THE ROLE OF TYRAMINE IN MODULATION OF SPIDER, *CUPIENNIUS SALEI*, MECHANORECEPTORS

by

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ABSTRACT

Efferent neurons containing GABA, glutamate, acetylcholine and octopamine (OA) innervate spider, *Cupiennius salei,* mechanosensory neurons. The spider leg transcriptome library contains several putative transmitter receptors including G-protein coupled α- and β-adrenergic OA and tyramine (TA) receptors.

I performed intracellular recordings from the strain detecting VS-3 neurons. TA caused a dose-dependent rise in action potential firing with an EC_{50} of 15.1 μM. VS-3 neuron sensitivity increased at all frequencies, but particularly in the high frequency range. Mianserin, an antagonist of β-adrenergic-like receptors, blocked the TA effect. Yohimbine, an antagonist of α-adrenergic-like receptors did not inhibit the TA effect, but reduced the baseline firing. These results indicate that the VS-3 neurons have at least two different adrenergic receptor types. The effects of TA and OA are similar, although OA is more potent. Thus, TA probably activates β-adrenergic OA receptors. The yohimbine effect indicates that α-adrenergic receptors maintain baseline sensitivity in VS-3 neurons.

LIST OF ABBREVIATIONS USED

5-HT	5-hydroxytryptamine (= serotonin)
8-Br-cAMP	8-bromoadenosine-3',5'-cyclic monophosphate
A	Sensitivity (at one hertz)
ACh	Acetylcholine
AChE	Acetylcholinesterase
AgCl	Silver chloride
AmOAR1	*Apis mellifera* (honeybee) octopamine receptor 1
AP	Action potential
AP/s	Action potentials per second
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
BmOAR1	*Bombyx mori* (silkmoth) octopamine receptor 1
C	Concentration
C. salei	*Cupiennius salei* (Central American wandering spider)
CA	California
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
cAMP	cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
Cl^-	Chloride ion
CHO	Chinese hamster ovarian cell line
CNS	Central nervous system
df	Degrees of freedom
EC_{50}	Half maximal concentration
Em	Membrane potential
f	Frequency
Fe	Femur
FL	Florida
GABA	γ-aminobutyric acid

$GABA_A$	ionotropic GABA receptor type A
G	Gain
GluCl	Glutamate gated chloride channel
G-protein	Guanine nucleotide-binding proteins
G_i	I type G-protein
G_q	Q type G-protein
G_s	S type G-protein
GPCR	G-protein coupled receptor
HEK-293	Human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O	Water
k	Fractional exponent
K^+	Potassium ion
KCl	Potassium chloride
MA	Massachusetts
MAO	Monoamine oxidase
Me	Metatarsus
$MgCl_2$	Magnesium chloride
n	Number of experiments
Na^+	Sodium ion
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OA	Octopamine
OAαR	α-adrenergic-like octopamine receptor
OAβR	β-adrenergic-like octopamine receptors
OAMB	Octopamine receptor in mushroom bodies
ON	Ontario
p-value	Estimated probability of rejecting the null hypothesis
P	Phase
Pa	Patella

PKA	Protein kinase A
R	Information capacity
RP-cAMPS	Adenosine-3',5'-cyclic monophosphate
SD	Standard deviation
SEM	Standard error of the mean
t	Time
TA	Tyramine
Ta	Tarsus
TAR1	Tyramine receptor type 1 = OA/TA receptor
TAR2	Tyramine receptor type 2
TAR3	Tyramine receptor type 3
Ti	Tibia
VS-3	Vorderseite-3 (Front side-3)

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Chapter 1. Introduction

Chapter 1.1. *Cupiennius salei*

Chapter 1.1.1. Origin

Cupiennius salei (Keyserling) are wandering spiders. French arachnologist Eugene Simon first coined the term *Cupiennius* in 1891. In 1963, Mechthild Melchers (University of Munich) described the spiders as a possible model organism for behavioral and physiological studies on spiders. Melchers came across the spiders by chance, when customs officials found them in a banana shipment of unknown origin. It was presumed to be from Central or South America at that time. Since then, *C. salei* have been among the most studied arachnids (Barth et al. 1988a; Barth 2002).

The size of the spiders varies depending on their nutrition. Average adult *C. salei* (older than 10 months) have body length of 2.7-4 cm, though the males have more slender bodies. Females have darker striations and fleshier legs than males (Figure 1.1.). Both male and female have an average leg span of 10-20 cm.

Chapter 1.1.2. Habitat

C. salei have been found in several regions of Central America, mainly the highlands of Guatemala and Eastern Mexico. They live in places that have an average temperature range of 15 - 20°C and a high humidity throughout the year. They are primarily arboreal and found in banana and agave plantations. They live on plants that have fleshy but structurally strong leaves (Barth et al. 1988b; Barth 2002).

Chapter 1.1.3. Feeding and Mating Habits

C. salei are nocturnal and do not spin webs. Instead, they wait in the shade at the base of the leaf stem, which is much cooler than the surroundings, during the day. They leave their retreat after sunset and await their prey, primarily depending on the vibrations caused by the prey on the leaves or movements of the air to detect them. The frequency range of these vibrations varies depending on the prey species. For example, vibrations caused by a cockroach have a wide bandwidth of 400-900 Hz while a fly produces a signal at about 100 Hz range. The spiders eat their catch right where they were caught and do not bring them back to their retreat. The retreat may be reused for more than a week or for just a day. The different types of prey include cockroaches, earwigs, flies, crickets and other small insects. Thus, *C. salei* plays an important role in their local ecosystem (Barth 2002).

