward the apex. The apical end of the shaft curves proximally at an angle increasing with hair length (reviewed by Barth 2002).

Another characteristic feature of trichobothria sensilla is their surface, which is covered by twig-like protuberances 1 to 6 μm long, giving them a feathery appearance. Despite their length, trichobothria have a tiny mass in the order of 10^{-9} g. This, along with their surface structure and soft articulation allows deflection of the hair shaft by the slightest air movement (Barth 2000). In an electrophysiological experiment, Barth and Höller (1999) determined that the airflow produced by a tethered fly at a distance of 55 cm could still be perceived by a trichobothrium. The varying length offers mechanical tuning to certain frequencies, with the longer hairs tuned to lower frequencies than the short ones (reviewed by Barth 2002). This frequency tuning is lost when the hair is directly attached to a stimulator, suggesting that fluid mechanics are responsible for the tuning (Barth and Höller 1999).

Each trichobothrium is innervated by three or possibly four sensory neurons. Although only three cell bodies are consistently observed in light microscopy (Anton 1991; Fabian-Fine et al. 1999b, 2002), electron microscopy showed evidence of a fourth dendrite in the outer dendritic segments (Anton 1991). Three dendrites are of similar size, while the fourth one is significantly larger and fills most of the dendritic shaft. The origin and function of the fourth dendrite are not known. Only one amplitude of action potentials could be recorded extracellularly (Barth and Höller 1999). In two other spider species (*Ciniflo ferox* and *Ciniflo similis*) a maximum of three phasic units could be physiologically distinguished, with different directional sensitivities (Harris and Mill 1977).

In *C. salei*, the sensory neurons do not fire action potentials spontaneously and their response is very phasic (Barth and Höller 1999). Thus, they do not respond to the position of the hair shaft, but rather to changes in position (Barth 2002). The trichobothria sensilla are directionally sensitive, meaning that they bend more easily in some directions than others. However, there is no evidence of different neurons in the same sensilla having different directional sensitivities.

1. 4. Spider slit sensilla

A special type of mechanosensillum that is not found in insects but is common in spiders is the slit sensillum or slit sense organ (reviewed by Barth 2004). *C. salei* has approximately 3,300 slits, most of them on the legs and pedipalps, but others widely distributed on the rest of the body. A slit consists of a 8 to 200 μm long and 1 to 2 μm wide cleft in the cuticle covered on the outside by a thin outer membrane. Two bipolar neurons innervate each slit. One of the sensory dendrites crosses the slit to attach to the outer

membrane via a specialized structure called the coupling cylinder and the other, shorter, dendrite stops near the entrance of the slit, close to an inner membrane (reviewed by Barth 2002). Both dendrite tips contain densely packed microtubules forming a 'tubular body' (reviewed by French et al. 2002). Pharmacological depletion of the microtubules has no effect on responses to mechanical stimuli of the sensory neurons (Höger and Seyfarth 2001) suggesting that the tubular body has no function in mechanosensation. Slit sensilla respond to strains in the cuticle produced by muscular tension, hemolymph pressure or substrate vibrations (reviewed by Barth 2002). The slits are found either singly, in small groups or arranged into lyriform organs. The lyriform organs resemble the shape of a lyre and they are made up of 5 to 30 slits (reviewed by Barth 2002) (Fig. 2A). Lyriform organs are only found on the appendages, most noticeably near the joints of walking legs (reviewed by French et al. 2002). *C. salei* has a total of 134 lyriform organs on the legs and pedipalps.

One of the lyriform organs has been given particular experimental attention in the past ten years. The VS-3 organ (nomenclature of Barth and Libera 1970) is found on the anterior side of each patella. It consists of 7 or 8 slits ranging in length from 15 to 120 μm (Seyfarth and French 1994). The sensory neurons have large elongated cell bodies and are arranged by pairs in a cluster, close to the cuticular slits (Fabian and Seyfarth 1997) (Fig. 2B). The lengths of the somata vary from approximately 20 to more than 100 μm, with the central slits having the largest neurons. The two neurons of each pair are usually of similar size except in one case; the neurons innervating the slit number 2 always have one soma that is significantly larger than its partner (Fabian and Seyfarth 1997) (Fig. 2B). The cell bodies, dendrites and initial axon segments of each neuron are tightly wrapped in a layer of glial cells up to 2.7 μm thick (Fabian-Fine et al. 1999a).

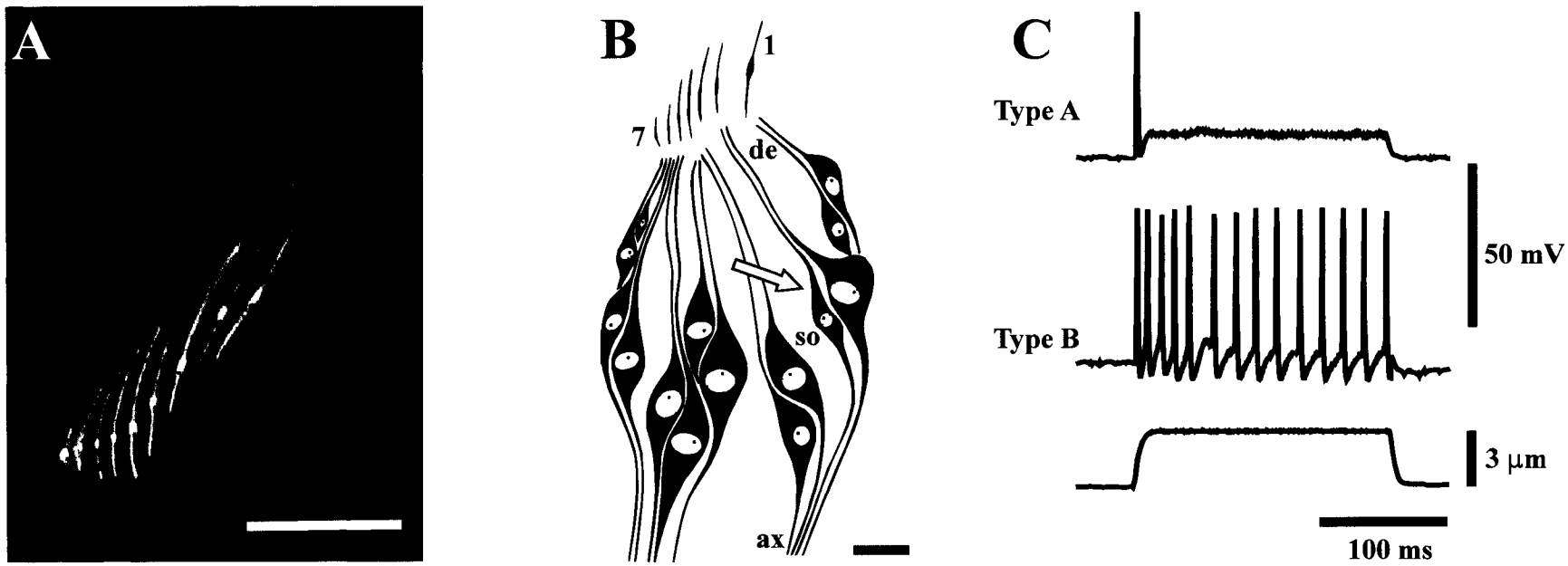

Figure 2:

Structure and intracellular responses of VS-3 neurons.
A: Photograph of the cuticular slits forming a lyriform slit sense organ. *B*: Schematic drawing of the arrangement of paired bipolar neurons in the VS-3 organ. The neuron pair innervating the second slit shows a significant size difference (arrow). ax = axons; so = somata; de = dendrites (modified from Fabian and Seyfarth 1997). Scale bars: 50 µm. *C*: Intracellular response to a 3 µm step mechanical stimulus in a rapidly adapting (Type A) and a slowly adapting (Type B) neuron (modified from Höger and Seyfarth 2001).

VS-3 neuron physiology has been well described (reviewed by French et al. 2002). The size and position of the neurons allow simultaneous intracellular recording and mechanical stimulation (Juusola et al. 1994). In addition, a preparation that makes it possible to perform current- and voltage-clamp recordings of the voltage- and ligand activated conductances has been developed (Sekizawa et al. 1999; Höger and Seyfarth 2001). In this 'hypodermis preparation' (see methods) the VS-3 organ is detached from the cuticle and placed on a coverslip allowing good access for pharmacological agents.

Intracellular recordings revealed that neurons from each pair differ in their adaptation properties. The Type A neuron adapts very rapidly and fires only one or two action potentials upon suprathreshold mechanical or electrical stimulation while the Type B neuron fires a burst of action potentials in response to similar stimuli (Seyfarth and French 1994) (Fig. 2C). These differences remain in the hypodermis preparation, in which cuticular components are not present, indicating that the elements that connect the dendritic tips to the cuticular slits are unnecessary for the adaptation behavior (Juusola and French 1998; Höger and Seyfarth 2001). The Type A and Type B neurons can not be distinguished morphologically except in the neuron pair innervating slit number 2 where the larger neuron has been shown to be Type B while the smaller neuron expresses the Type A behavior (Fabian and Seyfarth 1997; Fabian-Fine et al. 1999b).

Upon mechanical stimulation the receptor potentials of Type A neurons adapt slightly more rapidly than for Type B neurons (Juusola and French 1998). However, this alone can not explain the difference in the firing pattern. An investigation into the different voltage-activated conductances showed that the inactivation properties of voltage-activated Na^+ (I_{Na}) currents control the firing behavior of these neurons (reviewed by French et al. 2002). The

half-maximum inactivation was closer to the resting membrane potential in the Type B than in the Type A neurons. In addition, the I_{Na} recovered from inactivation faster in Type B (Torkkeli et al. 2001). In a simulation study using a Hodgkin-Huxley model it was demonstrated that changing only the slope of the I_{Na} inactivation curve and the time constant of recovery from inactivation was sufficient to completely switch between the two different firing patterns (Torkkeli and French 2002).

1. 5. Presynaptic modulation of mechanosensory neurons

Presynaptic modulation of sensory neurons is a common feature in both vertebrates and invertebrates. In most cases this takes place at the level of the centrally located synapses between sensory afferent axon terminals and postsynaptic neurons (reviewed by e.g. Watson 1992; Rudomin et al. 1998; Torkkeli and Panek 2002). Examples of the possible roles of presynaptic modulation are in preventing habituation or sharpening a receptive field (Watson 1992). Presynaptic inhibition by γ-aminobutyric acid (GABA) is the best studied type of modulation in both vertebrate and invertebrate preparations (e.g. Watson 1992; Rudomin et al. 1998). Another known transmitter in efferent fibers is glutamate (Watson 1992; Cattaert and Le Ray 1998; Cattaert et al. 2002), but its roles in presynaptic modulation are not well understood.

In *Aplysia* mechanosensory afferent terminals FMRFamide was also demonstrated to produce presynaptic inhibition (reviewed by Watson 1992). Acetylcholine (ACh), acting through muscarinic receptors also plays a role in presynaptic inhibition in insects (see below). In addition, immunolabeling against choline acetyl transferase (ChAT), the ACh producing enzyme, was shown in fibers presynaptic to sensory afferents in the dorsal horn

of the rat spinal cord (Ribeiro-da-Silva and Cuello 1990). Another neurotransmitter that was shown to induce inhibitory responses in crustacean mechanosensory afferents is histamine (El Manira and Clarac 1994; reviewed by Cattaert et al. 2002). Presynaptic facilitation appears to be a less common phenomenon. However, it was described in *Aplysia* in response to serotonin (Siegelbaum et al. 1982; Chang et al. 2000).

In addition to the modulation of their centrally located axons, crustacean and arachnid mechanosensory afferents also receive efferent innervation to their peripherally located parts. Well known examples of peripheral efferent innervation are the crustacean stretch receptors. In these receptors two types of efferent terminals were shown to contact the two sensory neurons and three types of synaptic contacts were distinguished based on their vesicle populations (Reviewed by Fabian-Fine et al. 2002). Immunoreactivity against GABA suggested that this transmitter was present in the efferent fibers (Elekes and Florey 1987a). There is also electrophysiological evidence for GABA acting as an inhibitory neurotransmitter at the stretch receptor synapses (reviewed by Cattaert et al. 2002). Octopamine on the other hand has an excitatory effect on the responses of most crustacean mechanosensory neurons (Pasztor and MacMillan 1990; reviewed by Cattaert et al. 2002 and Fabian-Fine et al. 2002).

The existence of peripheral chemical synapses in arachnids was initially described by Foelix (1975) in two spider species. More recently, morphological and electrophysiological studies described below have provided significant evidence of very complex efferent modulation of the peripheral parts of spider mechanosensory afferents.

1. 6. Efferent innervation of *C. salei* mechanosensory neurons

Using immunocytochemistry, together with light, confocal and electron microscopy, Fabian-Fine and colleagues described in great detail a complex network of efferent innervation of the *C. salei* mechanosensory neurons (Fabian-Fine et al. 1999a, b; 2000; 2002). An antibody against the synaptic vesicle protein synapsin was shown to label thin fibers along the leg nerves (Fabian-Fine et al. 1999a). Immunofluorescent punctae were linearly arranged along the sensory neurons, most prominently near the initial axon segments of the afferent neurons. Electron microscopical investigation confirmed that the antibody against synapsin bound to synaptic vesicles, and that numerous synapses existed between sensory neurons and fine nerve fibers running parallel to them. The fine fibers were determined to be efferent by cutting the leg nerve close to the CNS and observing their rapid orthograde degeneration (Fabian-Fine et al. 1999b). Electron microscopical study also indicated that synapses were not only present between the efferent fibers and sensory neurons, but they were found between the efferent fibers and glial cells, as well as between efferent fibers themselves. However, no synapses were found between sensory neurons (Fabian-Fine et al. 1999a, b). Simultaneous intracellular recordings from both neurons of a VS-3 pair also indicated that there was no coupling between the sensory neurons (Panek et al. 2002).

An antibody against GABA labled several efferent fibers innervating the VS-3 organ and other mechanosensory neurons such as tactile hairs, internal joint receptors and trichobothria (Fabian-Fine et al. 1999b). Three types of GABA-immunoreactive fibers were detected and GABA and synapsin immunoreactivity were often colocalized in the same fibers. However, 34 to 54% of the fine fibers showing synapsin immunoreactivity were not

labeled for GABA, suggesting that other transmitters could be present (Fabian-Fine et al. 1999b). This was confirmed by using an antibody against glutamate which also labeled several efferent fibers (Fabian-Fine et al. 2000).

Electron microscopy revealed at least four different types of peripheral synapses in the VS-3 organ (Fabian-Fine et al. 2000) (Fig. 3). Type 1 synapses contained small, clear, round vesicles with a mean diameter of 31.8 nm and occasional dense-core vesicles (75 to 120 nm in diameter) and/or granular vesicles (46 to 63 nm in diameter). Type 2 synapses contained large, clear, round vesicles with a mean diameter of 41.8 nm. In both of these synapse types the number of vesicles varied greatly. In type 3 synapses, a mixed population of small clear vesicles, large clear vesicles, dense-core vesicles and granular vesicles was observed. Finally, type 4 synapses were characterized by very large, clear, round vesicles 37 to 65 nm in diameter. Only a few vesicles were observed in this latter synapse type, and the corresponding presynaptic fibers were usually very small. Type 3 and type 4 synapses were only rarely seen, while type 1 and type 2 synapses were the most common. Electron microscopy with immunogold labeling using antibodies against GABA and glutamate revealed that at least some type 1 synapses contained GABA while some type 2 synapses contained glutamate (Fabian-Fine et al. 2000). The identity of the other synapse types has not yet been revealed.

In addition to the four different types of synapses, numerous combinations of synaptic contacts were shown to exist in the spider mechanosensilla (Fabian-Fine et al. 2000). They varied according to the synapse types and classes of contacts (whether contacts were made between efferent fibers and sensory neurons, efferent fibers and glia or between

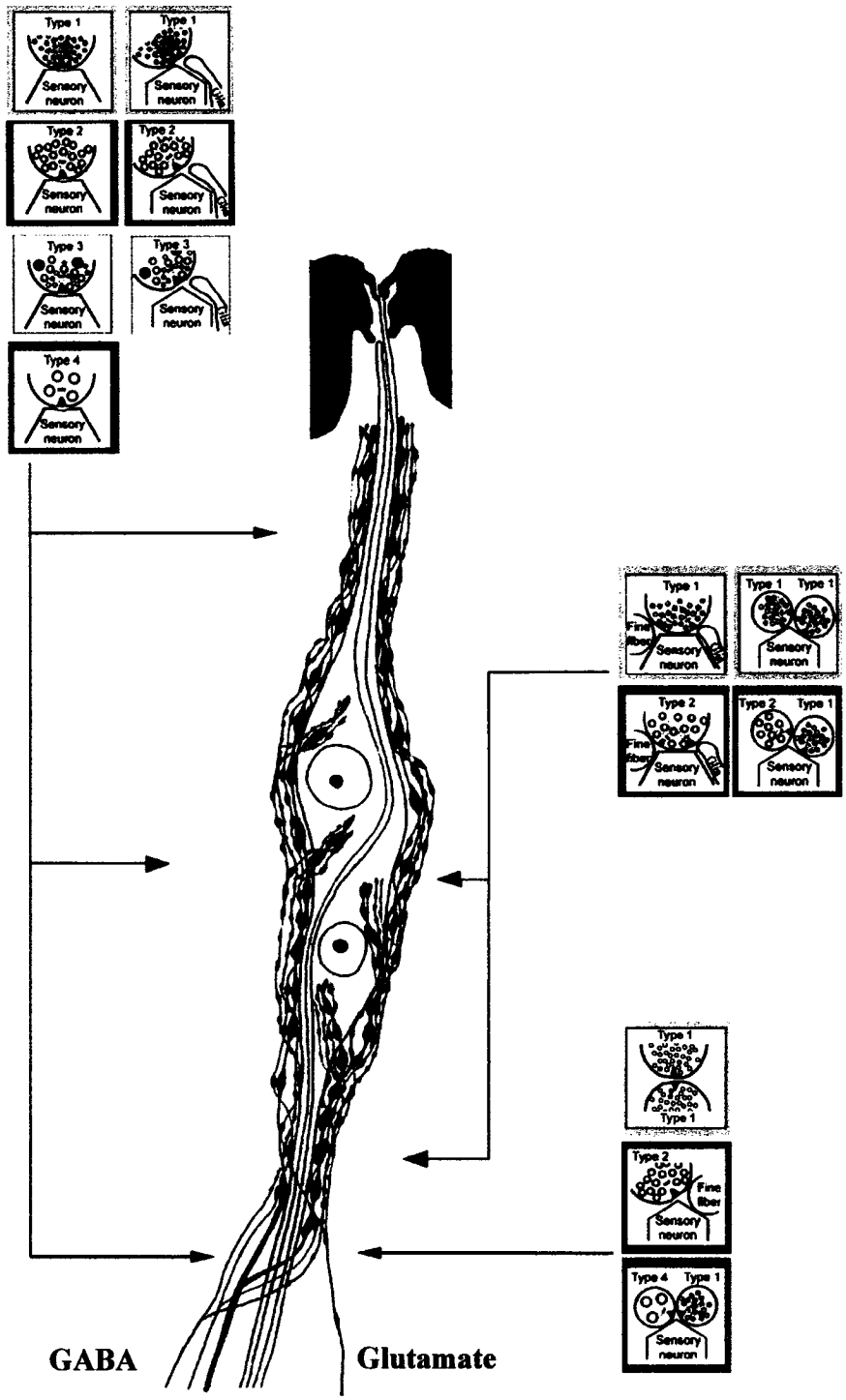

Figure 3:

Efferent innervation of the VS-3 neurons.
Distribution of some of the numerous different types of synaptic contacts on the dendrites, somata and axons of a pair of mechanosensory VS-3 neurons. Type 1 to 4 synapses are represented by different colors (inset). At least three types of efferent fibers are GABAergic (GABA), and at least one type is glutamatergic (Glutamate) (redrawn from Fabian-Fine et al. 2000).

efferent fibers themselves). They could be described as unidirectional contacts between a single pre- and postsynaptic element (monadic synapses); dyad synapses between three elements, with the two postsynaptic cells consisting of two neurons, two glial cells or one of each; reciprocal contacts where two efferent fibers contacted via type 1 synapses; and serial contacts where an efferent fiber synapsed onto another efferent fiber, which in turn was presynaptic to a sensory neuron or third efferent fiber (Fig. 3). The initial axon segment displayed the largest variety of synaptic contacts (Fabian-Fine et al. 2000). Such a complex array of microcircuits could allow an extensive and complex network of mechanisms for controlling mechanosensory inputs. So far, only the GABAergic control has been described in detail (Panek et al. 2002, 2003) and experimental work on glutamatergic modulation has been completed.

1. 6. 1. GABAergic control of spider mechanosensory neurons

Electrophysiological experiments quickly proved that GABA was an important player in the efferent control of VS-3 neurons. Application of GABA or muscimol, a specific agonist of ionotropic GABA receptors, on VS-3 neurons decreased their membrane resistance, depolarized them and abolished firing (Panek et al. 2002). These agonists also induced a rapidly activating but long-lasting inward current that was blocked by picrotoxin, but not by bicuculline (Panek et al. 2002). The pharmacology and kinetics of this current were similar to those described for the insect *rdl* (*r*esistance to *di*e*l*drin) GABA receptors (ffrench-Constant et al. 1991). These receptors have different molecular structures and pharmacology than either of the two types of vertebrate ionotropic GABA receptors, $GABA_A$ or $GABA_C$. However, all of these receptors are ligand gated Cl^- channels that produce fast

inhibitory responses (reviewed e.g., by Hosie et al. 1997; Borman 2000). GABA-induced inhibition of mechanosensory afferents via ionotropic receptors is a phenomenon that exists in all known mechanosensory afferents in vertebrates and invertebrates (e.g., Rudomin et al. 1998; Torkkeli and Panek 2002). In most cases it is described as presynaptic inhibition that occurs on the axon terminals close to the site of synaptic contacts with the second order neurons (e.g., Rudomin et al. 1998). In the spider mechanosensory afferents this inhibition was shown to occur in the peripherally located parts of the axons (Gingl et al. 2004).

Metabotropic $GABA_B$ receptors are coupled to Ca^{2+} and/or K^+ channels via a G-protein mediated pathway and they play important roles in long term modulation of synaptic transmission (reviewed by Bowery et al. 2002). In both vertebrate and invertebrate nervous systems the functional $GABA_B$ receptors are heterodimers that consist of two subunits, $GABA_BR1$ and $GABA_BR2$ (Mezler et al. 2001; Bowery et al. 2002). Immunocytochemistry with antibodies against these subunits suggested that $GABA_B$ receptors were also present in the spider mechanosensory neurons (Panek et al. 2003). Immunoreactivity against the R1 subunit was widespread in the sensory neurons and it was also observed in some glial cells. R2 subunit immunoreactivity was observed exclusively on the distal parts of all mechanosensory neurons (Panek et al. 2003). When specific agonists of $GABA_B$ receptors were applied in voltage-clamp experiments, they reduced the amplitude of the large transient Ca^{2+} current (Sekizawa et al. 2000; Panek et al. 2003) and induced a small but reversible increase in the amplitude of the outwardly rectifying K^+ current (Sekizawa et al. 1999; Panek et al. 2003). It is not clear what the functional significance of $GABA_B$ receptors is in the spider mechanosensory neurons, but they may cause a slow sustained inhibition when the neurons are subjected to repeated stimulation (Panek et al. 2003).

1. 6. 2. Glutamatergic control of spider mechanosensory neurons

An antibody against kainate type glutamate receptor subunits GluR 5, 6 and 7 (Ziff 1999; Dingledine and Conn 2000) labeled punctae along the long axis of the spider sensory neurons, most prominently near the initial axon segment (Fabian-Fine et al. 2000). Glial cells in the same axonal region also showed immunoreactivity against this antibody (Fabian-Fine et al. 2000). However, electrophysiological investigation has provided no evidence of kainate or other excitatory type glutamate receptors on the spider VS-3 neurons, suggesting that if these receptors are present they may be limited to the glial cells and efferent neurons. The sensory neurons were inhibited by bath applied glutamate, and this effect was present even when the efferent input was blocked (Panek and Torkkeli, in preparation). In addition, immunocytochemistry against inhibitory glutamate receptors has revealed that these receptors are present in the mechanosensory neurons and in some of the efferent neurons that innervate the sensory neurons (Torkkeli and Meisner, in preparation).

Inhibitory glutamate receptors that directly gate Cl^- channels ($GluCl^-$) are common in invertebrate nervous systems (Cleland 1996; Kehoe and Vulfius 2000). They are analogous to vertebrate inhibitory glycine receptors that also gate Cl^- channels but are not found in invertebrates (Cully et al. 1994, 1996). $GluCl^-$ channels are often found in the same neurons with ionotropic GABA receptors and in many cases GABA has been shown to interact with $GluCl^-$ channels (Etter et al. 1999). In the spider VS-3 neurons, glutamate-induced inhibition was less pronounced than the inhibition produced by activation of ionotropic GABA receptors. However, the glutamatergic inhibition affected all of the peripheral parts of the neuron including the most distal parts of the dendrites (Panek and

Torkkeli, in preparation).These results indicate that the spider mechanosensory neurons receive an extensive inhibitory control via multiple pathways.

1. 7. Acetylcholine as a potential transmitter at peripheral synapses in spider mechanosensilla

ACh is considered to be the main excitatory transmitter of mechanosensory neurons in arthropods (Trimmer and Weeks 1989; Judge and Leitch 1999; Fabian-Fine et al. 2002). Immunocytochemical evidence has also suggested that ACh is a transmitter in *C. salei* mechanosensory neurons. Immunoreactivity against ChAT was present in all sensory neurons of the VS-3 organ and the tactile hairs (Fabian and Seyfarth 1997). However, ChAT immunoreactivity was also found in cells lining the sensory axons, less frequent but similar in size and location to that seen with synapsin antibody, suggesting that some of the efferent fibers may also be able to synthesize ACh (Fabian-Fine et al. 2002).

Histochemistry for the ACh degrading enzyme acetylcholine esterase (AChE) revealed that only one of the two sensory neurons innervating each slit of the VS-3 organ and one of the three neurons innervating each tactile hair expressed AChE activity (Fabian and Seyfarth 1997). Only the smaller neuron of the two innervating slit number 2 was stained, suggesting that only the Type A neuron in this pair contained AChE. These observations along with the fact that cholinergic synapses generally correspond to type 1 synapses similar to those observed in the spider preparation suggest that ACh may act as a neurotransmitter at the spider peripheral synapses (Fabian-Fine et al. 2002), at least on Type A neurons. ACh could act via ionotropic nicotinic receptors, or have a slower effect

through metabotropic muscarinic receptors, as has been shown in other invertebrate mechanosensory neurons.

1. 8. ACh receptors

Two subclasses of acetylcholine receptors can be distinguished, the ionotropic nicotinic receptors (nAChRs) and the metabotropic muscarinic receptors (mAChRs) (reviewed by Breer and Sattelle 1987). Activation of nAChRs increase cation conductances with poor selectivity for different cations (Na^+, K^+ and Ca^{2+}). The mAChRs can act upon different cell signaling pathways leading to a decrease in intracellular cAMP concentration, activation of the phosphoinositol pathway or changes in ion permeability (reviewed by Breer and Sattelle 1987).

1. 8. 1. nAChRs

The molecular structure and pharmacology of vertebrate nAChRs is well understood. These receptors are found at the neuromuscular junction and in the CNS, although evidence of a physiological role in synaptic functions in the brain (Kandel and Siegelbaum 2000; Caulfield and Birdsall 1998) is limited. These receptors are part of the superfamily of neurotransmitter-gated ion channels that also includes ionotropic GABA, glycine and glutamate receptors (reviewed by Tomizawa and Casida 2001). nAChR is a pentameric receptor, with five homologous subunits assembling to form one functional receptor. Different subunit combinations can lead to different pharmacological profiles. Each subunit contains four transmembrane domains (M1-M4) (Leech and Sattelle 1993). In vertebrates 17 subunits are known. The mature skeletal muscle subtype at the neuromuscular junction

consists of two α1 subunits plus β1, δ and ϵ. This subtype is sensitive to the snake toxin α-bungarotoxin. The vertebrate neuronal nAChR subtype can be divided into α-bungarotoxin insensitive, made up of α2 to α6 and β2 to β4 subunits, and α-bungarotoxin sensitive where α7 to α10 subunits are present (reviewed by Tomizawa and Casida 2001; Millar 2003).

In contrast, insect nAChRs are not as well described. Similarly to vertebrate receptors they are permeable to cations (Leech and Sattelle 1993). They are found in the CNS at high densities, but insect neuromuscular junctions function using glutamate receptors (Usherwood et al. 1968). In *Drosophila*, four genes for α subunits (ALS, Dα2, Dα3, Dα4) and two genes for β subunits (ARD, SBD) have been identified. Four more candidate genes are predicted from genomic data. However, expression of these subunits in various combinations in *Xenopus* oocytes, human embryonic kidney cells (HEK293) or the *Drosophila* S2 cell line failed to produce any physiological responses. A functional response could only be obtained when any of the four *Drosophila* α subunits was coexpressed with rat or chick β2 subunits (reviewed by Tomizawa and Casida 2001).

In the peach-potato aphid *Myzus persicae* five genes for α subunits (Mpα1-5) and one gene for β subunits (Mpβ1) have been cloned. Homomeric receptors can be constructed from Mpα1 or Mpα2 subunits but their electrophysiological response is weak (reviewed by Tomizawa and Casida 2001). These same subunits can also assemble with Mpβ1, but the receptor is not functional. As for *Drosophila*, coexpression with rat β2 subunit is necessary for a functional receptor to be expressed. Therefore, it seems that additional subunits must exist. In the locust, *Locusta migratoria*, four genes for α-subunits (Loc α1-4) and one gene for β-subunits (Locβ1) have also been cloned. In the desert locust, *Schistocerca gregaria*, one cloned subunit (αL1) was expressed in *Xenopus* oocytes where it formed a functional

homomeric receptor that was activated by nicotine and blocked by α-bungarotoxin (reviewed by Tomizawa and Casida 2001).

Although the situation in vertebrates is relatively clear, with different subunits assembling in different combinations to form pharmacologically distinct nAChRs, it is not known what the subunit arrangement is in insects. However, electrophysiological evidence points towards the existence of different types of nAChRs with variable pharmacology (Lapied et al. 1990; reviewed by Tomizawa and Casida 2001).

1. 8. 2. mAChRs

Muscarinic receptors are highly conserved members of the G-protein coupled receptor superfamily (Venter et al. 1984; Van der Zee and Luiten 1999). They are the most abundant ACh receptors in the vertebrate brain (Breer and Sattelle 1987) where they are involved in motor control, thermoregulation, regulation of cardiovascular function and memory (Caulfield and Birdsall 1998). mAChRs are also present in the periphery where they are involved in smooth muscle contraction in the eye, intestine and airways, in glandular secretion and in modulation of cardiac output (Venter et al. 1984; Caulfield and Birdsall 1998). mAChRs are glycoproteins which, like other members of the G-protein coupled receptors, consist of a single subunit with seven transmembrane domains (Van der Zee and Luiten 1999). The C-terminus is located intracellularly and the N-terminus extracellularly (Dhein et al. 2001). Five mammalian genes have been cloned that encode mAChRs (M_1 to M_5) and mRNA of all subtypes is expressed in mammalian brain and peripheral tissue (reviewed by Khan et al. 2002). Pharmacologically, these receptors can also be distinguished by their affinity to various antagonists and allosteric agents (Caulfield and Birdsall 1998).

These subtypes display high sequence identity between mammalian species (>90% between human and rat for all subtypes; Dhein et al. 2001), but sequence divergence exists in the third internal loops between the $M_1/M_3/M_5$ subtypes and the M_2/M_4 subtypes that determine the coupling preferences of these two groups (Caulfield and Birdsall 1998). Indeed, the $M_1/M_3/M_5$ subtypes are coupled to a Gq protein activating the phospholipase C system, and the M_2/M_4 subtypes are typically linked to a Gi protein leading to an inhibition of adenylyl cyclase (Dhein et al. 2001; Khan et al. 2002). The M_2 receptor can also couple to K^+-channels as well as modulate Ca^{2+}-channels (Dhein et al. 2001). Moreover, when expressed in Chinese hamster ovary cells it was shown that the M_2 receptor could couple to both Gq and Gi pathways (Ashkenazi et al. 1987).

In *Drosophila*, a single mAChR has been cloned (Onai et al. 1989; Shapiro et al. 1989). Onai and colleagues (1989) cloned a *Drosophila* mAChR containing 788 amino acids. The sequence similarity with mammalian M_1 to M_5 receptors varied between 62% and 69% with a maximum of 88% in conserved transmembrane domains III and IV. Shapiro and colleagues (1989) described a spliced form of this receptor made up of 722 amino acids with 45 to 50% overall amino acid identity to mammalian M_1 to M_5 receptors and particular similarities with the $M_1/M_3/M_5$ subtypes in the amino terminus of the third cytoplasmic loop. This observation suggested an association of the *Drosophila* receptor with a Gq protein. This was further supported by functional expression in mouse Y1 adrenal carcinoma cells, in which this receptor bound the antagonists atropine and pirenzepine and induced an increase in intracellular inositol phosphates in response to the agonist carbachol.

1. 9. Presynaptic function of ACh

A presynaptic action of ACh has been described in both vertebrate and invertebrate preparations. The best known example in vertebrates is the efferent innervation of the cochlear hair cells that is provided by the olivocochlear bundle (Warr and Guinan 1979). It displays numerous efferent synapses on the outer hair cells (OHC), with several efferent endings at the base of each OHC (Spoendlin 1985). Histochemical and electrophysiological studies have shown that ACh is one of the efferent transmitters in these synapses (reviewed by Eybalin 1993). The hair cell ACh receptor belongs to the α-bungarotoxin sensitive nicotinic α9 and α10 gene family. Ca^{2+} influx through the nicotinic receptors was shown to activate nearby Ca^{2+}-gated K^+ channels, resulting in hyperpolarization and inhibition of the hair cell (reviewed by Fuchs 2002 and Millar 2003).

In insects, ACh has also been found to act presynaptically at the level of centrally located synapses. Release of ACh by locust synaptosomes was shown to be inhibited by mAChR agonists and potentiated by mAChR antagonists, suggesting that muscarinic receptors could act as autoreceptors on cholinergic afferent terminals (Breer and Knipper 1984). ACh was found to play a similar role in several insect mechanosensory neurons; for example, in the cockroach (*Periplaneta americana*) at the synapse between cercal sensory afferents and giant interneurons (Hue et al. 1989) and in the tobacco hornworm (*Manduca sexta*) at the synapse between planta hair afferents and the proleg retractor motoneurons (Trimmer and Weeks 1989). Immunocytochemical investigation and experiments with a fluorescence labeled pirenzepine probe indicated that mAChRs were also present in cultured antennal sensory neurons of pupal stage *Manduca* (Torkkeli et al. 2004). In the locust (*Schistocerca gregaria*) muscarinic modulation has been described at the synapse between

forewing stretch receptors and first basalar motoneurons, as well as between afferents from the campaniform sensilla and the fast extensor tibia motoneurons (Leitch and Pitman 1995; Parker and Newland 1995). In addition, in *Locusta migratoria* presynaptic muscarinic inhibition was observed between the forewing stretch receptor and first basalar motoneuron, but this was suggested to occur through mAChRs on GABAergic interneurons, which in turn made inhibitory contacts on the sensory afferents (Judge and Leitch 1999). Such interactions via GABAergic interneurons were also suggested as a possible mechanism in the cercal afferent-giant interneuron synapse of the cockroach (Le Corronc et al. 1991). The pharmacology of mAChRs involved in these presynaptic feedback mechanisms was similar to the vertebrate type M_2 receptors (Le Corronc et al. 1991; Leitch and Pitman 1995).

Muscarinic receptors have also been found on postsynaptic motoneurons (Trimmer and Weeks 1993; Parker and Newland 1995) where they have a slow excitatory function. The pharmacology of these receptors was different from the presynaptic mAChRs, and closer to the vertebrate type M_1 receptors (reviewed by Trimmer 1995).

1. 10. Octopamine as a potential transmitter at peripheral synapses in spider mechanosensilla

Octopamine is a monoamine that acts as a neurohormone, a neuromodulator and a neurotransmitter in invertebrates. The octopaminergic system is analogous to the vertebrate noradrenergic system, and octopamine is thought to modulate almost every invertebrate physiological process, including sensory perception (reviewed by Roeder 1999). In *C. salei*, octopamine-immunoreactive neurons have been found in the central nervous system (Seyfarth et al. 1993). Several octopamine-positive unipolar neurons were present in the

subesophageal ganglion with their primary neurites projecting into mid-dorsal neuropil where they arborised. Although very little evidence for octopamine-immunoreactive projections to the periphery was found, some single fine varicose terminals, confined to the roots of leg nerves, were labeled for octopamine. Some labeled somata were also present in the medio-ventral region of the subesophageal ganglion where GABA-immunoreactive cell bodies of similar size have been described (Schmid et al. 1990). Although no double labeling experiments were performed to test if these transmitters co-localized in the same neurons, it is tempting to suggest that some of the GABAergic efferent fibers found around peripheral mechanosensory neurons could also contain octopamine.

Octopamine may also act on mechanosensory neurons at the periphery by being transported via circulating hemolymph. Indeed, intensely stained varicosities were shown to be concentrated close to hemolymph spaces in the spider CNS, suggesting that octopamine may be released into the circulation. High pressure liquid chromatography (HPLC) investigation confirmed that ~30 x 10^{-9} M octopamine was present in the *C. salei* hemolymph (Seyfarth et al. 1993).

1. 11. Octopamine receptors

Octopamine receptors (OARs) are members of the superfamily of G-protein coupled receptors. They have seven transmembrane segments, with the N-terminus located extracellularly and the C-terminus located intracellularly (reviewed by Blenau and Baumann 2001). The primary structure of only very few OARs is known but the pharmacology of several receptors has been described (reviewed by Roeder 1999). Most of the OARs stimulate adenylyl cyclase leading to an increase in intracellular cyclic adenosine

monophosphate (cAMP) concentration. However, activation of protein kinase A (PKA), the next step of this cascade, was only demonstrated in a small number of preparations (Hildebrandt and Müller 1994). Some OARs are coupled to phospholipase C that activates intracellular calcium release from internal stores and activation of other OARs was shown to increase intracellular concentrations of both cAMP and calcium (reviewed by Roeder 1999). In addition, one type of OAR is thought to be coupled to Cl^- channels (Gerhardt et al. 1997b).

In recent years, several OARs have been cloned. These include two receptors in the pond snail *Lymnea stagnalis* (Gerhardt et al. 1997a, b) and one receptor in the sea-slugs *Aplysia californica* and *A. kurodai* (Chang et al. 2000). When expressed in HEK293 cells, activation of one of the *Lymnea* OARs (LymOA1) increased intracellular concentrations of both cAMP and inositol triphosphate (Gerhardt et al. 1997a). The second *Lymnea* OAR (LymOA2) had no effect on these second messenger concentrations but activated a voltage-insensitive Cl^- current (Gerhardt et al. 1997b). Activation of the *Aplysia* OAR (ApOA1) increased intracellular cAMP levels when expressed in HEK293 or Chinese hamster ovary cells (Chang et al. 2000).

OARs were also cloned from the fruit fly *Drosophila melanogaster* (Han et al. 1998), the honeybee *Apis mellifera* (Grohmann et al. 2003) and the cockroach *Periplaneta americana* (Bischof and Enan 2004). In *Drosophila*, the 637 amino acid OAR protein was first found in the mushroom bodies and named OAMB. However, it was later found in other *Drosophila* tissues including the thoracic ganglion, the female reproductive system and mature eggs in the ovary (Lee et al. 2003). When expressed in HEK293 cells, activation of this receptor induced an increase in both cAMP and intracellular calcium levels (Han et al.

1998). The honeybee OAR (AmOA1) is encoded by 587 amino acids. When this receptor was expressed in HEK293 cells, octopamine application increased intracellular calcium and cAMP concentrations. The cAMP increase was suggested to be a secondary effect induced by calcium release (Grohmann et al. 2003). The cockroach OAR (PaOA1) was found to be a 628 amino acid protein. When expressed in HEK293 cells, its activation increased intracellular levels of both cAMP and calcium. Here, the cAMP effect was shown to be independent of the increase in calcium level (Bischof and Enan 2004). Octopamine receptors from *Drosophila*, honeybee, cockroach and the LymOA1 of the pond snail are all closely related, with the greatest sequence conservation within their transmembrane domains (Grohmann et al. 2003; Bischof and Enan 2004).

1. 12. Physiological functions of octopamine

Octopamine acts upon numerous processes in invertebrate nervous and non-neural tissues (reviewed by Roeder 1999, 2002). In the CNS it controls the initiation and maintenance of different rhythmic behaviors, such as flight and running. It was also shown to be involved in memory, for example in mediating consolidation of olfactory memory in bees (Farooqui et al. 2003). Octopamine was also suggested to alter nicotinic receptor functions in the locust CNS, therefore decreasing cholinergic transmission between an afferent mechanosensory neuron and a motoneuron (Leitch et al. 2003). In the periphery, elevation of octopamine levels in the hemolymph within the first minutes of flight triggered the change from trehalose to fat as the primary energy source in locust (reviewed by Orchard et al. 1993). The power output of the flight muscles and the effectiveness of the heart were modulated as well (reviewed by Roeder 2002). In the locust reproductive system octopamine

was shown to increase the frequency of myogenic contractions and the amplitude of neurogenic contractions of the spermatheca (Clark and Lange 2003).

Many sensory functions are also modulated by octopamine. In the horseshoe crab *(Limulus polyphemus)* octopaminergic efferent neurons innervate all cell types in the compound and median eye as well as other photoreceptors (Battelle et al. 1999). These efferents have an endogenous circadian clock and they were shown to be responsible for the light adaptation of the eye. Other sensory processes, such as the olfactory response to pheromones in moth, were also shown to be enhanced by octopamine (reviewed by Roeder 1999).

There is significant evidence that mechanoreceptors in crustaceans and insects are modulated by octopamine. For example, the response of the lobster oval organ, a stretch-receptor that provides feedback from the ventilatory appendages to the CNS, was depressed by this monoamine (Pasztor and Bush 1987, 1989). However, in crayfish the sensory response of the same stretch receptor and of the muscle receptor organ was enhanced by octopamine (Pasztor and MacMillan 1990). The femoral chordotonal organs of the stick insect (Ramirez et al. 1993) and the locust (Matheson 1997) showed an increased activity of the position-sensitive (tonic) neurons but not of the movement-sensitive (phasic) neurons when exposed to octopamine. Octopamine also enhanced the response of the locust forewing stretch receptor involved in flight (Ramirez and Orchard 1990).

Except for the crustacean muscle receptor organ, none of the mechanosensory organs mentioned above was shown to have synaptic contacts on its peripheral parts. In insects, specialized dorsal and ventral unpaired median neurons (DUM and VUM neurons) are known to produce octopamine and are thought to release it into the circulation (Eckert et al.

1992; Achenbach et al. 1997; Roeder 1999). For example, some locust DUM neurons are activated during flight, and artificial stimulation of the same DUM neurons modulates the forewing stretch receptor in a manner similar to bath application of octopamine, suggesting that DUM neurons are involved in this modulatory process (Ramirez and Orchard 1990). DUM neurons are present in every ganglion of the ventral cord and are known to project efferent fibers to skeletal or visceral muscles (Bräunig and Eder 1998). However, it was also shown that a subgroup of DUM neurons in the third thoracic ganglion directly innervates a set of proprioceptors of the locust hindleg called the strand receptors (Bräunig and Eder 1998). Terminals of these DUM neurons were found in close proximity to the peripherally located dendrites of the mechanosensory neurons, whose cell bodies are located in the CNS. In this preparation, bath application of octopamine and stimulation of the appropriate DUM neuron increased the response of strand receptors to a mechanical stimulus, although in some cases the opposite effect was observed. These results suggest that DUM neurons may release octopamine locally, close to the peripherally located sensory endings, allowing a rapid and precise modulation of the sensory afferents.

1. 13. Objectives

The main objective of this thesis was to learn if the spider *C. salei* peripheral mechanosensory neurons and the efferent fibers that contact the sensory neurons are subject to cholinergic and/or octopaminergic modulation. Based on the previous evidence from ChAT and AChE labeling it seemed plausible that at least some of the spider mechanosensory neurons would have receptors for ACh and thus be able to respond to this transmitter. Since rapidly acting nicotinic ACh receptors have not been found in any

mechanosensory neurons other than the vertebrate cochlear hair cells, and given that there is ample evidence for muscarinic effects in several invertebrate mechanosensory neurons the main effort in the thesis was placed on investigations of a possible muscarinic system. I used specific fluorescent labeled antagonists against mAChRs and nAChRs to learn if these receptors were present in the spider mechanosensilla. To further investigate the presence of mAChRs I also performed immunocytochemical experiments with a specific antibody against these receptors. To investigate the physiological responses to cholinergic agonists I used both intra- and extracellular recording methods from two different mechanosensory organs in the spider leg, the VS-3 slit sensilla and the trichobothria sensilla.

Previous immunocytochemical evidence of octopaminergic neurons in the spider CNS and the well known role of octopamine as a modulator of mechanosensory afferents in many invertebrate species suggested that octopamine may serve as a modulator of the mechanosensory afferents in the spider *C. salei*. Therefore, I used an antibody against one of the recently cloned octopamine receptors to assess the presence of OARs in the spider mechanosensilla. I also carried out immunolabeling experiments with an antibody against octopamine to see if the octopamine-immunoreactive neurons have efferent fibers in the periphery, which would suggest a possibility for direct synaptic octopaminergic contacts on the peripheral parts of the sensory neurons. In addition, I performed extracellular recordings from trichobothria to learn the role of octopamine in the control of neuronal excitability of these mechanosensory neurons.

2. Materials and Methods

2. 1. Experimental animals

Central American wandering spiders (*Cupiennius salei*, Keys.) were kept in a laboratory colony at room temperature (22 ± 2°C). Newly hatched spiderlings were kept together in 4 liter glass jars and fed with fruit flies. After about 2 months the spiders were separated into individual containers and fed with crickets and cockroaches. All of the spider jars had about a 5 cm layer of moist soil on the bottom to maintain high humidity. Adult spiders (10 months or older) of both sexes were used for most experiments. However, female spiders were preferred for electrophysiological experiments. A laboratory colony of wild type Oregon-R *Drosophila melanogaster* was reared on a cornmeal diet at 22-25°C. Heads of adult flies were used for Western blot analysis.

2.2. Experimental preparations

For intracellular recordings, immunocytochemical and fluorescent probe experiments, spider legs were autotomized and dissected under spider saline containing (in mM): 223 NaCl, 6.8 KCl, 8 $CaCl_2$, 5.1 $MgCl_2$, 10 HEPES, and 3g/l of glucose, pH 7.8 (Höger et al. 1997). For immunocytochemical and fluorescent probe experiments "cuticular preparations" were initially used. The hairs were first removed from the patella and then the leg was split lengthwise. Muscles in the anterior part of the patella including the VS-3 organ were carefully removed leaving a thin membrane called the hypodermis, that contains the neurons, attached to the cuticle. For immunolabeling experiments of the tibial hair cells, the

tibia was shaved, then split lengthwise along the antero-posterior axis. The dorsal part of the tibia, containing the trichobothria hair sensilla, was then fixed. This allowed easier removal of the muscles without disruption of the hypodermis. Experimental protocols for immunocytochemistry and/or fluorescent probes were then performed. Once all steps of these protocols were complete, the hypodermis membranes were carefully detached from the cuticle and mounted on coverslips as described below.

For intracellular recordings, I used "hypodermis preparations". The hypodermis from the patella was detached from the cuticle and attached to a coverslip coated with 1 mg/ml poly-L-lysine (Sigma, Oakville, Ontario, Canada, P5899). In this preparation the mechanosensory neurons remained on top of the hypodermis membrane, allowing relatively easy penetration with the intracellular microelectrodes and pharmacological agents.

For octopamine immunocytochemistry and Western blot analysis, brains and/or peripheral tissue were taken from spiders sacrificed by deep CO_2 anesthesia following protocols approved by the Dalhousie University Committee on Laboratory Animals (00-297I).

2.3. Fluorescent probe experiments

Two fluorescent probes were used to detect ACh receptors on spider mechanosensilla: 200 nM Bodipy® 558/568 pirenzepine (Molecular Probes, Eugene, OR, B7437) in spider saline at pH 7.1 and 2 μg/ml Alexa Fluor 488 α-bungarotoxin (Molecular Probes, B13422) in spider saline at pH 8. For these experiments the cuticular preparations of the spider patella were incubated for 60 to 90 min in the probe, in the dark at room temperature. Following incubation, the preparations were washed three times with spider

saline. To improve the penetration of the fluorescent probes into the sensory neurons I performed five series of experiments using an enzymatic treatment prior to application of the probe, to detach the glial cells and efferent neurons from the sensory neurons. The cuticular preparations were treated with 1mg/ml EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid) (Sigma, E4378) in spider saline for 5 min followed by two rinses in normal saline. Preparations were then transferred into 1 mg/ml collagenase Type IV (Sigma, C5138) in spider saline at 37°C for 5 to 10 min, then rinsed three times in normal saline before incubation with the fluorescent probe.