Interestingly, similar to their mechanisms for catching prey, *C. salei* depend on vibration detection for sensing potential mates. This involves a back and forth parlay of taps on the leaves, between the male and female spiders, producing vibrations from the abdomen, pedipalps and legs. The male produces major vibrations by oscillations of their opisthosoma (posterior body) that are transmitted through the legs. They consist of 100-ms syllables followed by 100-ms pauses, with a prominent peak at 75-100 Hz. The pedipalps produce a brief signal at about 1 kHz. The female vibrations fall into a much lower frequency range (20-40 Hz) and lack the temporal pattern of male vibrations (Barth 2002).

Both these important aspects of the spider's life depend on mechanosensation, making it important for them to have well-developed mechanosensory organs. This has made them a preferred model to study mechanosensory neurons, as some of them are very large and give good access for electrophysiologists. Moreover, arachnids have cuticular

sensors in their exoskeleton that can be detached from the cuticle, making it easier to use in many experiments. These reasons, combined with the fact that *C. salei* can be bred and maintained in laboratory environment, make them a desirable model organism to work with.

Figure 1.1. *Cupiennius salei.* The photo shows an adult female *C. salei,* facing the camera. The two short pedipalps can be seen at the front. The white dotted circle marks the patella on a foreleg. The patella is where the VS-3 slit sensilla are found, in the anterior half. (Photo: Courtesy of Keram Pfeiffer).

Chapter 1.2. Arthropod Mechanosensilla

Mechanosensilla are specialized sensory organs that detect external or internal mechanical stimuli, such as touch, vibration and joint rotation, and translate it into

electrical signals (French 1988). For example, detecting movements of a prey and position of the body (proprioception) are aided by mechanoreceptors.

In arthropods, mechanoreceptors can be classified into two types. Type I are connected to exterior structures like hairs, while Type II are connected to internal structures, such as muscle. Type I have bipolar while Type II have multipolar sensory neurons (McIver 1986; French 1988).

There are three steps that occur during mechanotransduction: Coupling, transduction and encoding. Coupling refers to a deformation of the membrane of the sensory dendrite by a physical force, such as bending of a hair. This membrane deformation leads to transduction, an electrical current (receptor current) across the cell membrane. This current usually depolarizes the membrane and the signal is encoded to a train of action potentials (APs) that propagate via the axon toward the central nervous system (French 1988). Thus, a stimulus that the receptor received either from the external or internal environment has been encoded into electrical signals in the form of APs.

Chapter 1.3. *Cupiennius salei* Cuticular Mechanoreceptors

Spiders have extremely well developed mechanosensory organs that allow them to detect stimuli that are essential for their survival and reproduction. Dense hairs that detect touch cover the complete exoskeleton of many spiders. The density of these hairs in *C. salei* is 400 per mm^2. In addition, they have 90 filiform (trichobothria) hairs in each of their legs that are very sensitive airflow detectors. Stiff tactile spines are also found in the legs and each joint has several proprioceptive joint receptors. A special case of spider

mechanosensory organs are the strain detecting slit sensilla that are only found in arachnids (Barth 2002; Barth 2004).

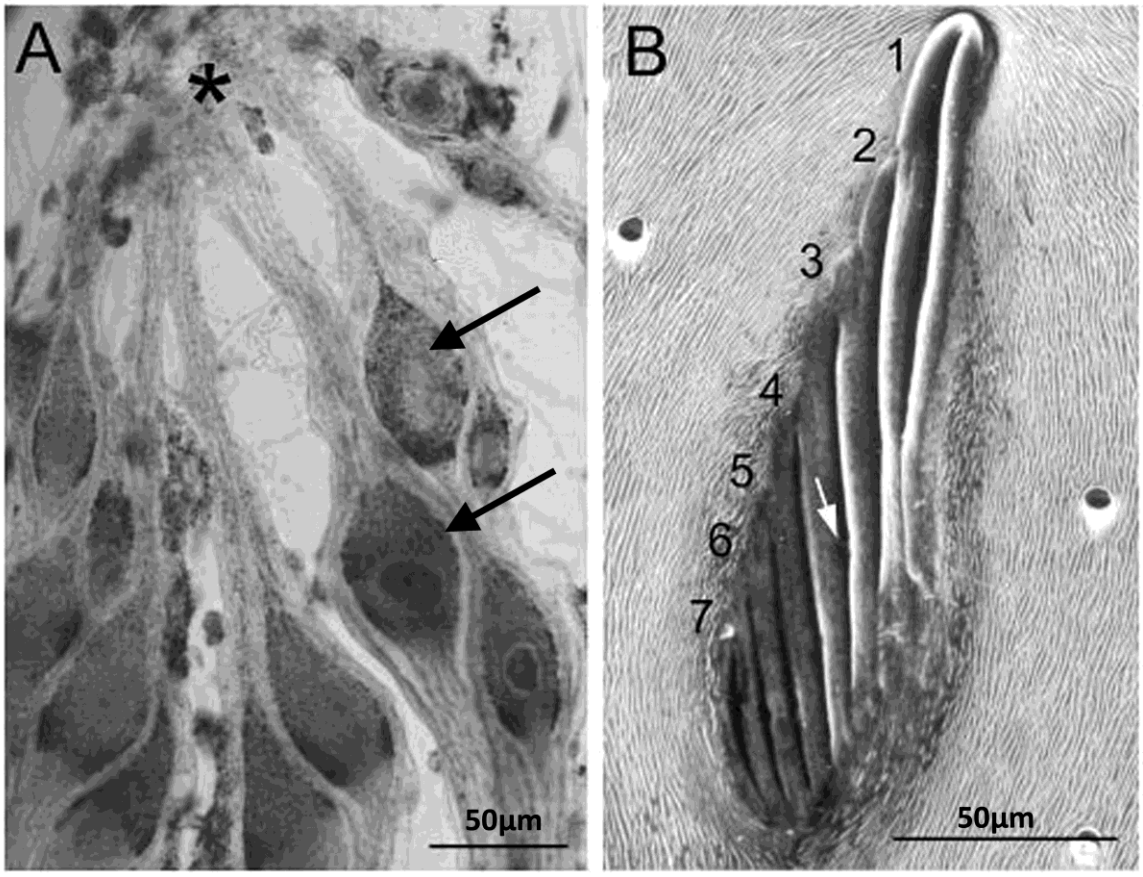

Figure 1.2. Organization of the VS-3 slit sensillum. A. Seven pairs of bipolar mechanosensory neurons in the spider patella hypodermis preparation, stained with toluidine blue. Arrows indicate somata of two neurons and asterisk is the dendritic tip region. **B:** Scanning electron microscopic image of the cuticular features shows the seven slits. The dendritic tips of the associated neurons insert near the center of each slit (white arrow). The leg is shaved and the round holes are locations of tactile hairs. Modified from (Fabian-Fine et al. 2000).

Chapter 1.4. Slit Sensilla

Spider slit sensilla are extremely sensitive strain detectors. They detect vibrations produced by mates, prey, and predators, and they are also used to detect skeletal strains caused by muscular activity and hemolymph pressure (Barth 2002). Slit sensilla are broadly classified into three types based on slit arrangement: single slits, groups of slits, and

compound “lyriform” sensilla in which the slit arrangement resembles a lyre (Figure 1.2.). Single slits are found all over the body surface, while the groups of slits are assembled in specific regions. The lyriform sensilla consist of 2-29 closely arranged slits composed in lyriform organs (Barth and Libera 1970; French et al. 2002).

The prevalence and percentage of slits vary greatly amongst arachnids. *C. salei* has close to 3300 slits (Barth and Libera 1970); most of them are on the legs and pedipalps. They have a total of 144 lyriform organs that are mainly found close to the joints (Barth 2002).

In a lyriform organ, each slit has a different stimulus threshold, but they respond to a similar overlapping range of amplitudes. In comparison with single slits, this gives lyriform organs a better ability to differentiate between stimuli and a higher sensitivity. Also, the range that the organ as a whole can respond to is higher than the sum of its parts. Thus, lyriform sensilla are more sensitive than single slits.

The slits are holes in the exoskeleton. These holes are covered by a 0.25 μm thick epicuticular membrane and bound by cuticular ridges (Barth 2002). There are three slit sensilla in the patella, two posterior and one anterior. The anterior slit sensillum is labeled VS-3 (VS = vorderseite = anterior side), and is activated when the tibia is deflected in the forward direction (Seyfarth 1978). The 7-8 slits of VS-3 are close to each other, each slit innervated by a pair of bipolar neurons (Figure 1.2.). In each pair, there are two types of neurons, they are both phasic neurons, but one adapts more rapidly to a step stimulus than the other (French et al. 2002). It is not possible to visually identify either type, since their positions vary in different legs. However, in the pair associated with the second slit, the

more rapidly adapting neuron is always significantly smaller than the more slowly adapting neuron (Fabian-Fine et al. 1999).

The VS-3 neurons are enwrapped by glial cells and the diameters of their somata vary in size from 20 μm to more than 100 μm (Figure 1.2.). The dendrites are attached to the slits and the mechanotransduction occurs in the dendritic tips (Höger et al. 1997). The VS-3 neurons are embedded in a thin hypodermis. A piece of cuticle that contains the slits and the neurons can be dissected from the patella and used as an experimental "cuticular preparation" (Juusola et al. 1994). With this preparation, the slits can be stimulated mechanically by pushing with a small glass probe during intracellular recording. The hypodermis can also be separated from the cuticle creating a "hypodermis preparation" where the dendrites are detached from the slits. This preparation can also be used for intracellular recordings and it allows rapid application and exchange of solutions for pharmacological experiments (Sekizawa et al. 1999). I used the hypodermis preparation in all experiments in this thesis.

Chapter 1.5. Efferent Innervation of Spider Mechanoreceptors

The mechanosensory neurons of all animals are regulated through efferent innervation from the central nervous system (CNS). In most cases, this modulation is presynaptic in the axon terminals that in vertebrates are located in the spinal cord and in arthropods in the central ganglia (Torkkeli and Panek 2002). In arachnids and crustaceans, synaptic modulation occurs also in the periphery (Fabian-Fine et al. 2002).

Chapter 1.5.1. Organization of Peripheral Synapses in *C. salei* Mechanosensilla

Occurrence of peripheral synapses in spiders was first demonstrated by Foelix (1975) and later described in many spider and scorpion species (reviewed by Fabian-Fine et al 2002). Using immunohistochemistry and immunoelectron microscopy and an antibody against the synaptic vesicle protein synapsin, Fabian-Fine et al. (1999; 2000) described the extent of synaptic contacts onto all peripherally located parts of the *C. salei* mechanosensory neurons. Synapses were shown to exist between efferent and sensory neurons, between neighboring efferent neurons as well as efferent neurons and glial cells.