Immediately after rinsing, the hypodermis was carefully detached from the cuticle and placed on a coated coverslip. The coverslip was inverted on a microscope slide (#0, VWR Scientific, West Chester, PA) and the neurons were observed under epifluorescence optics with an Axiovert 100 inverted microscope (Carl Zeiss, Oberkochen, Germany). Digital images were captured with an AxioCam (Carl Zeiss) camera and final images were enhanced using Adobe Photoshop 7 (Adobe Systems, San Jose, CA).

2.4. Western blot analysis

The brain tissue was removed from deeply anesthetized spiders by opening the prosoma ventrally and dissecting out the central nervous system. Tissue was rapidly frozen with liquid nitrogen and ground with a precooled mortar and pestle, dissolved in phosphate-buffered saline (PBS) and mixed by Vortex. PBS contained (in g/l): 8 NaCl, 0.2 KCl, 0.24 KH_2PO_4, 2.16 Na_2HPO_4 x $7H_2O$, pH 7.4. Laemmli sample buffer (Sigma, S3401) solution (1:1) and 4% protease inhibitor cocktail solution (Roche, Laval, Quebec, Canada, 1-607-

498) were added and the homogenate was kept frozen until used. The spider peripheral tissue was obtained from the hypodermis in the femur and patella. Membranes (16 to 24) were collected into a microtissue grinder tube (Fisher Scientific, Nepean, Ontario, Canada) on ice. The tissue was placed in Laemmli sample buffer and ground. Finally, 4% protease inhibitor cocktail was added.

The samples (5 to 30 µl each) were run on a SDS [sodium dodecyl sulfate]-acrylamide gel (Bio-Rad Laboratories, Mississauga, Ontario, Canada), and blotted to nitrocellulose membranes. Rainbow markers (BioRad, 161-0305) were used as molecular weight markers. The blot was incubated for 1 hour in blocking buffer which consisted of: Tris buffer saline [2-amino-2-(hydroxymethyl)-1,3-propanediol] (Roche, 604205), 1% Tween 20 (Fisher Scientific, 80107) and 5% skim milk powder, followed by overnight incubation with the primary antibody in blocking buffer at 4°C. Primary antibodies were: (1) M35 (Argene Biosoft, Varilhes, France, 10-217, 1:1,000 dilution), which is a monoclonal mouse antibody against mAChRs. (2) OAMB (a gift from Dr. K.-A. Han, Pennsylvania State University, PA; Han et al. 1998, 1:2,000 dilution), which is a polyclonal rabbit antibody against *Drosophila* octopamine receptors. The blot was then washed for 30 min in Tris buffer saline with 1% Tween 20, followed by incubation in a peroxidase-conjugated goat anti-mouse (Chemicon International, Temecula, CA, AP124P, 1:1,000 dilution) or an anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, 111-035-003, 1:300,000 dilution). Immunoreactive protein bands were visualized using an ECL plus chemiluminescent kit (Amersham Biosciences, Montreal, Quebec, Canada) according to the manufacturer's instructions. Briefly, the blot was incubated 5 min at room temperature in a mixture of the detection solutions A and B in a ratio of 40:1. The detection reagent was

then drained off and the blot was wrapped in SaranWrap before being placed in an X-ray film cassette for exposure of the autoradiography film.

2.5. Immunocytochemistry

2.5.1. M35 and $GABA_BR2$

Freshly dissected cuticular preparations were fixed in 4% paraformaldehyde (Fisher) in PBS for 10 min. After fixation, the preparations were rinsed three times for 10 min each in PBS. They were then incubated in 0.6% Triton X-100 (Sigma, T8787) in PBS for 1 h followed by 2 h in the blocking solution. The blocking solution consisted of: 5% normal goat serum (Gibco, Burlington, Ontario, Canada, 16210-064), 3% skim milk powder, 1% bovine serum albumin Fraction V (BSA) (Sigma, A9647) and 0.6% Triton X-100 in PBS. To detect the mAChRs the preparations were incubated in the M35 antibody (see Western blot analysis) at 1:200 dilution in the blocking solution, overnight at 4°C. For co-localization of mAChRs and $GABA_B$ receptors, I performed double labeling experiments using a guinea pig anti-$GABA_BR2$ antibody (Chemicon, AB5394) at 1:1,000 concentration simultaneously with the M35 antibody. After antibody incubation, the preparations were washed four times for 10 min each in 0.6% Triton X-100 in PBS followed by overnight incubation at 4°C, in the dark, in the corresponding secondary antibody in blocking solution. The secondary antibodies were a goat anti-mouse Alexa Fluor 488 (10 µg/ml) (Molecular Probes, A-11029) for the M35 and a goat anti-guinea pig CY-3 (1:1,000) (Jackson, 106-165-006) for the $GABA_BR2$. Tissue was then washed in 0.6% Triton X-100 in PBS in the dark, four times for 10 min each, followed by five 10 min rinses in PBS. The hypodermis membrane was then detached from the cuticle in the patella or the tibia and mounted on a microscope slide. The

mounting medium was prepared by dissolving 2.4 g Mowiol®40-88 (Sigma, 324590) to 6 g glycerol (BDH Inc., Toronto, Ontario, Canada). A volume of 6 ml of distilled water was then added and the solution was left for several hours at room temperature; 12 ml of 0.2 M Tris buffer (pH 8.5) was added and the medium was heated to 50°C for 10 min. It was then clarified by centrifugation at 5,000 x *g*. The mounting medium was then enriched with 2.5% DABCO [1,4-diazabicyclo(2,2,2)octane] (Sigma, D2522) to reduce fading.

For controls, the primary antibody M35 was omitted or replaced with mouse ascites fluid at the same concentration.

2.5.2. OAMB and synapsin

Cuticular preparations were fixed in 4% paraformaldehyde in PBS for 20 min, then washed three times for 10 min each in PBS. They were then incubated 1 h in 0.5% Triton X-100 in PBS, followed by 2 h incubation in blocking solution (5% normal goat serum, 3% skim milk powder, 1% BSA, 0.1% Triton X-100 in PBS). The primary antibodies were dissolved in the blocking solution and the preparations were incubated overnight at 4°C in this solution. Primary antibodies that were used were the OAMB antibody (see Western blot analysis) in 1:1,000 dilution and a monoclonal mouse anti-synapsin 1:100 (SYNORF1, a gift from Dr. E. Buchner, Universität Würzburg, Germany; Klagges et al. 1996). After antibody incubation the preparations were washed four times 10 min each in 0.1% Triton X-100 in PBS followed by overnight incubation at 4°C, in the dark, in the corresponding secondary antibody in blocking solution. Secondary antibodies were a goat anti-rabbit CY-3 (1:600) (Jackson 111-165-003) for the OAMB antibody and a goat anti-mouse Alexa Fluor 488 (10 µg/ml) (Molecular Probes, A-11029) for the anti-synapsin antibody. Preparations were

then washed four times 10 min each in 0.1% Triton X-100 in PBS, followed by five rinses for 10 min each in PBS. The hypodermis was then removed from the cuticle and mounted in Mowiol as described above.

For controls, the primary OAMB antibody was omitted or replaced with normal rabbit serum at the same concentration.

2.5.3. Octopamine

The tips of the legs of a deeply anaesthetized spider were cut off and the whole animal was fixed by perfusion through the heart. The fixative in these experiments consisted of 4% EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Sigma, E6383) in PBS. The legs were then removed, slit ventrally along the middle of each segment and placed into the same fixative for 2 h at 4°C. After four times 10 min washes in PBS, the legs were dissected to obtain cuticular preparations, then placed for 30 min in 0.5% Triton X-100 in PBS followed by 1 hour incubation in the same blocking solution as described in the previous section. Preparations were then incubated overnight at 4°C in a rabbit anti-octopamine primary antibody (Chemicon, AB1799) in blocking solution at concentrations of 1:500 or 1:1,000. Primary antibody incubation was followed by four 10 min washes in 0.1% Triton X-100 in PBS and the preparations were then incubated in the secondary antibody in blocking solution overnight at 4°C in the dark. The secondary antibody was a goat anti-rabbit Alexa Fluor 488 (Molecular Probes, A-11034) and it was used at a dilution of 10 μg/ml. The preparations were then washed four times 10 min in 0.1% Triton X-100 in PBS followed by five washes in PBS for 10 min each. The hypodermis preparations were mounted on a microscope slide in Mowiol.

For controls, the primary antibody was omitted or replaced with normal rabbit serum at the same concentrations.

The mounted preparations were examined under epifluorescence optics with an Axiovert 100 inverted microscope (Carl Zeiss) and under a laser scanning confocal microscope (LSM 510, Carl Zeiss) with an argon-krypton laser for Alexa Fluor 488 (488 nm) and a helium-neon laser for CY-3 (543 nm). Digital images were captured and analyzed, and the final images were prepared using Adobe Photoshop 7.

2.6. Electrophysiology

All electrophysiological experiments were performed on a gas-driven vibration isolation table (Technical Manufacturing, Peabody, MA) inside a Faraday cage.

2.6.1. Intracellular recordings

The coverslip carrying the hypodermis preparation was placed in a recording chamber and superfused with spider saline. A small vacuum pump attached to a running tap kept the saline level low. Drugs were applied manually with a syringe and needle or with a BPS-4 perfusion system and PR10 pressure regulator (ALA Scientific, New York, NY). Voltage- and current-clamp experiments were performed using the discontinuous single-electrode method (Finkel and Redman 1984) with an SEC-10L amplifier (NPI Electronic, Tamm, Germany). Borosilicate microelectrodes (Hilgenberg, Malsfeld, Germany; outer diameter of 1 mm, inner diameter of 0.5 mm) were pulled with a P-2000 horizontal puller (Sutter Instrument, Novato, CA). Microelectrodes were filled with 3 M KCl and their resistances were 40 to 80 MΩ in solution.

Switching frequencies of 20-25 kHz and a duty cycle of 1/4 (current passing/voltage recording) were used in all experiments. The neurons were observed with an upright microscope under bright-field optics (Axioskop 2 FS, Carl Zeiss). Microelectrodes were positioned with a micromanipulator (Burleigh Instruments, Victor, NY). The neurons were impaled by high-frequency oscillation ("buzzing") and allowed to stabilize for 15 min before the start of experiments. All experiments were controlled by an IBM-compatible computer with custom-written software via 16-bit analog-to-digital and 12-bit digital-to-analog converters (National Instruments, Austin, TX). The computer provided electrical stimulation and recorded current and voltage with sampling rates of up to 10 kHz. The membrane potential was low-pass filtered at 33.3 kHz and the current at 3.3 kHz by the voltage-clamp amplifier.

2.6.2. Extracellular recordings

A whole autotomized spider leg was fixed with beeswax onto a Styrofoam support. The dorsal side was placed upwards to expose the trichobothria sensilla on the proximal part of the tibia. The tip of the femur was closed with a beeswax plug. A hypodermic needle attached to plastic tubing (outer diameter 3 mm) was inserted in the side of the femur and fixed with beeswax (Fig. 4). A piece of cuticle was removed from the distal end of the tibia and the leg was perfused with spider saline via the tubing connected to a 20 ml syringe. A piece of paper tissue was used to direct the outflow of saline away from the tibia to a beaker placed under the preparation. To help the saline flow through the leg, the system was pressurized with a small air pump (Elite 799, Hagen, Montreal, Quebec, Canada). Smaller tubing (outer diameter 1mm) was inserted through the 3 mm tubing all the way to the

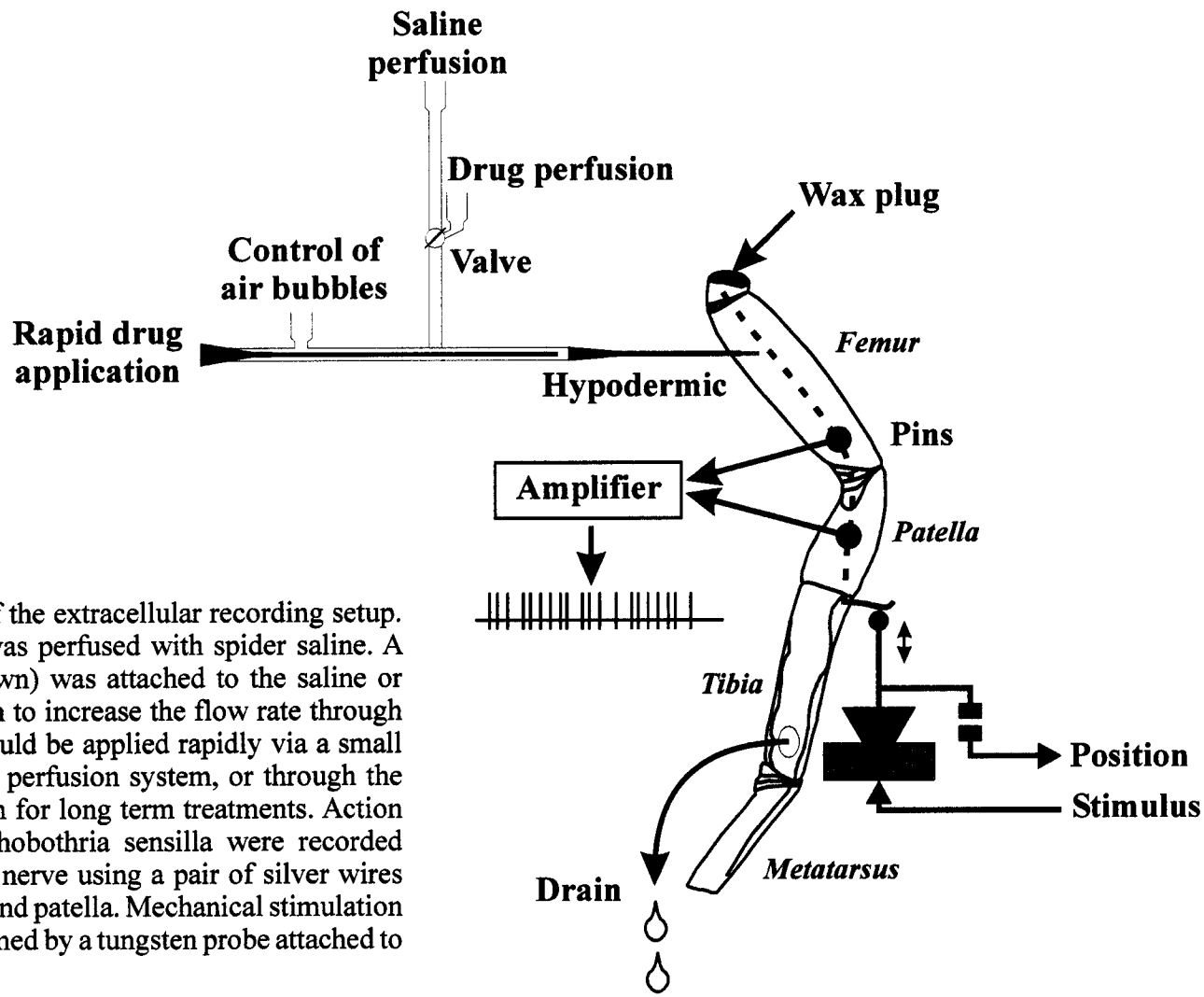

Figure 4:

Schematic diagram of the extracellular recording setup. A whole spider leg was perfused with spider saline. A small pump (not shown) was attached to the saline or drug perfusion system to increase the flow rate through the leg. Chemicals could be applied rapidly via a small tube inside the saline perfusion system, or through the drug perfusion system for long term treatments. Action potentials from thrichobothria sensilla were recorded through the main leg nerve using a pair of silver wires inserted in the femur and patella. Mechanical stimulation of the hair was performed by a tungsten probe attached to a loudspeaker.

hypodermic needle and connected to a syringe (Fig. 4). This inner tube was used for fast application of drugs. Drugs usually reached the target tissues within two or three seconds. To avoid build up of pressure during drug application, the air pump was disconnected from the system and reattached as soon as the application was finished.

A manual valve in the saline perfusion tube allowed replacement of normal spider saline with pharmacological agents that were needed for long term treatment of the preparation. This system was used to apply receptor or second-messenger system inhibitors. A fourth syringe could be attached to the main perfusion tube and was used mainly to remove air bubbles before they could enter the spider leg (Fig. 4).

Once the perfusion system was connected to the leg, hairs were removed from two areas on top of the femur and patella. Small amounts of petroleum jelly were applied to these areas, the perfusion system was turned off briefly, and two holes were pierced in the cuticle with a small needle. Silver wires (0.25 mm diameter, World Precision Instruments, Sarasota, FL) were inserted into the holes and fixed in place with a drop of beeswax. The perfusion system was immediately started again and the Styrofoam holder was placed on top of a 15 cm high column mounted on the vibration isolation table. The silver wires were connected to an AC amplifier (P55, Grass Instruments, West Warwick, RI) that allowed consistent recordings of action potentials (AP) from the main leg nerve. The signal was amplified 10,000 times, and low-pass filtered at 1 kHz by the amplifier.

Mechanical stimulation of the hair was achieved by a custom-built apparatus consisting of a tungsten wire (0.25 mm diameter, A-M Systems, Carlsborg, WA) mounted on a small (5 cm diameter) loudspeaker. The trichobothria sensilla could be observed with a dissecting microscope (SZ60, Olympus, Tokyo, Japan). One sensillum responsive to air

movements was chosen, and hairs surrounding it were carefully plucked with small forceps. The stimulator was positioned using a micromanipulator (ALA Scientific) and the tungsten probe was attached to the sensillum with petroleum jelly. Stimulator position was detected by a matched infrared LED-photodiode pair, which also provided second-order servo position control. The stimulus consisted of pseudorandom Gaussian white noise, generated by the computer via a 33-bit binary sequence algorithm, with a maximum amplitude of approximately 50 μm.

Action potentials (sampled with a time resolution of 100μs) were separated from the background noise by an algorithm, as described in French et al. (2001). The algorithm identified action potentials as an increase followed by a decrease of voltage with fixed amplitude within 1.5 ms. To ensure that the algorithm functioned properly, all separations were inspected visually. Action potential signals were digitally filtered by convolution with a $\sin(x)/x$ function to band-limit them to the range 0-500 Hz and produce a regularly sampled signal with 1 ms interval. Sampled mechanical displacement was digitally re-sampled by adjacent point averaging to give a 1 ms sample interval. The sampled signals were transferred to the frequency domain using the fast Fourier transform (Cooley and Tukey 1965) in segments of 512 sample pairs. Frequency response functions (gain and phase) between the input and the output were calculated by direct spectral estimation and plotted as Bode plots of phase and log gain versus log frequency. Coherence functions (Bendat and Piersol 1980) were calculated from the same data and plotted versus log frequency. All experiments were controlled by an IBM-compatible computer with custom-written software via 16-bit analog-to-digital and 12-bit digital-to-analog converters (National Instruments).

2.6.3. Chemicals

All chemicals were purchased from Sigma unless otherwise indicated. Drugs were initially dissolved in spider saline, aliquoted and kept frozen until shortly before the experiment. The following chemicals were used: ACh (Acetylcholine chloride, A6625), Muscarine (Muscarine chloride, M104), Nicotine (Nicotine hydrogen tartrate salt, N5260), Oxotremorine M (Oxotremorine methiodide, O100), Octopamine (DL-Octopamine hydrochloride, O0250), Tyramine (Tyramine hydrochloride, T2879), Phentolamine (Phentolamine methanesulfonate salt, P131), 8-Br-cAMP (8-Bromoadenosine-3',5'-cyclic monophosphate sodium salt, B7880) and Rp-cAMPS (Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer; Biolog Life Science Institute, Bremen, Germany, A002-05T).

2. 7. Statistical analysis

Statistical analyses were performed with the Prophet 6.0 software (AbTech Corporation, Charlottesville, VA). To test the effects of different cholinergic agonists on the frequency response parameters, I used the Mann-Whitney rank sum test and compared test values with control values at a given time. I also used a two-sample paired Wilcoxon signed rank test to compare differences over time in a single treatment category. To test the frequency response parameters following application of octopamine, I used a two-sample paired *t*-test.

3. Results

3. 1. Acetylcholine receptors on spider mechanosensilla

3.1.1. Visualization of ACh receptors with fluorescent probes

I initially assessed whether ACh receptors were present on spider peripheral mechanosensilla using fluorescently labeled ACh receptor antagonists as probes. This technique allowed me to test the binding of fluorescence labeled analogs of the nicotinic AChR antagonist, α-bungarotoxin, and the mAChR blocker, pirenzepine, on unfixed hypodermis preparations of spider patella.

Fifteen preparations with the α-bungarotoxin probe produced no positive binding, with or without collagenase pre-incubation, suggesting that nicotinic AChRs are not present in the spider mechanosensilla or leg nerves. However, clear punctate labeling was seen in the leg nerves when the pirenzepine probe was used (Fig. 5). The somata of the mechanosensory neurons are enwrapped by glial cells and efferent nerve fibers, making it difficult to visualize the probe binding in this area. To allow better visibility and to improve penetration of the fluorescent probe, 40 preparations were incubated in Type IV collagenase for 5 to 15 min prior to application of the pirenzepine probe. In this situation the binding was still clear in the nerve and punctate labeling was also observed in some VS-3 cell bodies (Fig. 6). However, strong autofluorescence was seen in the peripheral regions of the cell bodies. This fluorescence was also visible when the preparations were tested with 488 nm excitation wavelength (Fig. 6B) when the specific labeling was not seen. This autofluorescence was previously shown to be caused by lipofuscin granules that accumulate

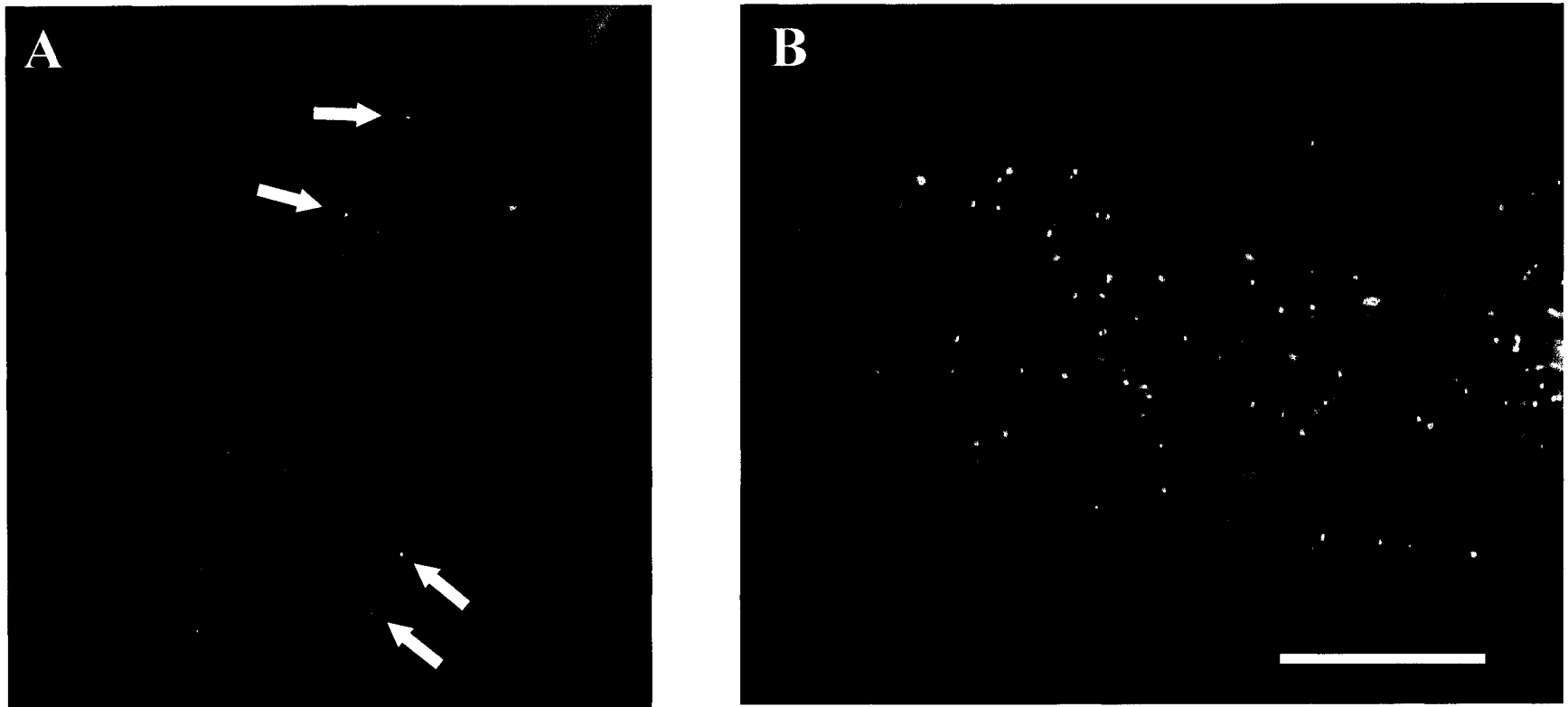

Figure 5:

Fluorescence labeled mAChR antagonist pirenzepine binding to the nerve in the spider patellar hypodermis.
A and *B*:The leg nerve on the spider patella incubated in 200 nM Bodipy® 558/568 pirenzepine probe displayed clear punctate staining (arrows). Scale bars: 20 μm.