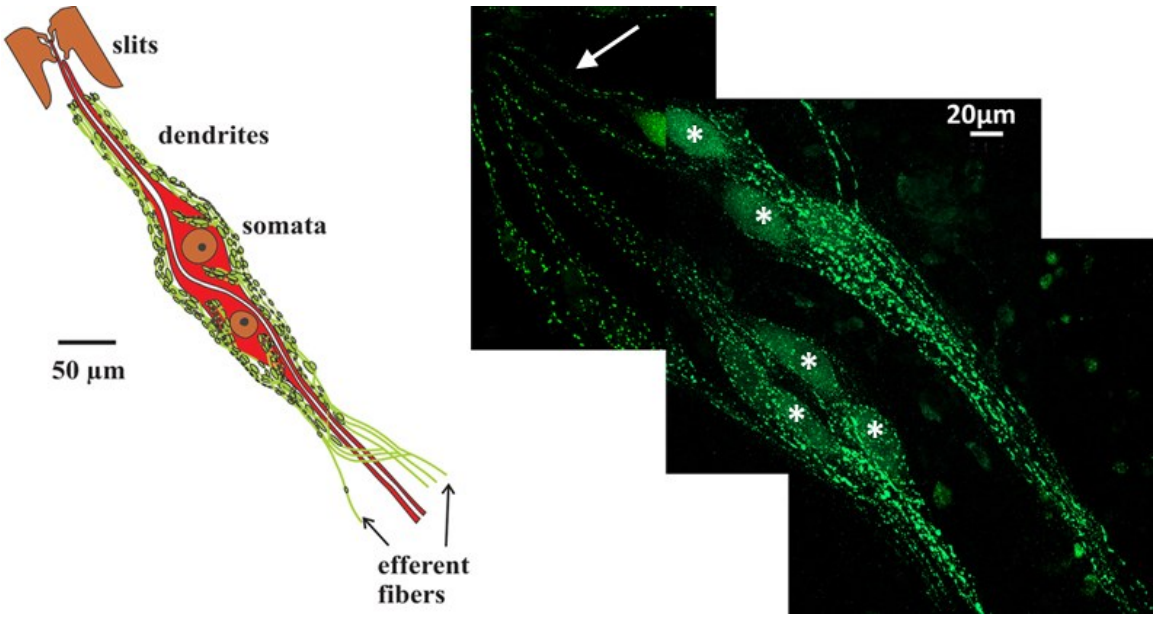

Figure 1.3. VS-3 neuron efferent innervation. On the left a schematic drawing of a single VS-3 neuron pair. The efferent fibers are shown in green and the VS-3 neurons in red. (Adapted from Fabian-Fine et al. 2000). On the right a confocal image of synapsin labeled efferent neurons on top of the VS-3 neurons (asterisks), arrow points to the dendrites (Courtesy of Shannon Meisner and Päivi Torkkeli).

Figure 1.3. shows synapsin immunostaining on the efferent fibers that surround the VS-3 neurons. Transmission electron microscopic examination showed that the efferent fibers that synapse with the VS-3 and other sensory neurons and glial cells contain several different types of synaptic vesicles. Some have electron-lucent vesicles; others contained clear vesicles, or granular or dense core vesicles. In some cases, there was a mixture of different vesicles inside the same efferent ending (Fabian-Fine et al. 2000). These findings suggest that several different transmitters may be released from the efferent neurons and some may contain more than one neurotransmitter or modulator.

Chapter 1.5.2. Neurotransmitters and Their Receptors in the *C. salei* Mechanosensilla

Choline acetyltransferase (ChAT) is the enzyme responsible for acetylcholine (ACh) synthesis. Immunoreactivity against ChAT was observed in all mechanosensory neurons of the spider leg (Fabian and Seyfarth 1997), indicating that these neurons use ACh as a neurotransmitter. One neuron of each pair also contained the ACh degrading enzyme acetycholineesterase (AChE) and both enzymes were present in some of the efferent fibers. This may indicate that ACh is also involved in efferent modulation of mechanosensory neurons. One of the sensory neurons in each pair also contained histamine that may serve as a co-transmitter in these neurons (Fabian and Seyfarth 1997).

Immunohistochemical investigation has revealed that some of the efferent neurons contain γ-aminobutyric acid (GABA), others glutamate, acetycholine or octopamine (Fabian-Fine et al. 2002; Widmer et al. 2005). Each of these transmitters also modulate the VS-3 and other mechanosensory neurons and many types of transmitter receptors have been located on the sensory neurons. GABA, a well-known inhibitory transmitter in all

animals, acts on both ionotropic and metabotropic receptors on VS-3 neurons (Panek et al. 2002; 2003). Activation of ionotropic $GABA_A$ receptors opens Cl^- channels, causes a large depolarization, decrease in membrane resistance and an increase in intracellular [Ca^{2+}]. During random noise stimulation, $GABA_A$ receptor agonists induce a brief inhibition followed by a long lasting excitation (Panek et al. 2002; 2008; Pfeiffer et al. 2009). Glutamate acts also on Cl^- channels (GluCl receptors) and causes a purely inhibitory response in VS-3 neurons (Panek and Torkkeli 2005; Torkkeli et al. 2012).

The spider mechanosensory and efferent neurons were strongly labeled by an antibody against muscarinic ACh receptors, but the role of these receptors has not yet been exposed. Interestingly, similar to $GABA_A$ receptor agonists, ACh inhibited a subgroup of the VS-3 neurons, likely also acting on Cl^- channels (Widmer et al. 2006).

The most important neuromodulator for this thesis is octopamine, which is considered the invertebrate counterpart of noradrenaline and acts on G-protein coupled receptors (Roeder 1999). Octopamine immunoreactive neurons were found in the *C. salei* central ganglia (Seyfarth et al. 1993) and on some of the efferent fibers innervating each mechanosensory organ in the spider leg (Widmer et al. 2005). Octopamine had an excitatory effect on the trichobothria neurons of the spider tibia (Widmer et al. 2005). The VS-3 neurons were also excited by octopamine and it slightly depolarized these neurons (Torkkeli et al. 2011). Recently, octopamine was shown to play a role in regulating mating tactic expression in the wolf spider *R. punctulata* (Hebets et al 2015).

Chapter 1.6. Tyramine and Octopamine

Biogenic amines tyramine (TA) and octopamine (OA) are the invertebrate analogs of adrenaline and noradrenaline (Roeder 1999). They are present in relatively high concentrations in neuronal and non-neuronal tissues of arthropods and other invertebrates. Both OA and TA act on a variety of G-protein coupled receptors. OA was first discovered in the salivary gland of an octopus (Erspamer and Boretti 1951). It has since been proven to act as a neurohormone that can be released into the circulation during stress or a neurotransmitter or modulator that is released locally close to the target cell (Evans and Maqueira 2005). OA modulates almost all physiological functions in invertebrates (Roeder 1999). TA was originally presumed to be only a metabolic precursor of OA, but has now been proven a neurotransmitter in its own right (Lange 2009).

Chapter 1.6.1. Biosynthesis of TA and OA

TA and OA are classified as biogenic monoamines. They are both synthesized from the amino acid tyrosine (Figure 1.4.). Tyrosine can be converted to TA by the enzyme tyrosine decarboxylase. TA is then converted to OA by tyramine-β-hydroxylase (reviewed by Roeder 1999; Lange 2009; Verlinden et al. 2010). An alternative pathway from tyrosine is conversion to dopamine. Dopamine can then be converted either to noradrenaline or to TA (Figure 1.4).

OA and TA act on their receptors for a short period followed by re-uptake using specific transporters or they can also be enzymatically inactivated. N-acetyl-transferase can inactivate OA after which it is processed by conjugases and the metabolites are completely

removed. Monoamine oxidase (MAO) enzymes that degrade biogenic amines in mammals are not believed to be important in removing OA in invertebrates (Verlinden et al. 2010).

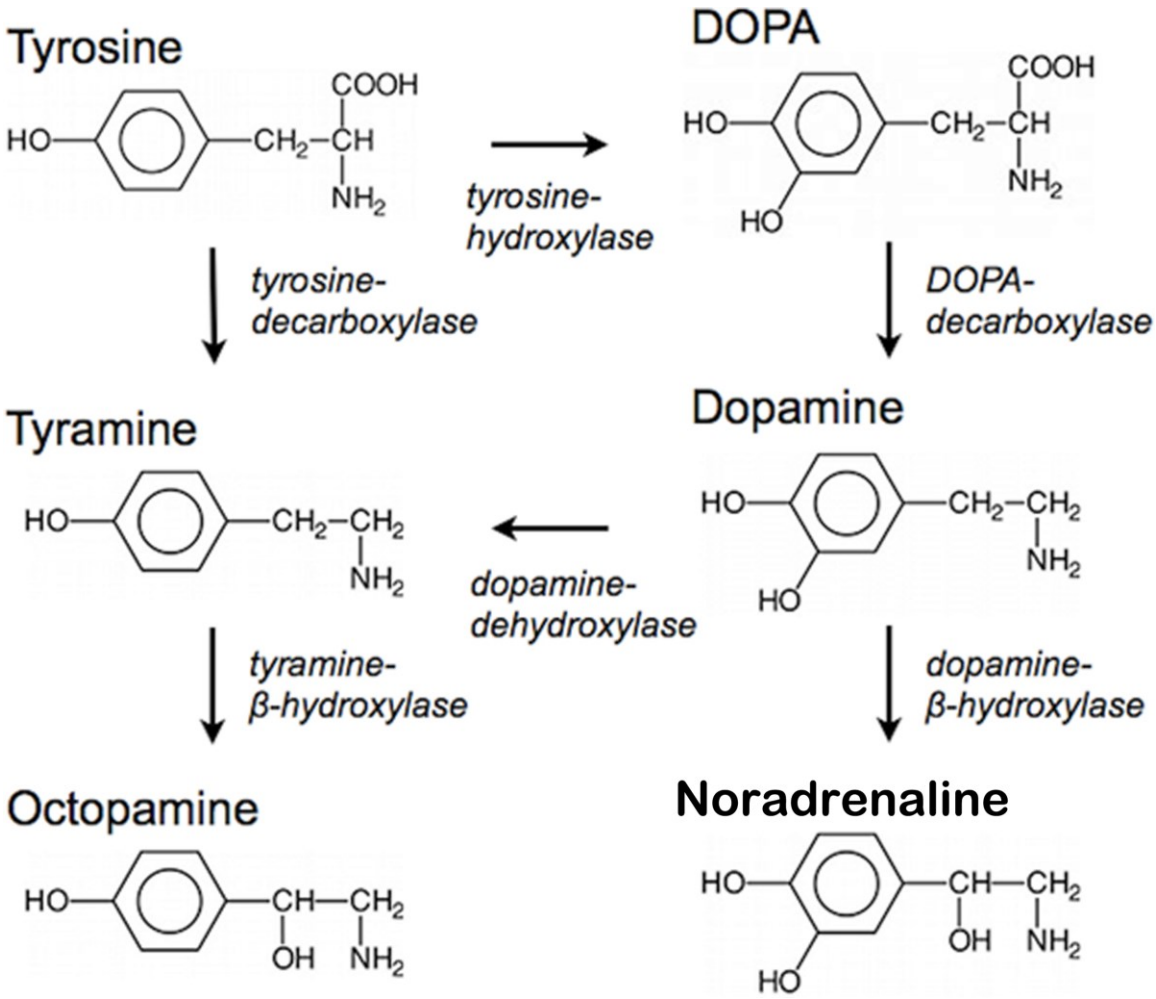

Fig 1.4. Biosynthesis of OA and TA. Tyrosine is transformed to TA by the enzyme tyrosine decarboxylase and TA can be converted to OA by tyramine-β-hydroxylase. The figure also shows the alternate pathway where tyrosine is converted to dopamine and norepinephrine.