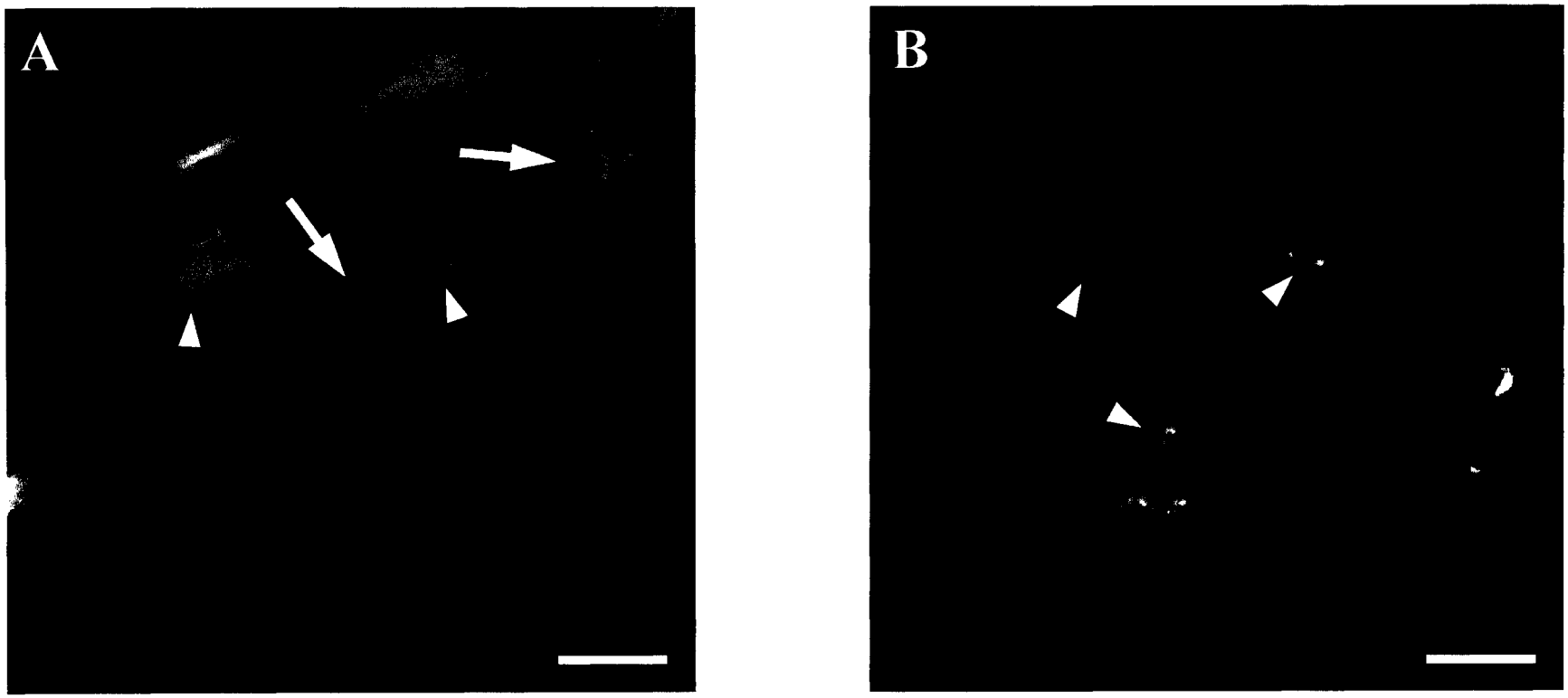

Figure 6:

Binding of the fluorescence labeled mAChR antagonist pirenzepine probe on collagenase treated VS-3 neuron cell bodies.
A: The preparations were incubated in 200 nM Bodipy® 558/568 pirenzepine probe. Punctate staining was visible in the somata (arrows). However, strong autofluorescence was observed in the peripheral areas of the neuronal cell bodies (arrowheads). *B:* The unspecific fluorescence was also seen with an excitation wavelength of 488 nm where no specific binding was visible. This autofluorescence was due to the lipofuscin granules that accumulate in the neurons (Fabian and Seyfarth 1997). Scale bars: 20 µm.

in the cell bodies of adult spiders (Fabian and Seyfarth 1997). These results suggest that mAChRs are present on the somata of the mechanosensory neurons as well as in the leg nerves. Since the leg nerves include afferent and efferent fibers as well as glial cells that can not be positively identified in unfixed whole-mount preparations it is not possible to identify which one of these cell types expressed the specific binding.

3.1.2. mAChR immunolabeling with M35 antibody

To further test if mACh receptors are expressed in the spider mechanosensilla I used a monoclonal antibody that was previously shown to bind to a common subunit in all five subtypes of vertebrate mAChRs (Carsi-Gabrenas et al. 1997). This antibody (M35) has been widely used to label vertebrate mAChRs (reviewed by Van der Zee and Luiten 1999) and it was also successfully used in some invertebrate preparations, both in Western blot analysis and in immunocytochemistry (Lammerding-Köppel et al. 1994; Wegener et al. 1996; Torkkeli et al. 2004). To assess the specificity of M35 in invertebrate tissue, a Western blot analysis was performed using extracts of *Drosophila* heads and spider brains. In addition, I used spider peripheral nervous tissue extracts from the hypodermis in the femur and patella. This antibody was also used in immunocytochemical analysis to see if M35 produced specific labeling in the spider mechanosensilla.

A. Western blot analysis

The M35 antibody labeled a clear band in all three tissues at about 75 kDa (Fig. 7), which is in the range of molecular weights (66 to 85 kDa) previously reported for mAChRs

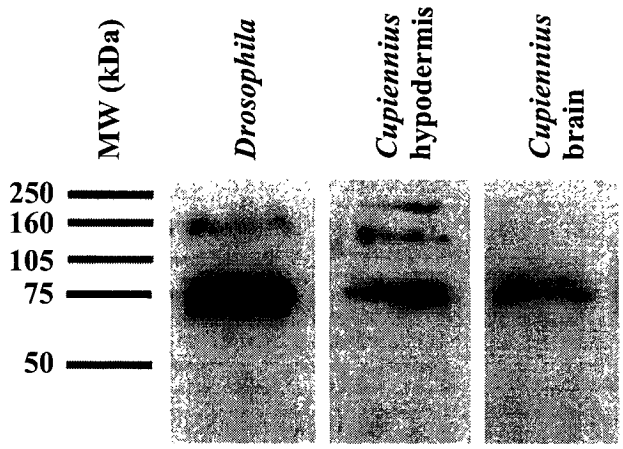

Figure 7:

Western blot analysis of mAChR proteins with M35 antibody.
M35 revealed a distinct band at ~75 kDa in *Drosophila* and spider tissue extracts. Fainter bands were also seen in extracts of the *Drosophila* head and the spider hypodermis, but not in the brain homogenates.

in different invertebrate species (Hwang et al. 1999; Torkkeli et al. 2004). Fainter bands could be seen at ~145 kDa for *Drosophila* head extracts and ~135 kDa and ~200 kDa for spider hypodermis homogenates. These could be due to receptor glycosylation or unspecific labeling, in particular in the hypodermis tissue where the total amount of protein was very low. However, G-protein coupled receptors have also been shown to form dimers (Bouvier 2001) and studies have demonstrated dimerisation of muscarinic receptors in Western blot analysis, at least under non-reducing conditions (Zeng and Wess 1999). It is possible that the band seen at 145 kDa represents a dimeric form of the protein, while the 135 kDa band could be a partial degradation product of the dimer.

B. Immunocytochemistry

In all patellar and tibial preparations tested, mechanosensory neurons of the VS-3 organs and tactile hairs showed strong immunoreactivity against the M35 antibody (Fig. 8A-C and F). Both neurons of each pair in the VS-3 organ and all three neurons that innervate the tactile hairs were identically labeled. M35 immunoreactivity was most intense in the sensory neurons and 1 μm optical sections through the somata suggested that it was present in the cell bodies of these neurons. Thin glial cells and fine efferent fibers surround all parts of the sensory neurons and could not be distinguished from each other, or from sensory neurons, even with the optical sectioning. It is possible that one or both of these structures were also immunoreactive against the M35 antibody. The nerve that leads to the VS-3 organ and other leg sensilla (Fig. 8B and F) was extensively labeled by the M35 antibody, suggesting that other structures in addition to the sensory axons were immunoreactive.

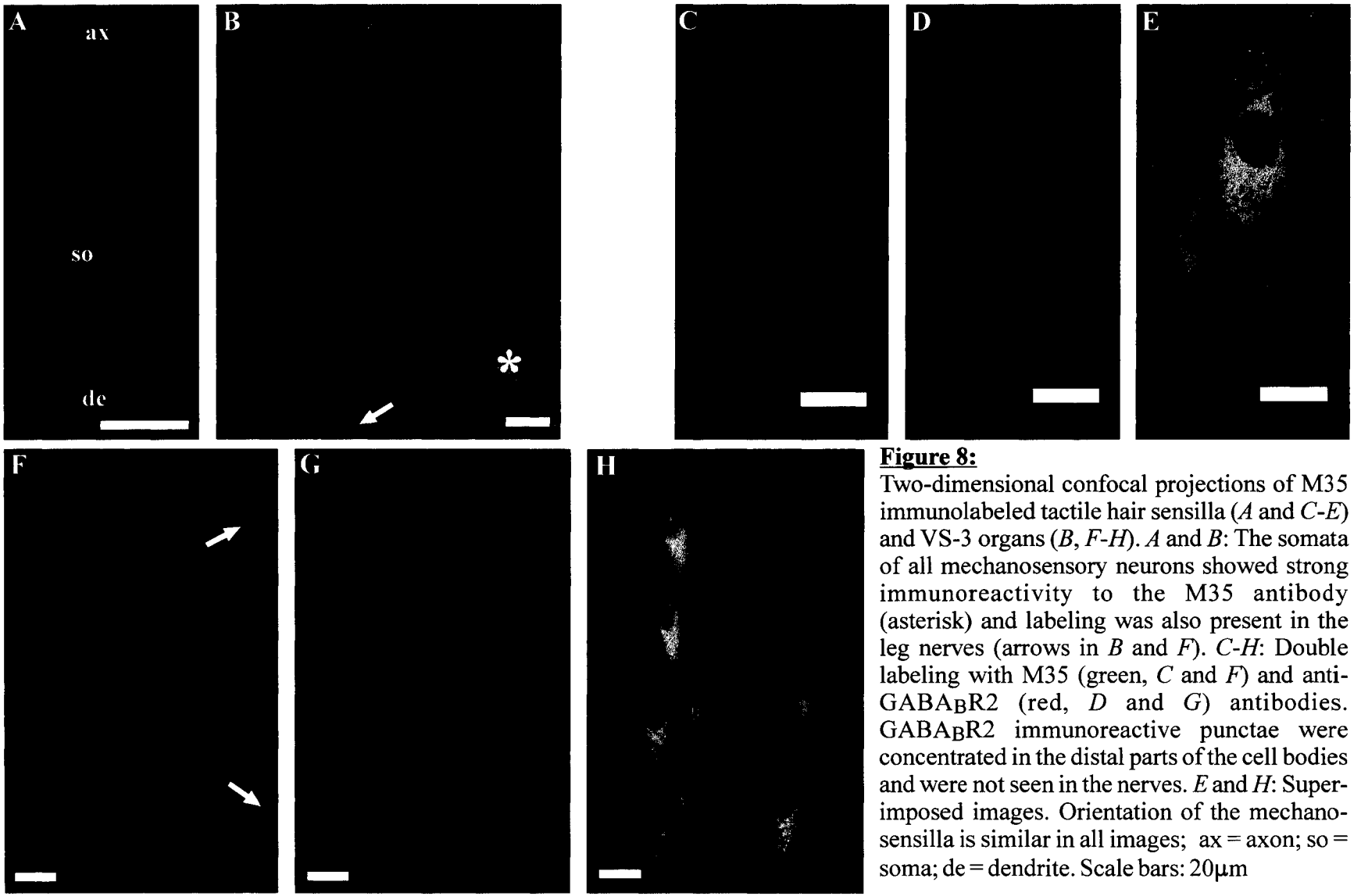

Figure 8:
Two-dimensional confocal projections of M35 immunolabeled tactile hair sensilla (*A* and *C-E*) and VS-3 organs (*B*, *F-H*). *A* and *B*: The somata of all mechanosensory neurons showed strong immunoreactivity to the M35 antibody (asterisk) and labeling was also present in the leg nerves (arrows in *B* and *F*). *C-H*: Double labeling with M35 (green, *C* and *F*) and anti-$GABA_BR2$ (red, *D* and *G*) antibodies. $GABA_BR2$ immunoreactive punctae were concentrated in the distal parts of the cell bodies and were not seen in the nerves. *E* and *H*: Superimposed images. Orientation of the mechanosensilla is similar in all images; ax = axon; so = soma; de = dendrite. Scale bars: 20μm

Control preparations in which the primary antibody was omitted or replaced by ascites fluid at the same concentration showed no labeling.

$GABA_B$ receptors have previously been shown to be present in spider mechanosensilla, and an antibody against the R2 subunit of these receptors specifically labeled the most distal parts of the sensory neuron cell bodies (Panek et al. 2003) . The same antibody was used here to confirm that the M35 immunoreactivity was co-localized in the same areas of the cell bodies. I performed double labeling experiments with the anti-$GABA_BR2$ antibody and the M35 antibody. $GABA_BR2$ immunoreactivity was only seen in the distal parts of the cell bodies and co-localized with the M35 labeling in optical sections through these parts (Fig. 8C-H).

The immunocytochemical observations, along with the fluorescent probe experiments, indicate that mAChRs are present in all mechanosensory neurons located in the spider patella and tibia. The extent of immunolabeling suggests that these receptors may also be present in the efferent fibers and/or glial cells that surround the sensory neurons.

3.1.3. Intracellular recordings from the spider VS-3 neurons

To investigate the possible role(s) of the mAChRs in regulating the excitability of mechanosensory neurons, I initially used sharp electrode intracellular voltage- and current-clamp recording techniques on hypodermis preparations of the VS-3 organ.

A. Voltage-clamp experiments

Single-electrode voltage-clamp experiments were performed to observe if agonists of ACh receptors produced currents in the VS-3 neurons. Agonist concentrations varied from

1 to 5 mM, resulting in estimated bath concentrations of 5 to 100 μM. Fifty-five experiments on 20 different cells were performed using acetylcholine as an agonist at holding potentials from -90 mV to +10 mV. In 12 experiments from two different cells I used muscarine as an agonist at holding potentials between -120 mV and -50 mV and in 12 experiments on four different cells nicotine was used as an agonist at holding potentials of -90 mV to -30 mV. To check that the drug application method functioned appropriately, I also applied GABA to the superfusion solution in some experiments. GABA has previously been shown to elicit large inward currents at holding potentials at or below -40 mV (Panek et al. 2002) and here it induced a similar large and long-lasting inward current indicating that the application system was functioning properly.

Although a small number of experiments produced a transient change in the membrane current in response to cholinergic agonists (Fig. 9A), no consistent currents were seen in the VS-3 neurons under voltage-clamp. The results of these experiments are summarized in figure 9 B. It shows that no responses were usually recorded and that in those cases where there was a change, it occurred in either the positive or negative direction. The fact that nicotine or ACh did not consistently produce currents further suggests that nicotinic AChRs are not present in the spider VS-3 neurons. Responses of muscarinic AChRs are more variable and these results do not rule out an effect of muscarinic agonists on the sensory neurons. However, it is likely that the changes that were recorded in 17 of 79 voltage-clamp experiments were artefactual and may have been caused by disturbance of the bath surface during application of the agonists. Control experiments where a drop of normal saline was used instead of agonists occasionally caused similar changes.

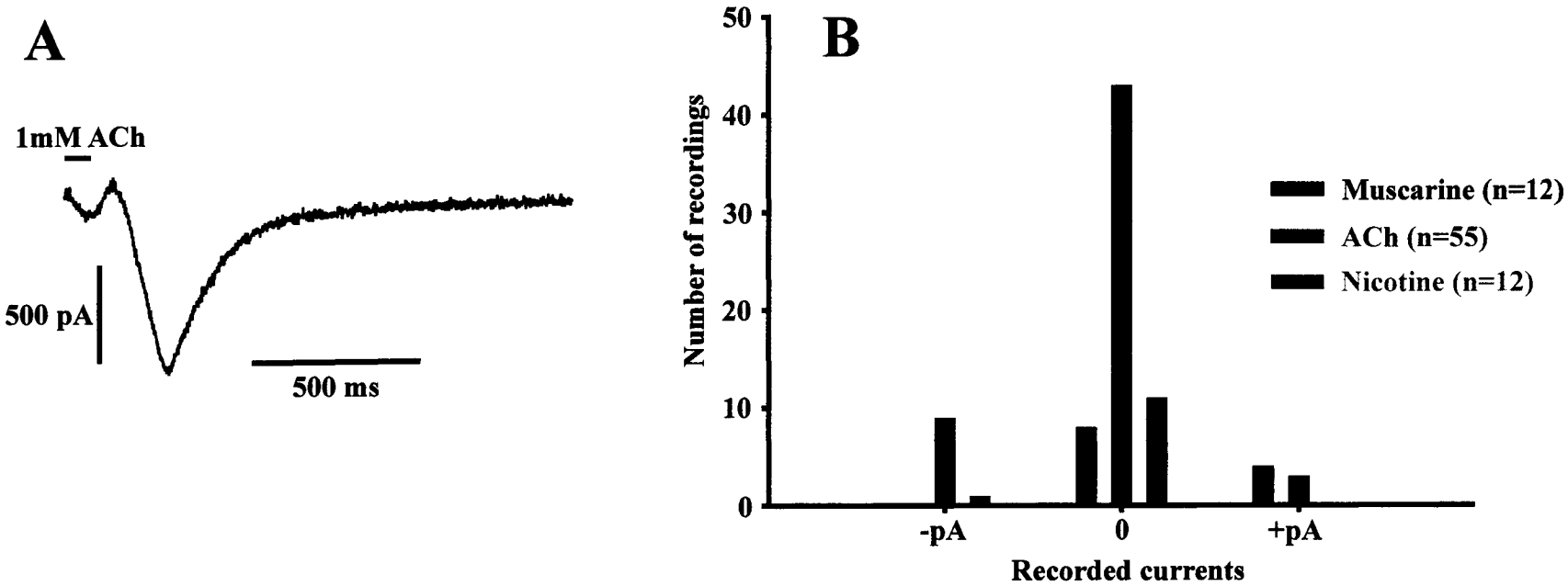

Figure 9:

Effect of cholinergic agonists on mechanosensory neurons of the VS-3 organ under voltage-clamp.
A: One of the 10 recordings showing an inward current following application of one drop of 1 mM ACh in the bath solution (holding potential -30 mV). *B*: Summary of observations following application of muscarine (black), ACh (red) or nicotine (green). In most cases no currents were recorded (62 experiments), only 10 negative and 7 positive currents were observed and showed no relationship to the holding potential at which the experiment was conducted. A total of 26 cells were tested at holding potentials of -120 mV to +10 mV.

B. Current-clamp experiments

I performed current-clamp experiments to further assess whether muscarinic agonists had an effect on the excitability of mechanosensory neurons in the VS-3 organ. The current-pulse protocol consisted of eight 200 ms pulses from -0.5 nA to 1.5 nA in 0.25 nA increments. The parameters that were recorded from these experiments were: 1) The membrane resistance that was estimated from voltage elicited with the -0.25 nA pulse; 2) the spiking threshold indicating the amount of current required to produce an action potential; 3) the maximum number of spikes elicited by the current pulses; 4) the level of steady membrane depolarization (=baseline depolarization) for positive current pulses; 5) the resting membrane potential; 6) the type of neurons (Type A, single-spiking neuron or Type B, multiple-spiking neuron).

Agonists were applied after a control recording in each experiment and a series of recordings were performed during 20 min after drug application followed by a washing period with spider saline. Agonists used were 100 μM to 5 mM ACh and 1 to 5 mM oxotremorine, a muscarinic agonist. Both were applied either by hand or using a pressurized perfusion system. Various volumes were tested, from 10 μl to 3 ml for ACh and from 0.1 to 1 ml for oxotremorine.

ACh was tested with 29 cells and oxotremorine with 13 cells. Eight of these cells were Type A neurons, nine were Type B neurons and in 25 cases the cell type could not be positively identified. There were no clear differences between the results with the two agonists and the results are pooled together in Table 1. This table shows that no differences between the two cell types could be seen in any of these parameters. In most experiments the threshold for eliciting action potentials increased by 0.25 nA or remained unchanged,

Table 1:

Summary of changes observed in current-clamp recordings following application of mAChR agonists. 29 cells were tested using ACh and 13 using oxotremorine. AP Threshold = the smallest current pulse that produced an action potential; Max # of AP = the maximum number of action potentials; R_{in} = the membrane resistance; Baseline depl. = the level of steady membrane depolarization for positive current pulses; E_m = the resting membrane potential (increase = depolarization and decrease=hyperpolarization) ; Type A = single-spiking neurons; Type B = multiple-spiking neuron; ? = type not known.

(n=42)	Increase				Decrease				No change			
	TypeA	Type B	?	**Total**	TypeA	Type B	?	**Total**	TypeA	Type B	?	**Total**
AP Threshold	5	3	10	**18**	1	3	2	**6**	2	3	13	**18**
Max # of AP	2	5	1	**8**	0	1	5	**6**	6	3	19	**28**
R_{in}	6	7	14	**27**	2	1	8	**11**	0	1	3	**4**
Baseline depol.	6	6	10	**22**	0	1	4	**5**	2	2	11	**15**
E_m	1	1	6	**8**	2	2	5	**9**	5	5	15	**25**

although in a small number of experiments there was a decrease. The maximum number of action potentials usually remained unchanged but small changes in both directions occurred in some experiments. Figure 10 A shows an example of a Type B neuron where application of 100 μM ACh decreased the firing rate.

The clearest change was in the membrane resistance, which increased in 27 experiments by an average of 38.6 ± 38.4 MΩ (mean ± s.d.) suggesting that the membrane conductance decreased after agonist application. In 17 cases this resistance increase was accompanied by an increase in baseline membrane depolarization. This effect was partly reversible in only six cases, five of these following oxotremorine application. In most cases the membrane resistance increase did not reverse and may have been caused by slight clogging of the electrode during the experiments. In a smaller number of experiments there was no change in either of these two parameters or both of them decreased. An example of the latter is shown in figure 10 B.

The neurons were held at their resting membrane potentials (about -70 mV) during these experiments. In eight recordings the membrane potential depolarized slightly and in four cases this change was reversible. In nine experiments the membrane hyperpolarized, three of these changes being reversible. In 25 experiments there was no change in the membrane potential. These results did not indicate that there was any repeatable response to muscarinic agonists in the VS-3 neurons, but suggested that either the effect was very small and only occurred in a subgroup of the neurons or that the mAChRs had a more long-term effect on cellular excitability.

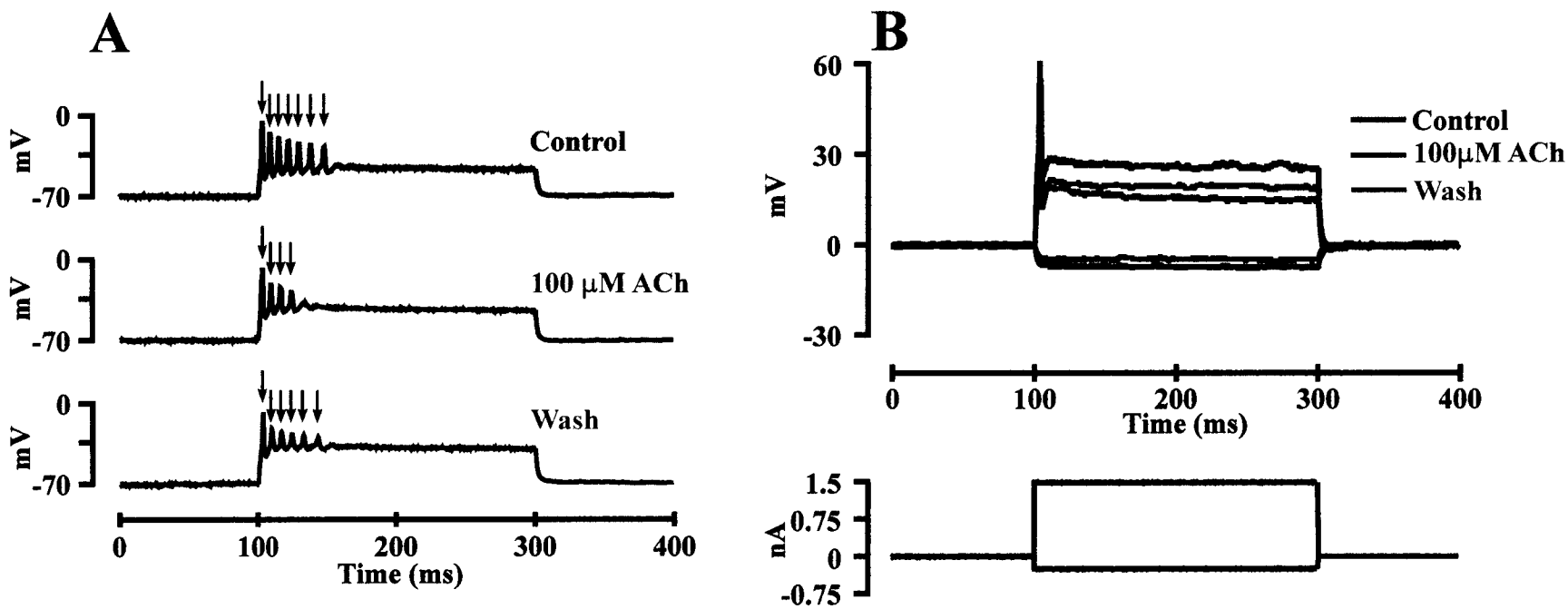

Figure 10:

Current-clamp recordings from VS-3 organ.
A: A Type B neuron responded to 1 nA current pulse by firing 7 action potentials (black). After application of ACh, the maximum number of action potentials decreased to 4 (red) and stayed lower as long as the drug was present in the bath. When the agonist was washed out, the number of action potentials increased again (green). *B*: Responses to current pulses of -0.25 nA and 1.5 nA. After application of 100 μM ACh (red) the membrane resistance decreased slightly, and the baseline depolarization decreased more sharply. When the drug was washed out, the effect reversed partially. The resting potential was -66 mV. It was subtracted from each trace to allow comparison.