Chapter 1.6.2. Physiological and Behavioral roles of OA and TA

Physiological roles of OA have been studied since Nathanson and Greengard (1973) found that low OA concentrations specifically stimulated adenylate cyclase enzyme in the

cockroach, *Periplaneta americana,* nervous tissue, leading to cAMP production. Since then OA has been shown to play many physiological and behavioral roles in a variety of invertebrate species and these have been reviewed by several authors (e.g., Roeder 1999; Roeder et al. 2003; Verlinden et al. 2010; Ohta and Ozoe 2014). Many of the roles of OA are related to stress and aim to adjust the body to situations that require energy (Adamo and Baker 2011). OA has been shown to regulate almost all muscles, including skeletal muscles in the leg, and flight muscles (Orchard et al. 1993) as well as muscles of the ovary and the contractile elements of the heart. Trachea, the fat body, and the hemocytes are also regulated by OA (Roeder 1999). In addition to the spider mechanoreceptor neurons, also locust, stick insect and cockroach mechanoreceptors and proprioceptors have been shown to be modulated by OA (Zhang et al. 1992; Ramirez et al. 1993; Bräunig and Eder 1998).

TA has been long studied to understand its role in invertebrates, more so since it was found to be a signaling molecule in its own right (Lange 2009). All octopaminergic neurons also contain TA, but some neurons contain only TA (Kononenko et al. 2009). These findings suggest that TA can have significant physiological roles independent of OA. Several reports suggest that OA and TA cause opposite behavioral effects in insects (Kononenko et al. 2009). However, some physiological effects are common for the two neuromodulators, including honeybee locomotion, blowfly and fruitfly flight (Ohta and Ozoe 2014).

Chapter 1.6.3. OA and TA receptors

OA and TA are believed to act on G-protein coupled receptors (GPCR). The original classification scheme for insect OA receptors was suggested by Evans (1981)

based on pharmacological characterization of responses to OA in locust leg muscles. In this scheme, OA receptors were classified as OA-1 or OA-2 receptors, with the latter group having several subclasses. OA-1 receptors mediate their actions by increase in intracellular [Ca^{2+}] while OA-2 receptor activation was linked to increase in intracellular cAMP. Even before any of the OA or TA receptors were cloned, it became obvious that multiple types of OA receptors are present, especially in a complex tissue (Evans and Robb 1993; Evans and Maquiera 2005).

Once several OA receptors were cloned and tested in expression systems a new classification scheme was suggested by Evans and Maqueira (2005). Since then, many more OA and TA receptors have been cloned in different invertebrate species and the classification has recently been modified by Ohta and Ozoe (2014) by addition of new subclasses. OA and TA receptors are now classified into five basic categories described below and summarized in the schematic Figure 1.5.

(i) <u>α-adrenergic like OA Receptor (OAαR)</u>

OAαRs share structural, functional and pharmacological similarity with vertebrate α_1-adrenergic receptors that mediate their effects by activating G_q proteins leading to increase in intracellular Ca^{2+}. This type of receptor was first cloned from *Drosophila,* and found initially in their mushroom bodies and was called OA receptor in mushroom bodies (OAMB) (Han et al. 1998). In addition to mobilizing intracellular Ca^{2+}, activation of this receptor also slightly increased cAMP concentration suggesting that it is also acting via G_s proteins (Han et al. 1998; Balfanz et al. 2005).

Orthologous receptors to OAMB have since been cloned from several other insect species (reviewed by Ohta and Ozoe 2014). When functionally expressed, their activation leads to rise in Ca^{2+} and cAMP levels. These effects are not linked to each other suggesting that OAMB receptors are independently coupled to G_q and G_s proteins (Huang et al. 2007; Verlinden et al. 2010; Beggs et al. 2011; Ohta and Ozoe 2014).

Recently, a new type of OA receptor, ChiloOAαR2 was cloned from the rice borer, *Chilo suppressalis* (Wu et al. 2014). This receptor is phylogenetically closest to vertebrate α_2-adrenergic receptors that act on G_i proteins and attenuate cAMP. When expressed in HEK-293 cells, activation of ChiloOAαR2 attenuated cAMP and stimulated Ca^{2+} release from intracellular stores.

All functionally expressed OAαRs can be activated by both OA and TA, to cause a change in cAMP and Ca^{2+} levels, but OA has a significantly higher potency than TA (Ohta and Ozoe 2014).

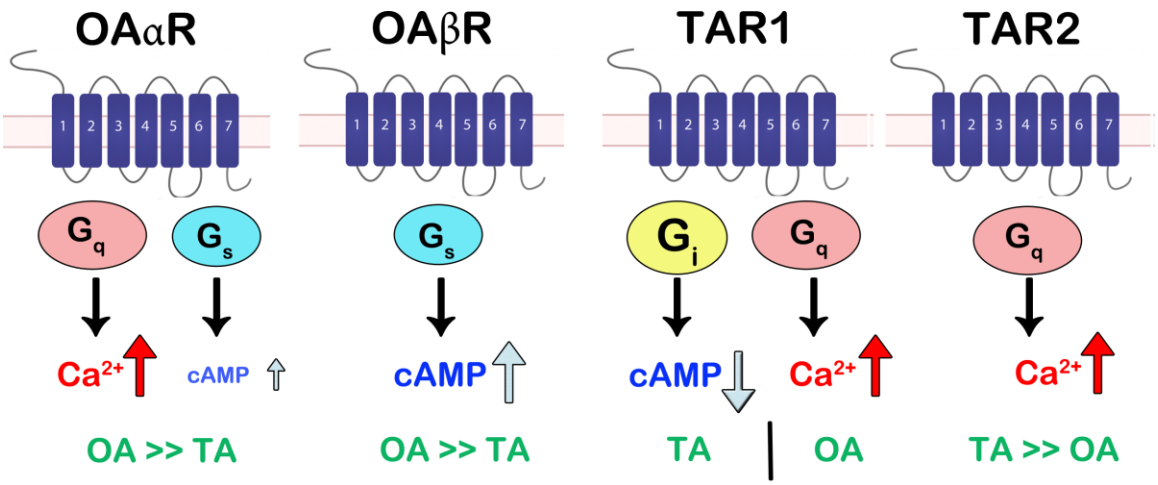

Figure 1.5. Schematic image of functional coupling of OA and TA receptors to G proteins and signal transduction. Potency of OA vs. TA to activate each receptor type is indicated below in green. (Courtesy of Päivi Torkkeli).

(ii) β-adrenergic like OA Receptor (OAβR)

Invertebrate OAβRs are orthologous to the vertebrate β-adrenergic receptors. They all induce an increase in intracellular cAMP concentration via stimulation of G_s proteins and have no effect on Ca^{2+} concentration (Evans and Maqueira 2005; Ohta and Ozoe 2014). OAβ receptors have been cloned from a large number of invertebrate species and most species have more than one type of OAβR. Receptors that have been tested in expression systems are activated by a significantly lower concentrations of OA than TA.

(iii) TA Receptor 1 or OA/TA Receptor (TAR1)

Two groups (Arakawa et al. 1990; Saudou et al. 1990) cloned the first *Drosophila* TA receptor simultaneously and since then orthologous receptors have been cloned from several invertebrate species. This receptor is often referred to as the OA/TA receptor because it can be activated by both agonists although they appear to act via different second messenger systems. TA is more effective in attenuating cAMP levels and OA more efficiently induced Ca^{2+} release from intracellular stores. The suggested mechanism behind this is that the receptor is linked to both G_i and G_q proteins, and selectively activates them based on which agonist is bound (Ohta and Ozoe 2014). This is similar to mammalian α_2 adrenergic receptors that are primarily coupled to G_i proteins and inhibit adenylate cyclase but can also stimulate phosphoinositide hydrolysis (Cotecchia et al. 1990).

(iv) TA Receptor 2 (TAR2)

Another TA receptor gene was cloned in *Drosophila* by Cazzamali et al. (2005). When expressed in Chinese hamster ovarian (CHO) cells, this receptor responded to a low

TA concentration by activating a G_q-protein, mobilizing Ca^{2+} from intracellular stores that triggered opening of Ca^{2+} activated Cl^- channels. Orthologous receptors have been found in several invertebrate species and those that have been tested appear to mobilize Ca^{2+} with no effect on cAMP (Huang et al. 2009). These receptors are all significantly more sensitive to TA than OA (Ohta and Ozoe 2014)

(v) TA Receptor 3 (TAR3)

Cazzamali et al. (2005) reported a third putative TA receptor type in *Drosophila*. A later comparative study showed that this receptor was clearly different to TAR2 and it was named TAR3 (Bayliss et al. 2013). Activation of TAR3 attenuated cAMP levels in a dose-dependent manner. TAR3 also caused an increase in Ca^{2+} levels. Thus, TAR3 seems to be linked to both G_i and G_q proteins. TA is more potent than any other amines in inducing either response. There has been no similar receptor reported in any other organism so far (Bayliss et al. 2013; Ohta and Ozoe 2014).

Chapter 1.6.4. OA and TA Receptor Pharmacology

Agonists bind to receptors and activate them while antagonists prevent the receptor activation. OA and TA receptor pharmacology has been investigated using general adrenergic antagonist and agonists (reviewed by e.g., Evans and Robb 1993; Verlinden et al. 2010; Ohta and Ozoe 2014). As described above, OA and TA can both serve as agonists of all these receptors, but their potency is variable. In some cases different agonists appear to activate a different signaling pathway and result in different cellular responses.

When the locust OA receptors, that were classified as OA-1 and OA-2 receptors, were investigated, their agonist and antagonist sensitivities were quite straightforward. For example, mianserin and metoclopramide blocked OA-2 receptors, but not OA-1 receptors while yohimbine blocked the OA-1 receptors but not the OA-2 receptors. Phentolamine blocked all types of receptors. However, it was more difficult to find drugs that would specifically act on only one of the OA-2 receptor subtypes (Evans and Robb 1993).

Now that many OA and TA receptors have been cloned, it has been possible to investigate their pharmacological profiles when the receptors have been functionally expressed in cell lines. Extensive reviews of pharmacological agents that have been tested have recently been published (Verlinden et al. 2010; Ohta and Ozoe 2014). In this thesis, I tested the efficiency of five antagonists: yohimbine, chlorpromazine, mianserin, metoclopramide and phentolamine.