3.1.4. Extracellular frequency response recordings from spider trichobothria sensilla

Since mAChRs might have relatively slow effects on mechanotransduction or excitability, a new extracellular recording setup was developed to allow longer-lasting recordings using mechanical rather than electrical stimulation (Fig. 4). A whole spider leg was perfused with saline and silver wires were inserted into the femur and patella to record action potentials propagating along the main leg nerve. This arrangement allowed recordings from the trichobothria sensilla that are located on the proximal part of the tibia (Fig. 11A). Agonists were applied through leg perfusion and their effects on the firing frequency were observed. The firing frequency was calculated in impulses/s using bins of 1 s. In all recordings, the firing frequency displayed a sharp decrease during the first 60 s of stimulation, before reaching a plateau (Fig. 11B). This is due to a normal adaptation process, the mechanisms of which are not yet known. Six cells were tested with ACh, seven with muscarine and 32 with oxotremorine, but there were no changes in the firing rate in any of these experiments.

To assess whether mAChR agonists had a slower, longer-lasting effect on the dynamic behavior or the information transfer rate of the mechanosensory neurons, I performed frequency response analysis. With this method, the neuron is considered as a linear system with an input, the stimulus, and an output, the action potentials. If the input is a sinusoid at a given frequency, the output will be a sinusoid with the same frequency but a different amplitude and phase. This change in amplitude and phase varies with the input frequency and can be plotted on a Bode plot as gain (the change in amplitude) and phase against each input frequency tested (Fig. 12).

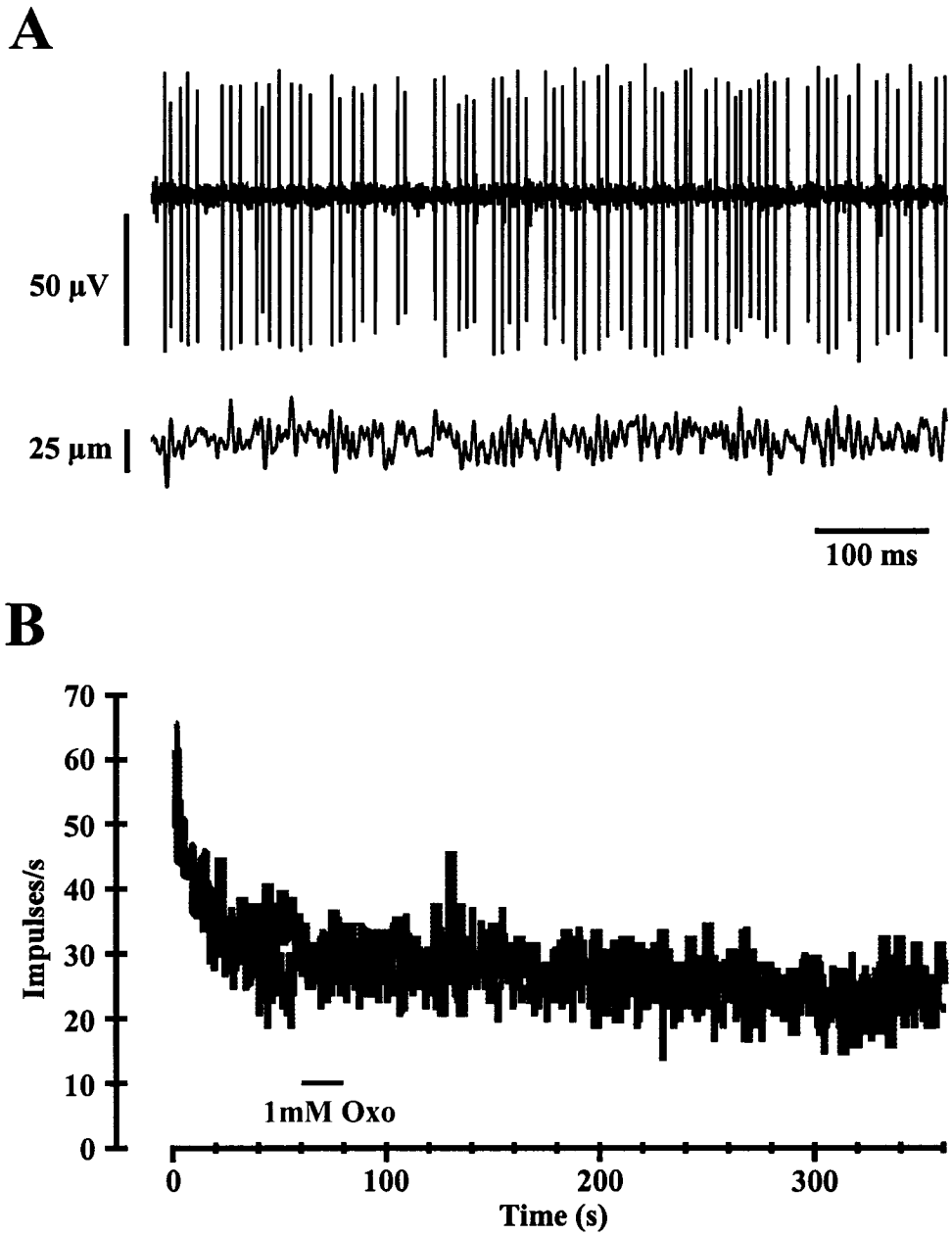

Figure 11:

Extracellular recordings from the mechanosensory neurons innervating trichobothria sensilla.
A: Typical recording showing the mechanical stimulation (bottom) of a trichobothrium and the resulting action potentials (top) recorded extracellularly from the main leg nerve. *B*: The firing frequency of a neuron (in Impulses/s) was calculated using 1 s wide bins. The green trace shows a control in which no agonist was applied. The frequency decreased sharply over the first 60s of the recording due to adaptation. When the muscarinic agonist oxotremorine (Oxo) was applied (blue), the firing frequency did not change.

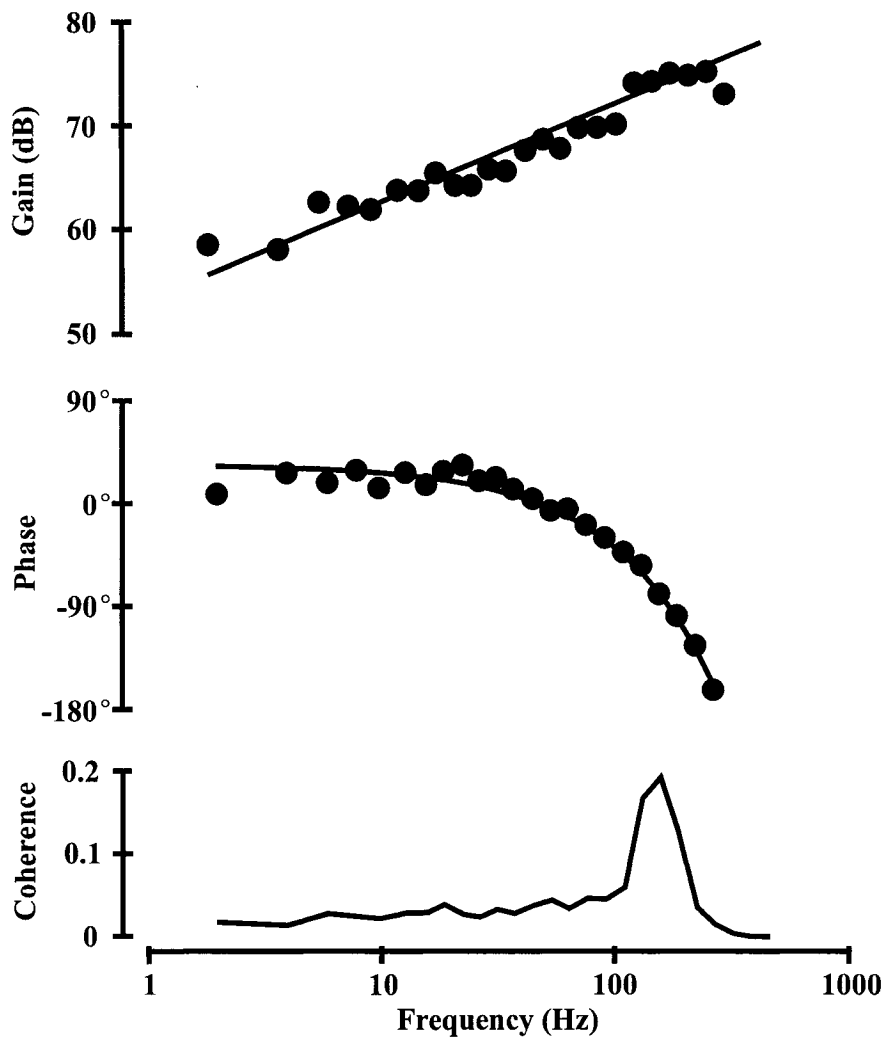

Figure 12:

Frequency response of a mechanosensory neuron innervating a trichobothrium.
Bode plot showing the gain, phase and coherence obtained by stimulating a trichobothrium neuron with pseudorandom white-noise displacement. The gain was fitted with a power law function and the coherence was used to calculate the information capacity of the neuron. The fitted parameters of the power law function and the information capacity were used to assess the effect of mAChR agonists on the trichobothria neurons.

In many sensory receptors the gain can be fitted by the power law relationship:

$$G(f) = Af^{k} \qquad \text{(Equation 3 - 1)}$$

where f is the frequency and A and k are fitted parameters describing the sensitivity and rate of adaptation of the neuron, respectively (Thorson and Biedermann-Thorson 1974). Preliminary experiments showed this to be true for the spider trichobothria receptors. The coherence function, $\gamma^2(f)$, was also calculated. It provides a measure of the linear correlation between input and output signals. In other words, it reflects how reliable the system is for a given input frequency. It also allows us to estimate the linear information transfer rate R, based on the Shannon formula (Shannon and Weaver 1949; reviewed by Borst and Theunissen 1999):

$$R = \int \log_2 \left\{ \frac{1}{1 - \gamma^2(f)} \right\} df \qquad \text{(Equation 3 - 2)}$$

Information transfer rate, measured in bits/s, is an estimate of the rate of information transmission through a communication system.

Experiments were conducted using 6-min long recordings. In order to avoid effects of the adaptation process, the first minute of each recording was discarded and the frequency response was calculated over the remaining 5 min. In each case, the first recording was a control measurement. After this initial recording, an agonist was applied and four successive recordings were done immediately following each other. Six cells were tested using 1 ml of

1 mM oxotremorine and five cells were tested using 1 ml of muscarine at concentrations of 100 μM (3 cases), 500 μM (1 case) and 1 mM (1 case) as agonists. The muscarine recordings were pooled. Seven cells were used as controls, in which no drugs were applied. In several cases more than one population of action potentials was observed and the frequency response analysis could not be performed on these recordings. Only cells in which fitting of the gain was accurate, as estimated by eye, were used here.

The fitted parameters A and k and the information transfer rate R were plotted against time for each treatment (Fig. 13). No significant differences could be seen between treatments at any point in time, using the Mann-Whitney rank sum test. To observe changes that occurred over time during each experiment, I used the two-sample paired Wilcoxon sign ranked test to compare each time period with the original recording (0 min). In the control recordings the power law exponent k and the information transfer rate R decreased significantly ($p<0.05$). This decrease was not observed in experiments in which the agonists were applied. However, a significant decrease ($p<0.05$) in information transfer rate was seen in the first recording after oxotremorine application as well. These changes in information transfer rate were due to a decrease in the coherence function that usually affected the whole frequency spectrum and not one particular frequency.

These results suggest that on a long time scale, mAChR agonists could affect the adaptation properties of the sensory neurons. Recordings performed without agonist application showed that when the neurons were stimulated over a long period of time the value of k decreased, indicating that they became relatively less sensitive to high frequency stimuli. Application of a mAChR agonist stopped this effect and maintained the sensitivity to high frequency at its original level. The information transfer rate was also affected. While it decreased in control recordings, it remained closer to its original level when oxotremorine

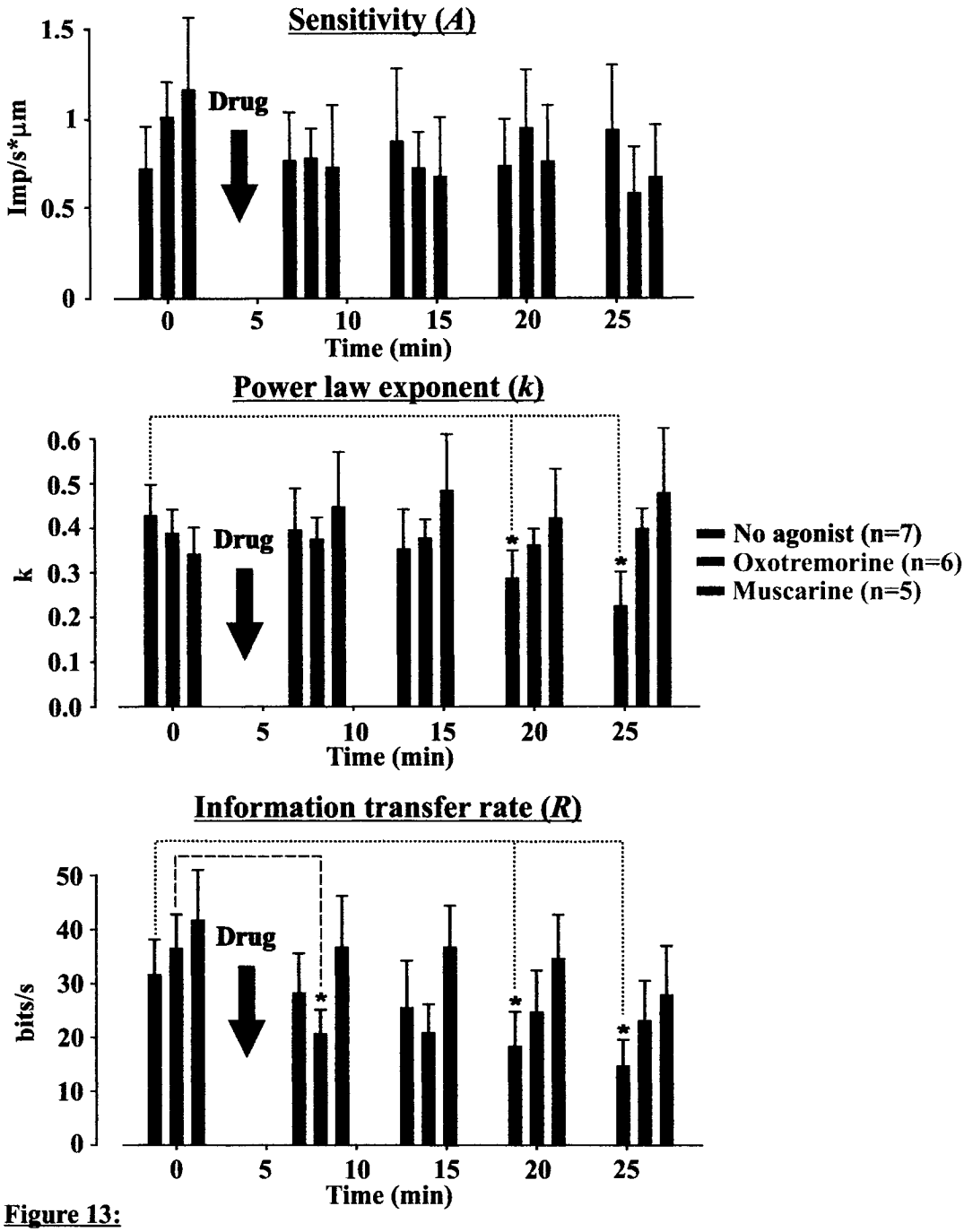

Figure 13:

Statistical analysis of the parameters obtained from the frequency response analysis for mAChRs. The means and standard errors for the fitted parameters *A* and *k* and the information transfer rate *R* are shown for the recordings without agonist application (black), and for recordings where oxotremorine (red) or muscarine (green) were applied at the time indicated in each graph. When no drug was applied the *k* and *R* values were significantly reduced ($p<0.05$) after 20 and 26 min. *R* also decreased significantly ($p<0.05$) immediately following oxotremorine application.

or muscarine were applied. Interestingly, immediately after oxotremorine was applied, the information transfer rate dropped significantly, before returning to its original level. This could indicate that stimulation of mAChRs also has a shorter term effect.

3. 2. Octopamine receptors on spider mechanosensilla

3.2.1. OAR immunolabeling with OAMB antibody

To assess the expression of octopamine receptors on the spider mechanosensilla, I used a polyclonal antibody against a specific type of OAR that is widely expressed in *Drosophila* mushroom bodies and therefore named OAMB (Han et al. 1998). However, this receptor has also been shown to be more widely expressed in the *Drosophila* nervous and reproductive systems (Lee et al. 2003). To test the specificity of the OAMB antibody in the spider tissue Western blot analysis was performed using extracts from spider brain. This antibody was also used to test for specific labeling on the spider mechanosensilla.

A. Western blot analysis

The OAMB antibody produced a distinct band at ~75 kDa (Fig. 14A) corresponding to the calculated molecular weight of *Drosophila* receptors of 72 kDa (Han et al. 1998). Because of the significant unspecific labeling that was observed at higher molecular weights this experiment was repeated several times using different primary and secondary antibody concentrations. The 75 kDa band was observed in each blot, but the unspecific labeling was also always present. Although this antibody was previously used with success in immunohistochemistry, its developers failed to produce identifiable bands in Western blots with *Drosophila* tissue (Kyung-An Han personal communication).

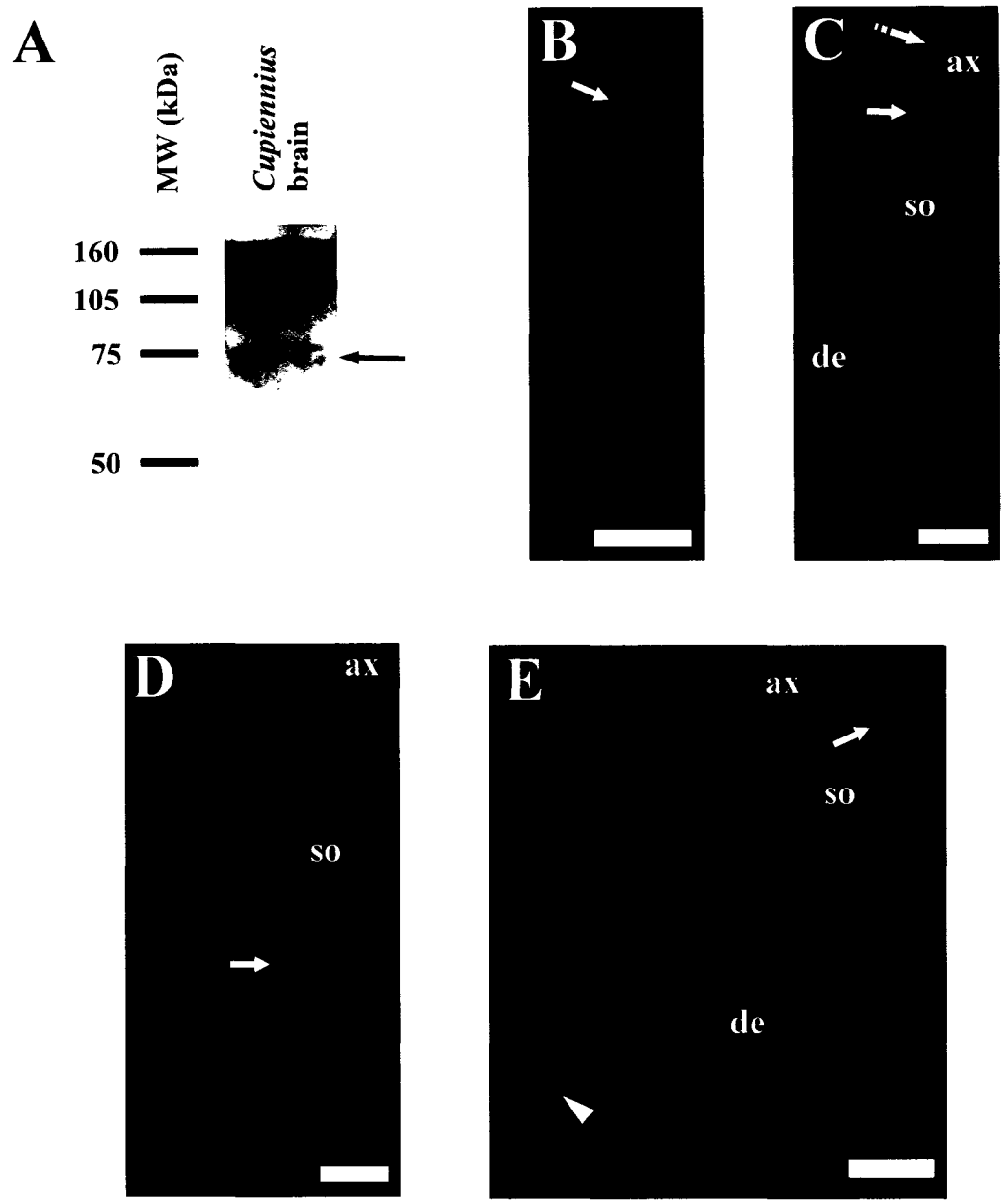

Figure 14:

A: Western blot analysis of octopamine receptor proteins in spider brain tissue extract using a polyclonal unpurified antibody developed against a cloned *Drosophila* octopamine receptor (OAMB). A distinct band is seen at ~75 kDa (arrow), closely matching the calculated molecular weight of the *Drosophila* receptor. *B-E*: Two-dimensional confocal projections of OAMB immunolabeled tactile hair sensilla (*B* and *C*) and VS-3 organs (*D* and *E*). All sensory neurons showed strong immunoreactivity to the OAMB antibody. Labeling was strongest in the proximal part of the somata (arrows in *B-E*) but could also be seen in other parts of the cell bodies (arrowhead in *E*) and in the axons (dashed arrow in *C*). ax = axon; so = soma; de = dendrite. Scale bars: 20 μm

B. Immunocytochemistry

The OAMB antibody was used for immunocytochemical analysis of whole-mount preparations of the spider patella and tibia. Immunoreactivity was seen in mechanosensory neurons of the VS-3 organ and of tactile hairs (Figs. 14B-E). The labeling was strongest in the proximal parts of the somata, but it was also occasionally present in other parts of the cell bodies and in the axons (Figs. 14C and E). Despite the large amount of unspecific labeling seen in Western blot analysis, the immunocytochemical results were very specific. The cellular localization was consistent and the labeling had a typical rod-like shape. Control preparations in which the primary antibody was omitted or replaced by normal rabbit serum at the same concentration showed no labeling.

To test whether efferent nerves could form synapses with OARs and to see if any of the OAMB immunoreactivity was present on the efferent nerve fibers, double labeling was performed using OAMB antibody and a monoclonal antibody developed against *Drosophila* synapsin (SYNORF1, Klagges et al. 1996). This antibody labels specifically the synaptic vesicle protein synapsin I on presynaptic neurons and it was previously used on the spider preparations to label peripheral efferent fibers (Fabian-Fine et al. 1999a, b; Panek et al. 2003). It binds specifically to the efferent neurons but not to the glial cells or sensory neurons (Fabian-Fine et al. 1999b). Clear synapsin immunoreactivity was detected in the fine fibers surrounding the axons, cell bodies and dendrites of the sensory neurons (Figs. 15A and B). The synapsin labeling was often co-localized with OAMB immunoreactivity on the areas around the proximal parts of the cell bodies and the axons, suggesting that synaptic contacts between efferent fibers and OARs may exist. However, OAMB labeling was found exclusively on the sensory neurons and was not seen on the efferent fibers.

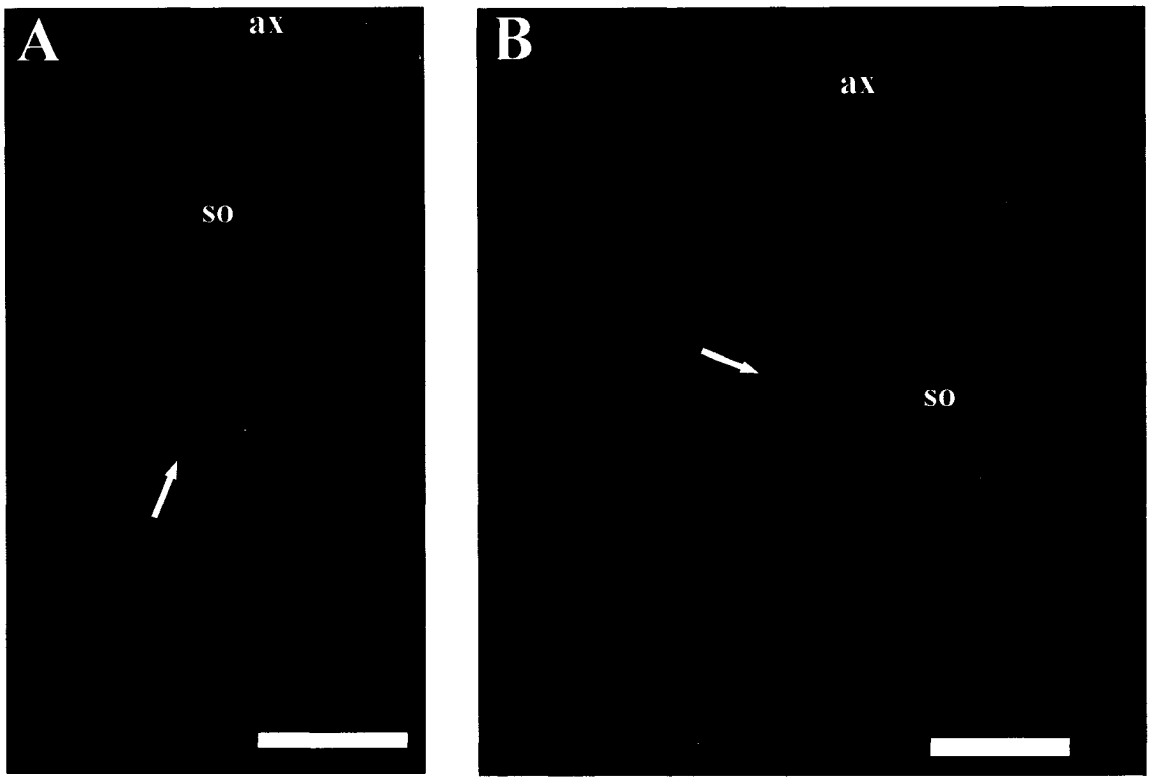

Figure 15:

Double immunolabeling of the VS-3 organ with anti-octopamine receptor antibody OAMB (red) and anti-synapsin antibody SYNORF1 (green). The typical OAMB labeling was seen in the proximal part of the somata and in the axons, while synapsin immunoreactivity was present on the fine fibers surrounding the sensory neurons. Some of the synapsin labeling on the efferent fibers was located close to OAMB labeling (arrows). OAMB immunoreactivity was only seen in the sensory neurons and was not observed in the efferent fibers. ax = axon; so = soma. Scale bars: 20 μm

3.2.2. Immunolabeling with anti-octopamine antibody

Octopamine immunoreactive neurons have previously been found in the spider CNS (Seyfarth et al. 1993). In the locust some of the hindleg proprioceptors were shown to be directly innervated by octopaminergic DUM neurons (Bräunig and Eder 1998). To see if octopamine could be released locally from the efferent fibers surrounding the spider mechanosensory neurons, I tested an antibody against octopamine that shows no cross-reactivity with tyramine or other monoamines (Chemicon Data Sheet #AB1799). In whole-mount preparations some fine octopamine immunoreactive fibers were seen in the leg nerve. One branch passed over the neuron cell bodies innervating tactile hairs and proceeded between them (Fig. 16A and B). Single octopamine immunoreactive fibers also passed over VS-3 neuron axons (Fig. 16C and D) and in some cases they were seen on top of cell bodies. The diameter of these immunoreactive fibers varied from 0.5 μm to 1.6 μm, corresponding to the reported size for fine efferent fibers of 0.2 μm to 3 μm (Fabian-Fine et al. 1999).

3.2.3. Octopamine effects on the excitability of spider trichobothria neurons

A. Octopamine effect on firing rate

The same extracellular setup that was used for testing the effects of muscarinic agonists on spider mechanosensory neurons was used here to test for effects due to the octopamine receptor agonists octopamine and tyramine. Three- to six-minute long recordings were performed using white noise mechanical stimuli and recording responses before and after agonist application. In the control recordings without agonist application the firing rate decreased during the first minute, then remained at a constant level for the rest of the recording period. Octopamine was applied 30 to 60 s after the beginning of the recording

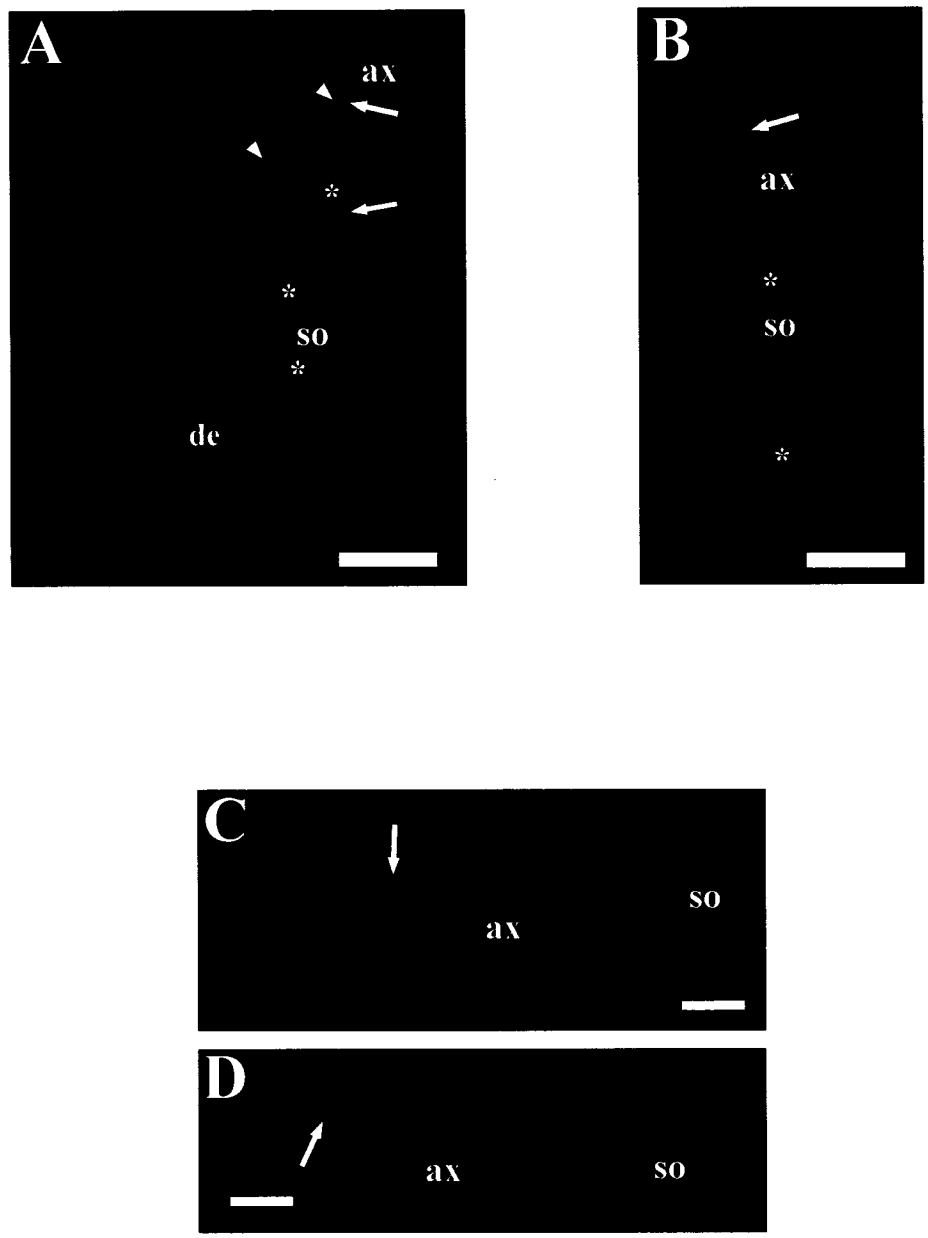

Figure 16:

Two-dimensional confocal projections of octopamine immunolabeled tactile hair sensilla (*A* and *B*) and VS-3 organs (*C* and *D*). One fine fiber surrounding the cell bodies (asterisks) showed strong immunoreactivity (arrows). Only two of the three cells are visible in *B*. Some of the octopamine immunoreactive fibers had more than one branches (arrowheads in *A*). Ax = axon; de=dendrite; so = soma. Scale bars: 20 μm

through the leg perfusion. Immediately following octopamine application there was a sharp increase in firing frequency that nearly reached the level of the initial frequency before adaptation (Fig. 17A). Tyramine, the precursor of octopamine, had a similar effect to octopamine by increasing the firing frequency of the trichobothria neurons (Fig. 17B).

B. Dose-response relationship

The firing frequency remained elevated for a period of time proportional to agonist concentration. With 1 mM octopamine, the increased frequency was still seen 5 min after application. To quantify how octopamine concentration affected firing frequency, an exponential decay function was fitted to the first 30 to 60 s before drug application in each recording (Fig. 18A, red). The fitting was performed by a minimum error procedure, and its accuracy was always verified by eye. This was considered as the base level for each neuron, and was subtracted from the original trace (Fig. 18A, blue). The resulting trace (Fig. 18A, black) was used to estimate the peak response by averaging its 20 highest points. The calculated peak responses were used to plot a dose-response curve (Fig. 18B). Thirty-six cells were tested with octopamine applied through the perfusion at concentrations ranging from 100 nM to 1 mM . However, the final concentration of octopamine at the site of action was not known, but assumed to be lower due to dilution with saline in the perfusion system and in the spider leg. The response to octopamine was clearly dose dependent.

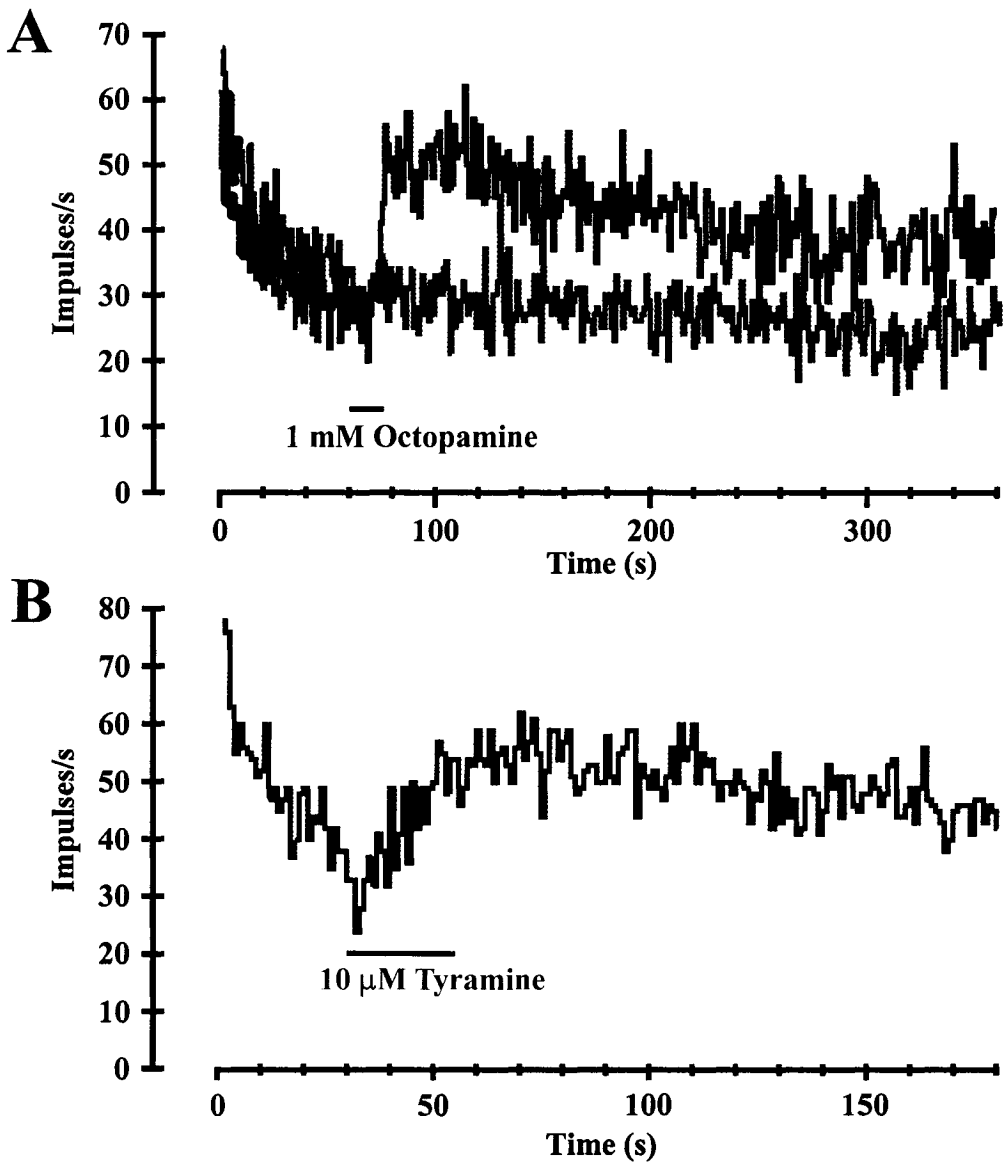

Figure 17:

Firing frequency of thrichobothria neurons in response to mechanical stimulation under control conditions and after application of octopamine receptor agonists.
A: Control recording without agonist application showing a typical response to mechanical stimulation that decayed rapidly during the first minute of stimulation (green). After application of 1 mM octopamine the firing frequency increased rapidly (blue). At this concentration, the effect was still seen 5 min after application. *B*: The trichobothria neurons also responded to tyramine, the precursor of octopamine, with an increased firing frequency.

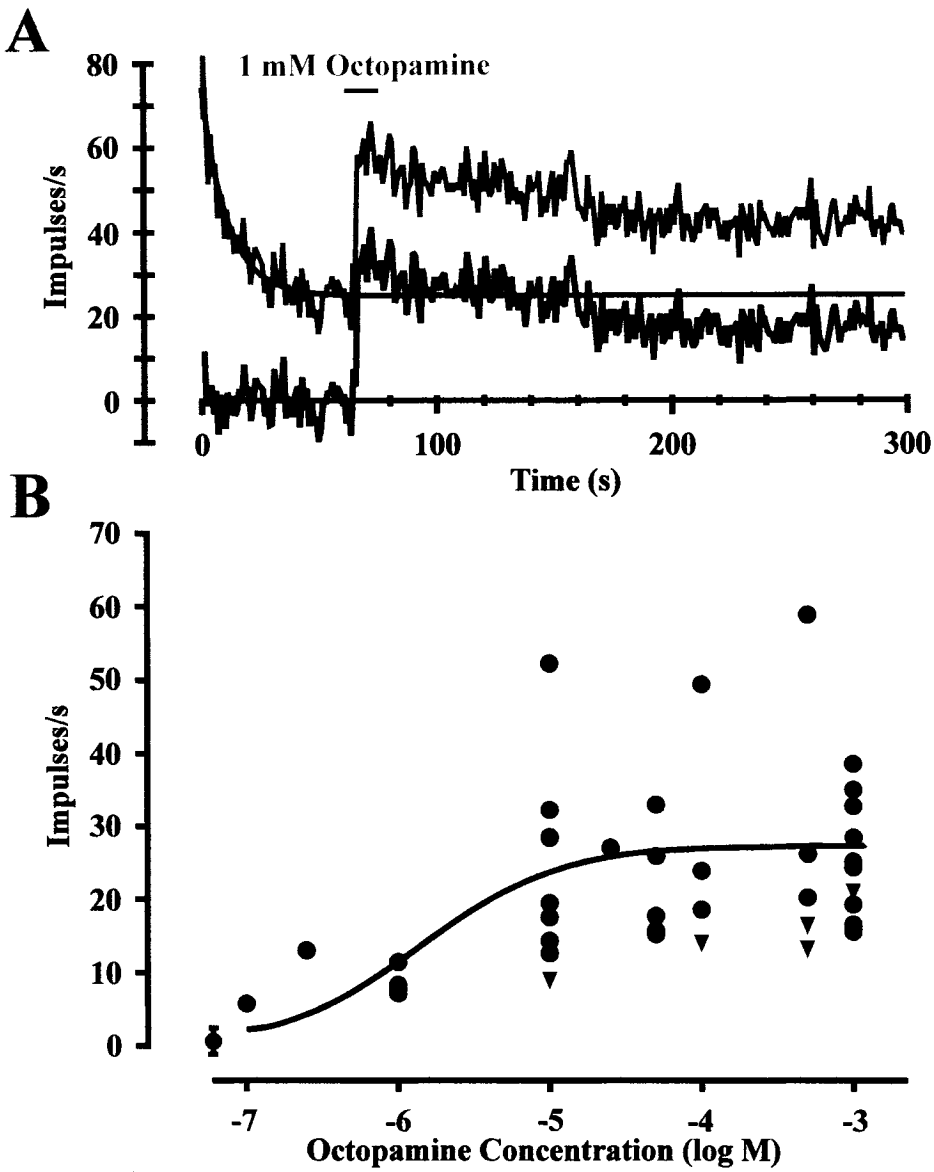

Figure 18:

Dose-response curve of octopamine induced responses.

A: An exponential decay function was fitted to the adaptation period prior to octopamine application (red). This function was then subtracted from the original trace (blue). The peak response was calculated by averaging the 20 highest points of the resulting trace (black). *B*: Peak-responses were plotted against the logarithm of the agonist concentration and data points were fitted with the Hill equation. The EC_{50} was 1.39 µm and Hill coefficient 0.96. The green point represents the peak value (mean ± s.d.) of five control recordings in which no drug was applied. The peak responses for tyramine were found in the lower range of octopamine responses (▼).

To estimate the half-maximal concentration, the dose-response curve was fitted with a Hill equation:

$$y = \frac{y_{max}[C]^n}{\left([C]^n + [EC_{50}]^n\right)} \qquad \text{(Equation 3-3)}$$

where y is the peak response, y_{max} is the maximal peak response, $[C]$ is octopamine concentration, $[EC_{50}]$ is the half-maximal effective concentration and n is the Hill coefficient. The calculated EC_{50} was 1.39 µM, corresponding to previous studies (Ramirez and Orchard 1990; Gerhardt et al. 1997a; Han et al. 1998; Van Poyer et al. 2001).

The Hill coefficient n was 0.96. This suggests that the octopamine receptors are non-cooperative and that one agonist molecule binding to the receptor is sufficient to activate the response. Similar findings have been made for molluscan OARs (Gerhardt et al. 1997a).

The peak-responses of three neurons in which tyramine was used as an agonist were also plotted on the dose-response curve. These values were in the lower range of octopamine responses (Fig. 18B), suggesting that tyramine is slightly less potent than octopamine in increasing the firing frequency of the trichobothria neurons. Other studies have also shown tyramine to be a less potent agonist of OARs (Evans 1981; Ramirez and Orchard 1990; Gerhardt et al. 1997a; Han et al. 1998; Chang et al. 2000; Van Poyer et al. 2001; Grohmann et al. 2003; Bischof and Enan 2004;).

C. Octopamine receptor frequency response analysis

To test the effect of octopamine on the dynamic behavior and information transfer rate of the trichobothria neurons, frequency response analysis was performed from nine experiments in which 100 nM to 1 mM octopamine was applied. The first 30 to 60 s of the recording prior to drug application was discarded and frequency response analysis was performed on the remaining record. It consisted of at least a 150 s recording period, during which the firing frequency increased in response to octopamine. For each cell, initial control recordings were performed in normal spider saline. The frequency response analysis was done on the same time window for the control recordings as for the experiments with octopamine.

The parameters from frequency response analysis of the control and octopamine recordings were compared within each cell using the two-sample paired *t*-test. The fitted gain parameters A and k increased after octopamine application, but only the increase in A was statistically significant (Fig. 19). In addition, there was a statistically significant increase in the information transfer rate R following agonist application (Fig. 19).

D. Phentolamine effect on the firing frequency and octopamine response

Phentolamine is an effective antagonist of octopamine receptors that has been widely used in invertebrate neural and non-neural preparations (Evans 1981; Ramirez and Orchard 1990; Roeder 1992; Howell and Evans 1998; Degen et al. 2000; Clark and Lange 2003). However, at high concentrations phentolamine has also been shown to have a local anaesthetic effect through interaction with voltage activated Na^+ channels, thus directly inhibiting neuronal excitability (Ramirez and Pearson 1990).

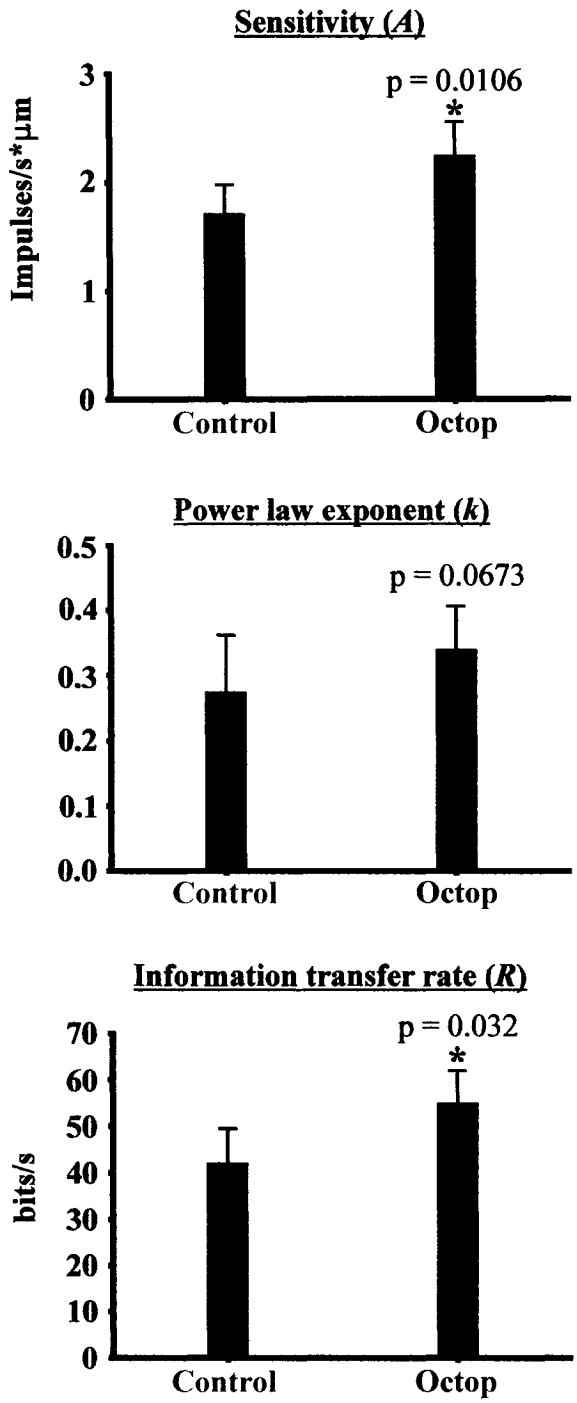

Figure 19:

Statistical analysis of the parameters obtained from frequency response analysis for OARs. The means and standard errors for the fitted parameters A and k and for the information transfer rate R are shown here. Nine cells were used in each case. The sensitivity (A) and information transfer rate (R) increased significantly following octopamine application ($p<0.05$, two-sample paired t-test).

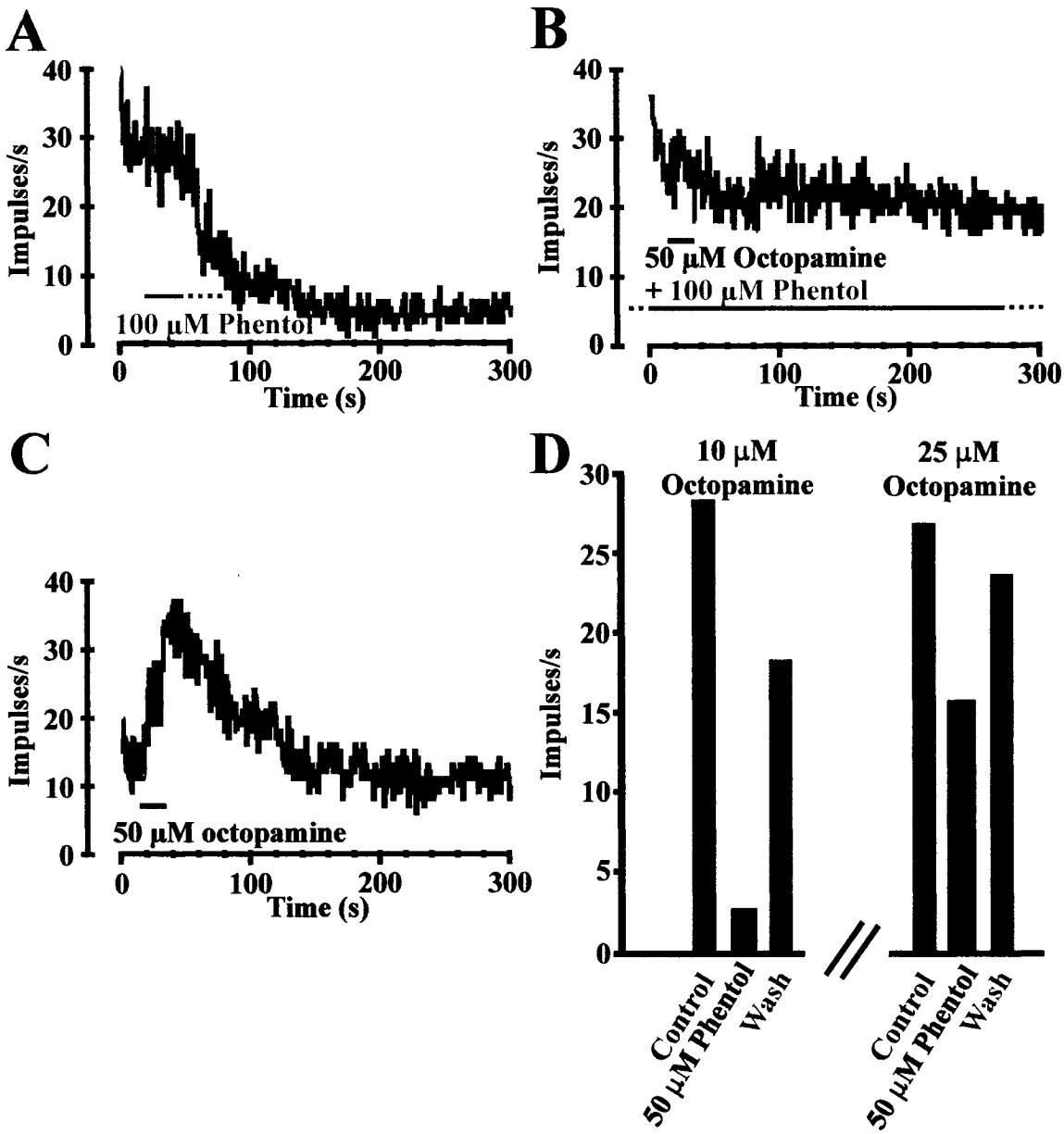

Figure 20:

Effect of phentolamine (Phentol) on the firing frequency and octopamine response of spider trichobothria neurons.
A: 100 µM phentolamine was added to the perfusion solution at the time indicated. This reduced the firing frequency of the mechanically stimulated neuron. After 12 min of incubation with phentolamine, a mixture of 50 µM octopamine and 100 µM phentolamine was applied (*B*). The typical stimulatory effect of octopamine did not occur. Following a 12 min washing period with spider saline, 50 µM octopamine was applied alone (*C*). The typical increase in firing frequency was observed. *D*: Peak responses for two different cells following application of 10 µM or 25 µM octopamine. Octopamine was applied alone (red), after 10 min incubation with 50 µM phentolamine (purple), and after a 15 min washing period with spider saline (green).

Phentolamine applied alone to the spider leg during mechanical stimulation (Fig. 20A) reduced the trichobothria neuron firing frequency in seven of 14 experiments, even at 50 μM concentration. This is a significantly lower concentration than described earlier for the anaesthetic effect of phentolamine on locust and cockroach mechanoreceptors, for which 200 to 500 μM was needed to inhibit the neurons (Ramirez and Pearson 1990). In spider, this inhibitory effect was more acute when phentolamine was applied through the rapid drug application system (see Fig. 4) rather than through the drug perfusion system. When the neurons were continuously perfused with phentolamine for 10 to 12 min followed by application of a mixture of octopamine and phentolamine the typical stimulatory octopamine effect was blocked in five of nine experiments (Fig. 20B and D). Following a washing period of 12 to 15 min with spider saline, application of octopamine alone increased the firing frequency (Fig. 20C and D). These results suggest that phentolamine can block the OARs in the spider trichobothria neurons, but that it also has a direct inhibitory effect on these neurons.

E. Second-messenger pathway from the spider OAR

Most of the known OARs activate adenylyl cyclase in response to octopamine binding (Fig. 21A) (reviewed by Roeder 1999, 2002). For example, the functionally expressed *Drosophila* receptor OAMB increased cAMP concentration when stimulated (Han et al. 1998). However, the next step of the second messenger pathway, the activation of PKA, was only shown *in vivo* in the honey bee antennal lobe (Hildebrandt and Müller 1995) and the jumping muscle of the locust (Walther and Zittlau, 1998). Here, I tested whether a cAMP analog, 8-Br-cAMP, would activate the second messenger pathway of OARs in the

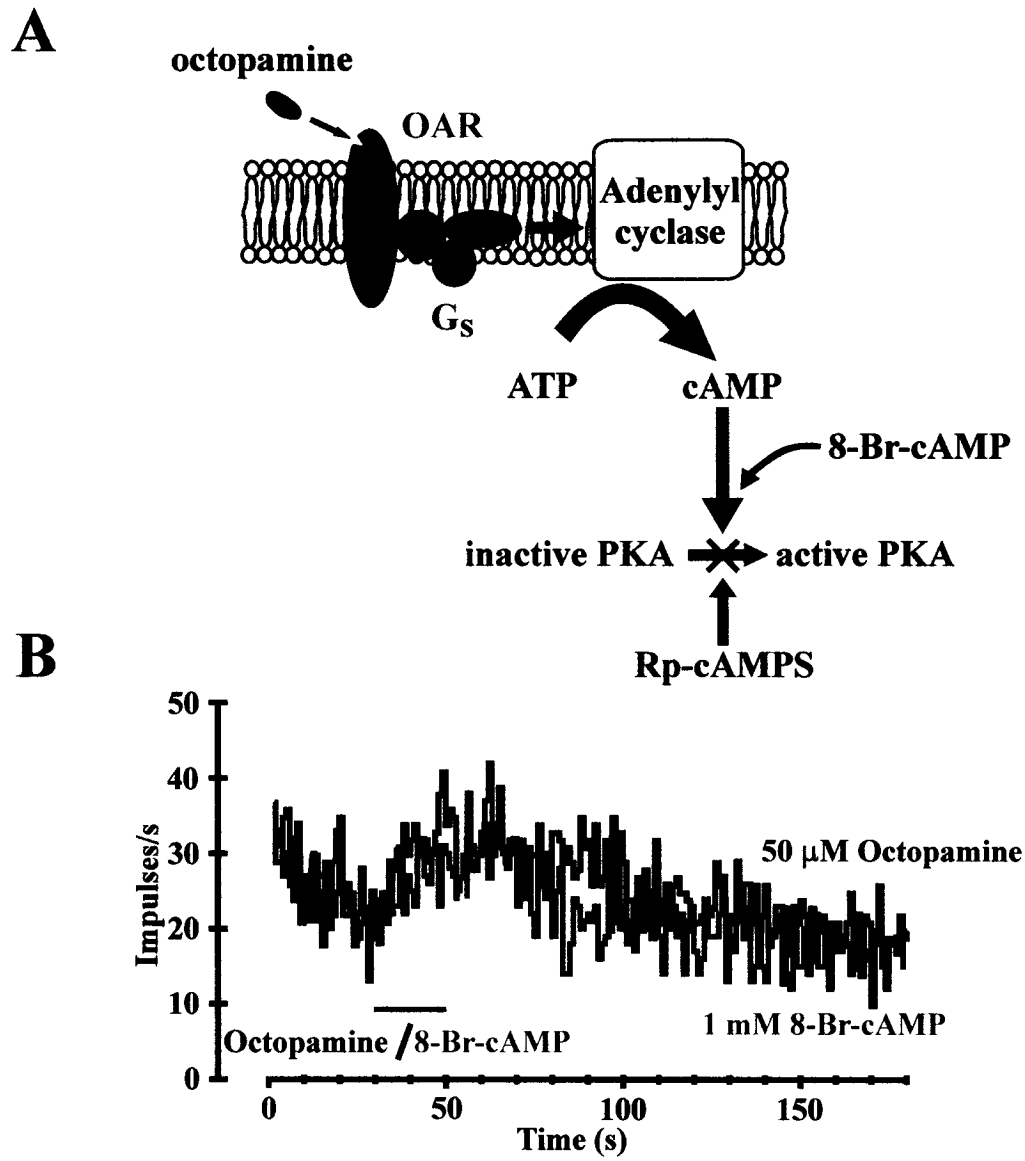

Figure 21:

Second messenger pathway of OARs in the spider mechanosensory neurons.
A: Schematic representation of the putative second messenger pathway activated by ligand binding to OARs. Binding of octopamine to the OAR activates the associated G_S protein, allowing one of its subunits to bind to and activate adenylyl cyclase. The activated adenylyl cyclase catalyzes the formation of cAMP from cytosolic ATP. The second messenger cAMP activates protein kinase A (PKA). To test whether this pathway was used by OARs in the spider neurons, an analog of cAMP (8-Br-cAMP) and an inhibitor of PKA (Rp-cAMPS) were used. *B*: Application of 1 mM 8-Br-cAMP to the perfusion solution induced an increase in the firing frequency of the trichobothria neuron. The time course of this response was similar to the response of the same neuron to an application of 50 µM octopamine.

spider trichobothria neurons. I also tested whether a phosphodiesterase resistant competitive inhibitor of PKA type I and II, Rp-cAMPS, would have an inhibitory effect on the trichobothria response to octopamine. Both of these substances are membrane permeant.

When 1 mM 8-Br-cAMP was applied to the perfusion, the firing frequency increased in all four experiments performed. The time course of this response was very similar to that observed with octopamine (Fig. 21B) suggesting that the octopamine effect could be mediated via cAMP.

To test the effect of Rp-cAMPS, a control recording was performed in which octopamine was applied to a preparation perfused with normal saline. It produced a typical increase in firing frequency of the trichobothria neurons (Fig. 22A, blue). Rp-cAMPS was then applied and perfused for 15 min, followed by a new octopamine application (Fig. 22A, red). The response to octopamine decreased sharply. On average, the decrease in amplitude of the peak responses to octopamine following incubation with Rp-cAMPS was approximately 50% in the three experiments performed here (Fig. 22B). The Rp-cAMPS effect was not reversible and the firing frequency in response to octopamine remained decreased even after a prolonged washing period. These results suggest that OARs in the spider trichobothria neurons are positively coupled to adenylyl cyclase and act via a PKA mediated pathway.

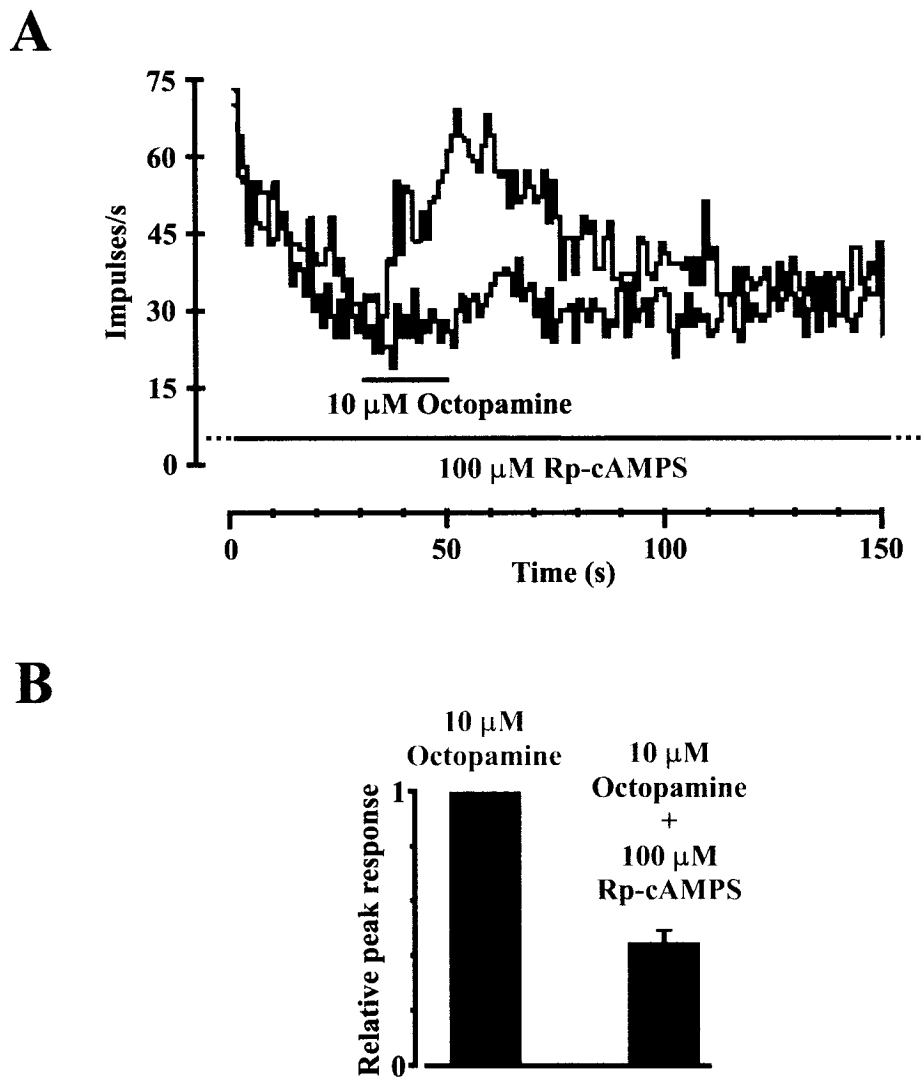

Figure 22:

Effect of the PKA inhibitor Rp-cAMPS on the tirchobothria neuron octopamine response. *A*: A typical response to 10 μM octopamine is shown in blue. When the same neurons was perfused with 100 μM Rp-cAMPS for 15 min, a new application of 10 μM octopamine produced only a small increase of the firing frequency (red). *B*: The relative peak response to octopamine before (blue) and after Rp-cAMPS application (red) in three different preparations is shown here. The peak responses decreased by more than 50% (0.45±0.04; mean ± s.e.).

4. Discussion

4.1. ACh at spider peripheral synapses

Previous studies on *C. salei* have suggested that ACh may be released from efferent neurons innervating peripheral parts of the mechanosensory neurons (Fabian and Seyfarth 1997; Fabian-Fine et al. 2002). I investigated if ACh receptors were present in spider mechanosensilla and what their potential effects on excitability of the mechanosensory neurons were. Although efferent innervation through specific types of nicotinic AChRs was demonstrated in vertebrate outer hair cells (reviewed by Fuchs 2002), similar findings have not been described in any other vertebrate or invertebrate mechanoreceptors. In contrast, muscarinic AChRs were shown to have a role in the regulation of ACh release in invertebrate sensory afferents at centrally located synapses (reviewed by Trimmer 1995). In this study I focused mainly on muscarinic receptors and found evidence for the presence of mAChRs in the peripheral nervous system of the spider *C. salei*.

4. 1.1. Visualization of ACh receptors on spider mechanosensilla with fluorescent probes

The presence of ACh receptors in spider mechanosensilla was initially assessed by binding of fluorescent antagonists of either muscarinic or nicotinic receptors. In vertebrate preparations the labeled muscarinic antagonist Bodipy® pirenzepine was shown to bind preferentially to M_1 receptors with similar affinity as the unlabeled pirenzepine (Hammer et al. 1980; Wang et al. 1994; Goutsouliak et al. 1997). However, pirenzepine also displays

relatively high affinity for the other subtypes of mAChRs (reviewed by Caulfield and Birdsall 1998).

Pirenzepine was described as the antagonist of choice for discriminating between nicotinic and muscarinic receptors (Benson 1992). It was shown to inhibit mAChRs in many invertebrate preparations. For example, muscarinic receptors or receptors with mixed pharmacological profiles in the cockroach fast coxal depressor motoneuron and DUM neurons (Bai and Sattelle 1994; Grolleau et al. 1996), the tobacco hornworm proleg retractor motoneurons and central neurons (Trimmer and Weeks 1993; Qazi et al. 1996), dissociated neurons of the thoracic ganglia of locust (Benson 1992), a midge non-neuronal epithelial cell line (Wegener et al. 1996) and motoneurons of the lobster cardiac ganglion (Freschi 1991) were all shown to be inhibited by pirenzepine. The cloned *Drosophila* mAChR was also shown to bind pirenzepine effectively (Shapiro et al. 1989; Blake et al. 1993; Reaper et al. 1998).

The Bodipy® pirenzepine fluorescent probe was previously used to label mAChRs in cultured mammalian neurons (Wang et al. 1994) and cardiomyocytes (Goutsouliak et al. 1997) as well as neurons and non-neural cells in primary cell cultures of *M. sexta* (Torkkeli et al. 2004). In the spider, the probe binding was seen as clear punctate labeling in the leg nerve that included afferent sensory fibers and efferent fibers (Fig. 5). A similar punctate labeling pattern was observed with the same probe in cultured neurons of *M. sexta* (Torkkeli et al. 2004). The labeling was also seen on the cell bodies of the mechanosensory neurons after they were pre-incubated in collagenase to remove glial cells, which might prevent the penetration of the large fluorescent pirenzepine molecule (MW 747.09 kDa).

The fluorescent nicotinic antagonist α-bungarotoxin did not show any labeling in the spider preparations on either the nerve fibers or the cell bodies, with or without collagenase treatment. Although very little is known about nAChRs in spiders, α-bungarotoxin has been found to have an effect on many other invertebrate preparations. Unlike vertebrate neuronal nAChRs, most of which are α-bungarotoxin insensitive (reviewed by Tomizawa and Casida 2001), neuronal nAChRs in invertebrates bind this toxin (reviewed by Leech and Sattelle 1993). Such α-bungarotoxin sensitive nAChRs are often present in neurons postsynaptic to sensory afferents. For example, a radioligand binding study found these receptors in the antennal lobes of *M. sexta* where neurons postsynaptic to the antennal olfactory neurons are located (Hildebrand et al. 1979). α-bungarotoxin was also shown to block synaptic transmission between cockroach mechanosensory cercal afferents and giant interneurons (reviewed by Breer and Sattelle 1987), between locust stretch receptor neurons and motoneurons (Leitch and Pitman 1995), and between locust filiform hair receptor neurons and the A4I1 interneuron (Gauglitz and Pflüger 2001), indicating that α-bungarotoxin sensitive receptors are present postsynaptically in these preparations. In addition, α-bungarotoxin blocked ACh receptors in cockroach DUM neurons (Grolleau et al. 1996), in locust thoracic ganglia neurons (Benson 1992), and in cultured Kenyon cells of crickets, *Drosophila* and honeybees (Cayre et al. 1999; Goldberg et al. 1999; Déglise et al. 2002; Su and O'Dowd 2003). However, ACh-induced current was only partially blocked by α-bungarotoxin in the honeybee preparation (Goldberg et al. 1999) suggesting that a subpopulation of the receptors are insensitive to this antagonist. Some α-bungarotoxin resistant nAChRs were also described in cultured cockroach DUM neurons (Lapied et al. 1990; Courjaret and Lapied 2001) and abdominal ganglia neurons (Fickbohm and Trimmer

2003) as well as in *M. sexta* motoneurons (Trimmer and Weeks 1989). However, some of these α-bungarotoxin-insensitive receptors responded to both muscarinic and nicotinic agonists and are not considered to be real nicotinic acetylcholine receptors but rather muscarinic receptors with mixed pharmacological profiles (David and Pitman 1996).

Absence of α-bungarotoxin binding activity in the spider preparation suggests that spider mechanosensilla do not have nicotinic ACh receptors that are sensitive to this antagonist. These results do not exclude the possibility of other types of nicotinic or mixed pharmacology receptors being present in this preparation. However, the spider VS-3 neurons did not respond to nicotine in electrophysiological experiments, which supports the idea that nAChRs are not present.

4.1.2 Anti-mAChR immunoreactivity

The presence of mAChRs on the somata of spider mechanosensory neurons and nerve fibers was further demonstrated using M35 antibody developed against mAChRs extracted from calf forebrain homogenates (André et al. 1983, 1984, 1987). This antibody has been successfully used in many vertebrate preparations (Amadeo et al. 1995; Calaza and Gardino 2000) including humans (Grando et al. 1995; Sailer et al. 2000) as well as in insect preparations (Lammerding-Köppel et al. 1994; Wegener et al. 1996; Torkkeli et al. 2004) and sea urchin eggs (Piomboni et al. 2001). M35 recognizes all five human mAChR subtypes equally well (Carsi-Gabrenas et al. 1997) suggesting that the epitope for M35 is located on a highly homologous region (reviewed by Van der Zee and Luiten 1999). Cross reactivity of monoclonal antibodies developed against rat brain mAChRs was demonstrated in several species from human to *Drosophila* (Venter et al. 1984), indicating that the epitope of these

receptors is highly conserved. A cloned *Drosophila* mAChR showed 45% to 69% sequence identity with mammalian mAChRs and up to 88% identity in the transmembrane domains (Onai et al. 1989; Shapiro et al. 1989).

Although originally thought to be ineffective in Western blot analysis (André et al. 1984, 1987), the M35 antibody showed a clear band at 75 kDa in *Drosophila* head extracts and in spider central or peripheral tissue homogenates (Fig. 7). A similar observation was made previously in *Chironomus tentans* (Wegener et al. 1996) and *M. sexta* (Torkkeli et al. 2004), suggesting that current Western blot methods can better conserve the epitope than at the time of the initial investigation (André et al. 1984). In *C. tentans*, the molecular weight of the receptor protein was ~80 kDa (Wegener et al. 1996) and in *M. sexta*, a distinct band was seen at 85 kDa (Torkkeli et al. 2004) similar to the estimated molecular weight of *Drosophila* mAChR (Venter et al. 1984; Onai et al. 1989). In *Caenorhabditis elegans* a 585 amino acid mAChR with an estimated molecular weight of 66.5 kDa (Hwang et al. 1999) and an alternatively spliced isoform of the same receptor with 611 amino acids and an estimated mass of 69.5 kDa (Park et al. 2003) have been cloned. The 75 kDa band seen in the spider brain homogenates is in good agreement with the previously described range of molecular weights for mAChRs.

Using immunocytochemistry, the M35 immunoreactivity was found in the somata of mechanosensory neurons (Fig. 8). The somatic location was confirmed in double labeling experiments with an antibody against $GABA_BR2$ that was previously shown to specifically label the distal parts of the sensory neuron cell bodies (Panek et al. 2003). Optical sectioning with confocal microscopy showed immunoreactivity to both antibodies in the same sections of the somata, although M35 immunoreactivity was significantly more widespread. Only the

nuclei of the mechanosensory neurons were not labeled with M35. A similar distribution of M35 immunoreactivity has been described using electron microscopy in vertebrate cortical cells, where labeling was widespread throughout the cytoplasm but was not seen in the nucleus (Buwalda et al. 1995; Van der Zee and Luiten 1999).

M35 labeling also appeared in the nerve trunks in the spider leg (Fig. 8). It was not possible to distinguish whether the labeling was in the axons of the sensory and/or the efferent neurons or in glial cells that are also present in the nerve trunks. Similarly, the somatic labeling was so extensive that it was difficult to determine whether it was also present in glial cells surrounding the neurons. M35 labeling was previously found in glial cells of the mammalian CNS (reviewed by Hösli and Hösli 1993) and in *M. sexta* antennal cultures (Torkkeli et al. 2004).

4.2. Electrophysiological recordings: muscarinic agonists effects on spider mechano-receptor neurons

4.2.1. Voltage-clamp experiments

ACh, muscarine and nicotine were applied to voltage-clamped spider VS-3 neuron preparations at different holding potentials to test whether these agonists induced a current in the mechanosensory neurons. The results of these recordings were not consistent. There were no responses that could have been caused by nAChRs, which usually elicit large, rapid currents in response to agonist application (Marder and Paupardin-Tritsch 1978; Lee and O'Dowd 1999). However, desensitization could also have prevented an ACh effect on nicotinic receptors (Katz and Thesleff 1957).

Responses of muscarinic receptors, on the other hand, are more complex and several possibilities were investigated. A slow inward current with a peak at about -30 mV was detected in other invertebrate preparations in response to muscarinic agonists (Marder and Paupardin-Tritsch 1978; Freschi 1991; Benson 1992; Trimmer 1994). In the lobster this current was shown to be carried mostly by Na^+ through voltage-dependent cation channels (Freschi and Livengood 1989). In the experiments with spider preparations this type of current could not be observed. In the crayfish motoneurons a net inward current resulting from the closure of K^+ channels was seen in response to muscarinic agonists (Cattaert et al. 1994) that resembled the vertebrate M-current (Brown 1988). This type of current was not present in the spider VS-3 neurons. In some invertebrate preparations a biphasic response to muscarinic agonists with faster and slower components was observed (Lapied et al. 1992; Cattaert et al. 1994; David and Pitman 1996), suggesting that mAChRs could also act through a faster mechanism. None of these types of responses was observed in the spider preparation.

4.2.2. mAChR agonist effects on the excitability of spider mechanosensory neurons

To learn if muscarinic agonists regulate the neurons' excitability, they were used in intracellular current-clamp experiments from the VS-3 neurons and in extracellular recordings from trichobothria hair cells. Oxotremorine was previously used to study muscarinic receptors in many vertebrate (Elgoyhen et al. 1994; Lippe et al. 2002; Williams and Messer 2004; Fukudome et al. 2004) and invertebrate models (Marder and Paupardin-Tritsch 1978; Trimmer and Weeks 1989, 1993; Benson 1992; Bai and Sattelle 1994; Cattaert et al. 1994; Trimmer 1994; Cayre et al. 1999; Goldberg et al. 1999; Qazi and Trimmer

1999a, b; Gauglitz and Pflüger 2001). In the spider VS-3 neurons preparation, oxotremorine or ACh did not significantly change neuronal excitability. The most commonly observed effect was an increase of membrane resistance, sometimes accompanied by an increase in the baseline depolarization that was reversible in only six recordings (Fig. 10). One of these neurons could be clearly described as a Type B neuron, the identity of the remaining cells was unclear. This observation is interesting since one of the effects of muscarinic agonists is a closure of K^+ channels (Cattaert et al. 1994), which would lead to an increase in membrane resistance and baseline depolarization as observed here. However, the effect described here was too inconsistent to be conclusive.

Muscarine and oxotremorine effects on firing frequency of the trichobothria neurons were then tested using pseudorandom white noise mechanical stimulation (Fig. 11). Neither of the agonists had an effect on the firing frequency of the mechanosensory neurons. The sharp decrease in firing frequency seen in the first minute of stimulation was due to a normal adaptation process and was not influenced by application of muscarinic agonists.

4. 2. 3. mAChR agonist effects on the dynamic behavior of the trichobothria neurons

To test whether application of muscarinic agonists had a long term effect on the dynamic behavior of these mechanosensory neurons, frequency response analysis was performed. I also calculated the neurons' information transfer rate, a commonly used measure from communication theory that has also been applied to biological systems (reviewed by Rieke et al. 1997). The gain of the frequency response increased with increasing frequency, showing the typical high-pass nature of these rapidly adapting mechanosensory neurons, similar to what was observed in the cockroach femoral tactile

spine (French 1984) and the spider VS-3 organ (French et al. 2001). The power law relationship fitting the gain could be described by two fitted parameters, the sensitivity A and the fractional exponent k. When all data were pooled at time 0 before drug application, the average value for k was 0.39 ± 0.15 (mean ± s.d.; n=18), similar to what was observed for Type A neurons in the VS-3 organ in response to mechanical stimulation (French et al. 2001).

The information transfer rate recorded here for the pooled data at time 0 was 36.2 ± 16.9 bits/s (mean ± s.d.; n=18). This value was lower than that measured in the VS-3 organ Type A neuron with the same estimation method following mechanical stimulation (French et al. 2001), where information transfer rate was estimated at 64 bits/s. In the VS-3 neurons, using a different estimation technique for the information transfer rate a range of 190 to 370 bits/s was obtained (Juusola and French 1997), and it was suggested that the maximum information capacity for these neurons is 200 to 300 bits/s (French et al. 2001). For other spiking neurons, such as the cockroach tactile spine neurons, an information capacity of up to 500 bits/s was measured, but under artificial stimulation conditions (French and Torkkeli 1998). In non-spiking neurons, information capacities of 1,000 bits/s in fly photoreceptors (de Ruyter van Steveninck and Laughlin 1996) and of 4,600 bits/s in crab proprioceptors (DiCaprio 2004) were also reported.

Here, information transfer rate was measured from the coherence function, which describes the linear correlation between the input and the output of the system (Bendat and Piersol 1980). Therefore, it is underestimated, because it only measures linear encoding (French et al. 2001). A low information transfer rate can result from nonlinearities or added noise to the system. Several factors could have influenced my measurements. Since each

sensillum is innervated by three neurons, perfect separation of the action potentials was not always possible. In addition, the stimulation method is not perfectly reliable because the tungsten probe could not be tightly attached to the hair. Thus, a small shift of the stimulator could have occurred, leading to slightly different movements at the base of the hair where the sensory dendrites are attached. Comparison with other preparations, in particular with the VS-3 neurons, is difficult because of the use of different stimulators and recording methods. Moreover, the firing rate has to be taken into account, because information capacity increases with firing rate (French et al. 2001). In the trichobothria neurons, the firing rate was low when averaged over the period of time used for frequency response analysis. The pooled value at time 0 was 13.6 ± 7.4 AP/s (mean ± s.d; n=18), and the estimated value for the information that can be carried by each action potential was 3.2 ± 1.7 bits/AP. This is in the range of 2 to 4 bits/AP observed in VS-3 neurons with mechanical stimulation (French et al. 2001).

Application of muscarinic agonists to trichobothria neurons had no effect on the parameter A, but maintained the parameter k at higher values over time than in control recordings (Fig. 13). This means that the high-pass characteristics of the neurons were maintained for a longer period of time following agonist application. In contrast, adaptation processes or fatigue decreased the sensitivity of the neurons to high frequency stimuli in control experiments. Similarly, the information transfer rate R remained at a constant level in experiments with muscarinic agonists, while it decreased after 20 and 26 minutes in control experiments. This could be explained by a more pronounced decrease in firing frequency over time in control experiments than in experiments with muscarinic agonists, since information capacity was shown to be proportional to firing rate (French et al. 2001).

However, the firing rate decreased similarly in all situations, suggesting instead that the signal-to-noise ratio decreases over time in control experiments, in which no agonist is applied.

4.3. Octopamine at spider peripheral synapses

Octopamine modulates many physiological processes of insects, including their sensory transduction (reviewed by Roeder 1999; Bräunig and Pflüger 2001). Octopamine is released from ganglionic DUM and VUM neurons, and many insect sensory neurons have been shown to be modulated by octopamine released into the hemolymph (e.g. Ramirez and Orchard 1990, Matheson 1997). However, a special class of proprioceptors in locust was shown to be directly innervated by DUM neurons and modulated by octopamine (Bräunig and Eder 1998). Octopamine immunoreactive neurons have also been found in the *C. salei* central nervous system (Seyfarth et al. 1993), but there is no previous research describing the role of octopamine in modulating spider sensory neurons. In addition, it is not known whether octopamine in spiders can be released locally close to the target cells. Here, I investigated the presence and function of octopamine receptors in spider mechanosensilla. The findings in this thesis provide new evidence for a role of octopamine in the spider peripheral nervous system.

4.3.1. Octopamine receptors in the spider nervous system

To test if OARs were present in spider brain or mechanosensilla I used an antibody developed against a cloned *Drosophila* OAR (OAMB) (Han et al. 1998). Antibodies against OARs are not commercially available but other antibodies developed against *Drosophila*

proteins have been successfully used in *C. salei* (Fabian-Fine et al. 1999a,b). The OAMB antibody is directed against the third cytoplasmic loop of the receptor, a 542 base pair fragment of the OAMB cDNA (Han et al. 1998). The same antibody has shown positive immunolabeling in the *Drosophila* mushroom bodies (Han et al. 1998), but the OAMB receptor was also found in the thoracicoabdominal ganglion and the female reproductive system using in situ hybridization (Lee et al. 2003).

Western blot analysis from spider brain tissue extracts consistently labeled a band at approximately 75 kDa (Fig.14A). This is in agreement with the predicted molecular weight of the *Drosophila* OAMB of 72 kDa (Han et al. 1998). The OAMB antibody and a specific antibody against honeybee OARs both displayed a band at 78 kDa in homogenates from honeybee antennal lobes (Farooqui et al. 2003) and at 48, 60, 72 and 78 kDa from extracts of the whole honeybee brain (Farooqui et al. 2004). In the spider brain homogenates Western blots produced some unspecific labeling but the 75 kDa band was clearly visible at all concentrations tested, suggesting that spider OARs bind the OAMB antibody. The unspecific binding was probably due to the fact that this antibody was not purified.

Immunocytochemistry of spider peripheral mechanosensilla with OAMB antibody produced clearest labeling in the most proximal parts of the somata and initial axon segments (Fig. 14B-E). The rod-like shape of the immunoreactivity and its localization in the cell was similar in all types of leg mechanosensory organs. These results suggest that octopamine receptors in the spider mechanosensory neurons are concentrated close to the axon hillock. Previous research has shown that spider VS-3 neuron dendrites have voltage activated Na^+ channels and that they generate action potentials under mechanical stimulation (Seyfarth et al. 1995; Gingl and French 2003). However, these neurons still fire action potentials when

stimulated electrically after the dendritic parts are severed (e.g. Sekizawa et al. 1999) suggesting that there is an additional site for spike generation. Therefore, location of octopamine receptors at the proximal somata and initial axon segment would allow modulation of the neuron's excitability before the signal is propagated to the axon terminal, but would not affect earlier stages of signal transduction.

Interestingly, ionotropic GABA receptors are probably also located at the initial axon segment of the VS-3 neurons. Recent investigation showed that muscimol and GABA, both agonists of these receptors, had no effect on the dendritic or somatic action potentials but readily inhibited the axonal action potentials (Gingl et al. 2004). The location of octopamine receptors in the same region suggests that it is an important site for rapid modulation of sensory information. More slowly acting metabotropic GABA receptors have been found exclusively on the most distal parts of the VS-3 neuron somata and the dendrites (Panek et al. 2003).

Until now, the OAMB antibody has been used for immunocytochemistry only in *Drosophila* brain slices (Han et al. 1998; Lee et al. 2003). The results presented here suggest that it is also suitable for use in immunocytochemistry in other arthropod species. This should come as no surprise, given the high degree of amino acid sequence similarities between cloned OARs from different species (Grohmann et al. 2003). A study in bees and locusts, two species separated by 330 million years of evolution, showed a congruent distribution of OARs in the CNS and similar pharmacology of neuronal OARs, suggesting that the octopaminergic system has been conserved throughout a large part of arthropod evolution (Degen et al. 2000).

4.3.2. Is octopamine released locally from the efferent fibers?

An antibody against the *Drosophila* synaptic vesicle protein synapsin (Klagges et al. 1996) produced clear immunoreactivity in the efferent fibers surrounding the spider mechanosensory neurons (Fig. 15). Similar results have been obtained previously in the same spider preparation (Fabian-Fine et al. 1999a, b; Panek et al. 2003). This antibody was also used as a synaptic marker in other invertebrates (Sadek et al. 2002; Frambach et al. 2004). In the spider preparation, anti-synapsin immunoreactivity was most abundant around the axons of the sensory neurons close to the cell bodies and adjacent to anti-OAMB labeling in double labeling experiments (Fig.15), suggesting that some of the presynaptic sites may release octopamine.

The possibility that octopamine could be released from the spider peripheral efferent fibers locally was further investigated using immunolabeling against octopamine (Fig. 16). An antibody against octopamine conjugated to keyhole limpet hemocyanin using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) as a coupling agent was used. This antibody was previously used successfully in the chemosensory system of *Drosophila* larvae (Python and Stocker 2002). Here, it produced clear labeling in some of the spider peripheral efferent fibers. Unlike synapsin immunoreactivity, which was concentrated in varicosities, the octopamine antibody labeled longer segments of the efferent fibers as would be expected for a transmitter. Only one thin (~1 μm in diameter) immunoreactive fiber contacted each neuron. However, the octopamine labeling was not continuous throughout the thin efferent fibers. Similar observations have been made previously with a different octopamine antibody in secondary neurites of octopaminergic neurons in the spider CNS (Seyfarth et al. 1993).

These results strongly suggest that octopamine is contained in small efferent fibers and released locally to modulate spider mechanosensory neurons. Similar direct innervation was previously demonstrated in locust in which octopaminergic DUM neurons innervate directly some hindleg proprioceptors (Bräunig and Eder 1998). However, the morphological part of that investigation involved cobalt staining, so that octopamine immunoreactivity was not shown directly in the DUM neuron terminals.

4.4. Electrophysiological recordings: octopamine agonists effects on spider mechanoreceptor neurons

In response to the application of octopamine, mechanically stimulated trichobothria neurons greatly increased their firing frequency (Fig. 17). The response was fast, starting within a few seconds after the start of drug application. The exact time for the drug to reach the site of action varied with the perfusion flow rate and was not known precisely, but was estimated to be two to three seconds. Excitatory responses to octopamine were observed in other mechanoreceptor preparations. The firing rate of the locust forewing stretch receptor in response to mechanical stimulation increased 9 to 12 seconds after octopamine application (Ramirez and Orchard 1990). Likewise, the position sensitive neurons of the stick insect femoral chordotonal organ were activated starting approximately 15 seconds after agonist application, and reaching a peak two minutes after application (Ramirez et al. 1993). Similar observations were made in the femoral chordotonal organ of the locust, in which octopamine application caused an increase in tonic spiking within 30 seconds of application, reaching a peak after approximately three minutes (Matheson 1997). However, in both the stick insect

and the locust femoral chordotonal organ the phasic mechanosensory neurons were not influenced by octopamine (Ramirez et al. 1993; Matheson 1997).

In some cases octopamine has also been shown to have an inhibitory effect. For example, in a lobster stretch-receptor called the oval organ, octopamine was shown to depress the response to mechanical stimulation (Pasztor and Bush 1987, 1989). In no case in the spider was such a decrease in firing frequency observed. The trichobothria neurons consistently responded to octopamine application with an increase in firing frequency.

When tyramine was applied to the preparation a similar excitatory effect was seen. Tyramine is the precursor of octopamine. The enzyme tyrosine decarboxylase acts on the amino acid tyrosine to form tyramine. Tyramine in turn is the substrate of the enzyme tyramine-β-hydroxylase that produces octopamine (reviewed by Roeder et al. 2003). In several other preparations tyramine has been shown to have similar effects as octopamine but with a lower affinity for OARs. This is the case in the locust forewing stretch receptor (Ramirez and Orchard 1990) and extensor-tibiae neuromuscular preparation (Evans 1981) and was also confirmed in functional studies of cloned octopamine receptors of *Aplysia*, *Lymnaea*, *Drosophila*, honeybee and cockroach (Chang et al. 2000; Gerhardt et al. 1997a; Han et al. 1998; Grohmann et al. 2003; Bischof and Enan 2004). The affinity of tyramine for OARs was calculated in bees and locusts (Roeder 1995; Degen et al. 2000) and was found to be five to seven times lower than that of octopamine. However, in some examples, tyramine is thought to act upon specific receptors and have an opposite effect to octopamine (reviewed by Blenau and Baumann 2001 and Roeder et al. 2003) as was shown in the locomotor behavior of *Drosophila* larvae (Saraswati et al. 2003). Here, tyramine always increased the firing frequency of the mechanosensory neurons.

4.4.1. Dose-response relationship for octopamine

Since the firing frequency of the neurons varied in control conditions, the response to agonist application was standardized by fitting an exponential decay to the adaptation period of the recording. This fitting represented the firing frequency of a given neuron when no agonist was applied and was subtracted from the test recording to obtain the peak response to octopamine (Fig.18). Although the variability was still high, this technique allowed comparison between different neurons. The results presented in this study are probably slightly underestimated, because the fitting reached a constant value 30 to 60 seconds after the beginning of the recording, that is likely to be higher than the real plateau value. In addition, the octopamine concentration used in the dose-response plot represents the concentration applied to the perfusion system. The final concentration at the site of action is probably somewhat lower. Despite these issues, the half-maximal effective concentration calculated here corresponds to that found in previous studies. In other invertebrate preparations, the EC_{50} for octopamine responses, estimated by counting spikes or measuring changes in cAMP levels, varied from 0.2 to 5 μM (*Drosophila*: Han et al. 1998; Van Poyer et al. 2001; locust: Ramirez and Orchard 1990; *Lymnaea*: Gerhardt et al. 1997a). The Hill coefficient of 0.96 is also in agreement with the demonstrated value for a cloned OAR in *Lymnaea*, where various agonists were shown to have a Hill coefficient not significantly different than one (Gerhardt et al. 1997a).

4.4.2. Octopamine effects on the dynamic properties

The frequency response parameters for thrichobothria mechanosensory neurons were calculated before and after octopamine application (Fig. 19). Both fitted parameters of the

gain A and k increased, but only A was statistically different from the control value. This means that the mechanosensory neurons became more sensitive and fired more action potentials throughout the whole frequency spectrum. As expected from the observed increase in firing frequency, the information transfer rate also increased significantly following octopamine application.

4.5. Phentolamine effect on octopamine response

To assess the specificity of the octopamine response, I used the OAR antagonist phentolamine (Evans 1981). Phentolamine blocked the effect of octopamine but it also inhibited the firing frequency when applied alone at a concentration of 50 μM or more (Fig. 20). Phentolamine is known to act as a local anaesthetic in vertebrates by blocking conduction of action potentials along the axon (Northover 1983). However, it was also demonstrated that phentolamine inhibits insect mechanoreceptors at lower concentrations (200 to 500 μM) than those necessary to inhibit axonal conduction (Ramirez and Pearson 1990). Phentolamine is thought to act upon Na^+ channels, and it is suggested that, in insect mechanoreceptors, these channels are more sensitive to phentolamine in the encoder region than along the axon (Ramirez and French 1990; Ramirez and Pearson 1990). In spider, the concentration that was needed to inhibit the trichobothria neurons was four to ten times lower than previously reported in insects (Ramirez and Pearson 1990). One possible explanation for this high sensitivity is that the stimulus frequency used here was high. Indeed, the anaesthetic effect of phentolamine has been shown to be use-dependent, meaning its efficiency increased with stimulus frequency (Ramirez and Pearson 1990). Another possibility is that phentolamine in spider may act not only on voltage-activated Na^+ channels

but also on the mechanosensitive ion channels that are also selective to Na^+ (Höger et al. 1997). It is also possible that phentolamine could block the response of mechanosensory neurons to endogenous octopamine. However, under the experimental conditions used here, the it is likely that saline perfusion washed out humoral octopamine.

4.6. Second-messenger pathway of octopamine

To test whether the second-messenger pathway of spider octopamine receptors involved cAMP and the activation of a protein kinase A (PKA), two sets of experiments were undertaken. First, a membrane permeant analogue of cAMP, 8-Br-cAMP, was applied to the preparation while recording from mechanically activated trichobothria sensory neurons. 8-Br-cAMP has been previously used to assess the involvement of cAMP in the second-messenger pathway of various invertebrate preparations. For example, it was used in the crayfish neuromuscular junction, where serotonin is involved in enhancing the synaptic transmission via a cAMP pathway (Beaumont and Zucker 2000), in the silkworm prothoracic glands, where the release of the ecdysteroid hormone involved cAMP signaling (Dedos and Birkenbeil 2003), in the grasshopper brain, where locally applied 8-Br-cAMP was shown to elicit stridulation (Wenzel et al. 2002) and in locust, where the effect of octopamine on ionic currents of the jumping muscle could be mimicked by this membrane permeant cyclic nucleotide (Walther and Zittlau 1998). Concentrations of 8-Br-cAMP used in these experiments ranged from 300 μM to 5 mM. In the spider trichobothria neurons application of 1 mM 8-Br-cAMP increased the firing frequency, similar to what was observed when octopamine was applied (Fig. 21). This suggests that cAMP is involved in the second messenger pathway of OARs in spider.

To further test this hypothesis, I used Rp-cAMPS, a potent competitive inhibitor of PKA, that is membrane permeant and more resistant to phosphodiesterase than other cAMP analogs (Wang et al. 1992). Rp-cAMPS binds to PKA and keeps it in a locked conformation, unable to release its catalytic subunit (Dostmann 1995). This PKA inhibitor was also used in previous invertebrate studies. It was shown to block peptidergic activation of the locust DUM neurons by the tachykinin-related peptide, locustatachykinin (Lundquist and Nässel 1997), while in grasshopper it was used to inhibit stridulation induced by local application of muscarine in the protocerebrum (Wenzel et al. 2002). Rp-cAMPS clearly decreased the spider octopamine response, further suggesting that cAMP and PKA are involved in this response in spider mechanosensory neurons. This is in agreement with the idea that most OARs act through the cAMP/PKA pathway (reviewed by Roeder 1999, 2002). However, the experiments performed here cannot exclude the possibility that OARs may also act via a Gq protein and the phospholipase C pathway. In this case, phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is involved in the release of calcium from intracellular stores while DAG activates the protein kinase C (reviewed by Blenau and Baumann 2001). Indeed, some evidence suggests that OARs can act simultaneously through both cAMP and IP_3 pathways. When expressed in heterologous systems, cloned octopamine receptors showed variation in their second messenger coupling. In *Aplysia* a cloned octopamine receptor (ApOA1) was shown to activate specifically the PKA pathway, with no increase in IP_3 levels (Chang et al. 2000). However, in *Lymnaea* stimulation of the cloned octopamine receptor LymOA1 was shown to induce an increase in both inositol phosphates and cAMP (Gerhardt et al. 1997a). In addition, the three insect OARs cloned so far all

induced an increase in cAMP as well as in intracellular calcium when stimulated (Han et al. 1998; Grohmann et al. 2003; Bischof and Enan 2004). However, the mechanism of intracellular calcium increase was not investigated in detail and cannot be attributed with certitude to the IP_3 pathway.

5. Conclusions and future directions

In this study I wanted to discover if spider peripheral mechanosensory neurons are subject to cholinergic and/or octopaminergic modulation. This study provided no evidence for the presence of nAChRs in the spider mechanosensilla, but immunocytochemical experiments showed that mAChRs were present. However, neither their precise locations nor firm evidence of functional synapses with efferent fibers and/or glial cells could be established. To answer these questions, an electron microscopical study would probably be necessary.

The role of mAChRs in the spider mechanosensilla remained uncertain even after extensive series of electrophysiological experiments. It is possible that mAChRs are involved in regulating long-term adaptation, as suggested by the results from frequency response analysis. However, it is also possible that muscarinic receptors have other functions in these sensilla. They could modulate the neurons' response to other transmitters, as observed in the rat hippocampus (Markram and Segal 1990) or play a role that is not directly related to the regulation of neuronal excitability, such as triggering gene expression.

Immunocytochemical experiments showed that octopamine receptors were present in all mechanosensory neurons of the spider leg. They were concentrated in the proximal parts of the somata, in areas rich in efferent innervation and synaptic contacts. At least one efferent fiber adjacent to each mechanosensory neuron was immunoreactive to octopamine and contacted the sensory neurons, suggesting that intimate local release of octopamine occurs. Again, an electron microscopical study would probably be necessary to precisely assess the distribution of octopaminergic efferent fibers and their contacts with sensory neurons.

In electrophysiological experiments, octopamine strongly increased the sensitivity of mechanically stimulated neurons, as did tyramine. Phentolamine, an antagonist of octopamine receptors, blocked the octopamine effect but it also inhibited the neurons' excitability alone, suggesting a local anesthetic effect on Na^+ channels, as well as inhibition of octopamine receptors. Using a specific cAMP analog and a PKA blocker I demonstrated that octopamine receptors in the spider leg mechanosensory neurons act via the cAMP/PKA pathway.

Two major questions emerging from this work deserve further investigation in the future: 1) Do OARs also increase intracellular Ca^{2+} levels and if so, is this happening through the IP_3 pathway? Specific drugs affecting intracellular Ca^{2+} stores and/or Ca^{2+} channels could be used here, along with a specific phospholipase C inhibitor. 2) What are the precise ionic events resulting from octopamine application? Intracellular recordings from VS-3 neurons should be helpful in determining which ion channels are affected by octopamine.

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