Mianserin has been shown to bind strongly to all types of *Drosophila, Apis mellifera* and *Chilo suppressalis* OAβ receptors and it inhibits cAMP production at nanomolar concentrations (Maquiera et al. 2005; Wu et al. 2012; Balfanz et al. 2014). Significantly less efficient antagonists for these receptors (in rank order) were metochlopramide, phentolamine and chlorpromazine. Yohimbine had no effect on any of the OAβ receptors tested. Interestingly, the cAMP production of *Bombyx mori* BmOAR2 receptor was inhibited by only one antagonist, chlorpromazine, while none of the other four antagonists had any effect (Chen et al. 2010).

In binding studies, yohimbine has been shown to have a high affinity to two insect TAR1 and three OAαR1s. It is also a potent inhibitor of the cAMP attenuation of five different TAR1 receptors and of cAMP production in one OAαR1 (Ohta and Ozoe 2014).

Yohimbine blocks the Ca^{2+} rise in the *Bombyx mori* BmOAR1, and BmTAR2 and in the *Locusta migratoria* TAR1 type receptor (Poels et al. 2001; Huang et al. 2009; 2010). Thus, although yohimbine is generally considered an effective inhibitor of TA receptors (Lange 2009), it also appears to be an efficient inhibitor of OAαRs.

The pharmacology of only four OAαRs has been investigated and these studies have only tested a small number of antagonists. Therefore, the results are somewhat inconclusive. While yohimbine has been a very efficient inhibitor of all three receptors that have been tested, mianserin and metoclopramide were completely ineffective in inhibiting OA induced Ca^{2+} rise in BmOAR1 (Huang et al. 2010). Mianserin inhibited OA induced Ca^{2+} rise at relatively high concentrations in AmOA1 receptor (Beggs et al. 2011), but the efficiency of other antagonists in the present list were not tested. Note that phentolamine has only been tested in a binding study on one of the OAα receptors and it had a high affinity (Bischof and Enan 2004).

In binding studies the *Drosophila* and locust TAR1 receptors had significantly lower affinity to (in rank order) chlorpromazine, phentolamine and mianserin than to yohimbine (Arakawa et al. 1990; Saudou et al. 1990; van den Broeck et al. 1995). Chlorpromazine also blocked the cAMP reduction in three different insect TAR1 receptors while mianserin had this effect on only one of the four receptors that have been tested (Ohta and Ozoe 2014).

Bombyx mori TAR2 receptor is the only receptor in this category that has been tested. In addition to yohimbine, the OA induced Ca^{2+} rise was inhibited by chlorpromazine and mianserin, but at significantly higher concentrations than yohimbine (Huang et al. 2009).

In conclusion, it is clear that most of the adrenergic antagonists may bind and inhibit all types of OA and TA receptors, but there are large differences in their affinities. The β-adrenergic type receptors are most efficiently inhibited by mianserin with metoclopramide coming as a distant second and yohimbine has not effect on these receptors. Yohimbine is effective in inhibiting all three α-adrenergic type receptors; OAαR, TAR1 and TAR2. Phentolamine is a most efficient general inhibitor of all types of OA and TA receptors while chlorpormazine is less potent but also rather unselective.

Chapter 1.7. Objectives

The main objective of this thesis was to learn if and how the biogenic amine tyramine (TA) modulates the spider, *Cupiennius salei*, mechanosensory VS-3 neurons. Another goal was to find out on what types of receptors TA might act and whether more than one type of receptor is involved. There is significant evidence for the role of octopamine (OA) in modulating the spider mechanosensory neurons but much less is known about TA, even though it has a variety of physiological and behavioral roles in other invertebrates (Lange 2009).

Previous work has found OA immunoreactive neurons in the spider CNS and in some of the efferent fibers that innervate mechanosensory neurons in the legs (Seyfarth et al. 1993; Widmer et al. 2005). The axo-somatic region of all mechanosensory neurons in spider leg patella were immunoreactive to an antibody against *Drosophila* OAα1 type receptor (Han et al. 1998; Widmer et al. 2005). The VS-3 neurons respond to OA with a small depolarization and an increase in sensitivity to mechanical and electrical stimuli (Torkkeli et al. 2011). TA has only been tested in extracellular experiments on trichobothria

neurons in the spider tibia where it also enhanced their sensitivity, but was less potent than OA (Widmer et al. 2005). Recently, TA immunoreactive neurons have been found in the spider CNS in cells projecting to the legs (Ruth Fabian-Fine personal communication).

C. salei brain and hypodermis transcriptome libraries contain at least seven complete and three partial biogenic amine receptors (French 2012). These receptors include α- and β-adrenergic like OA receptors, TAR1 and TAR2 receptors and dopamine receptors. Therefore, it is likely that more than one type of adrenergic receptors are expressed in the mechanosensory neurons, the efferent neurons and/or other cells in the hypodermis.

My goals were to use electrophysiological and pharmacological methods to explore if TA modulates the VS-3 neurons, how it regulates the neurons' sensitivity, what types of receptors it acts on and whether they are the same or different receptors than those activated by OA. VS-3 neurons detect strains and vibrations that occur in the spider's environment or are caused by their body movements. These movements contain different frequencies at different behavioral situations. I used pseudorandom white noise stimulation in my experiments to investigate TA effects on different frequency components.

Physiological effects of biogenic amines on several invertebrate preparations have previously been shown to vary based on the agonist concentrations suggesting that different signaling pathways may be activated when agonist concentration exceeds specific levels. One of my goals was to establish the dose-response relationship of TA effects on VS-3 neuron sensitivity and membrane potential and to explore if different TA concentrations might cause different physiological effect.

TA and OA receptor pharmacology is relatively well documented in insect tissue and for several functionally expressed receptors (Evans and Robb 1993; Verlinden 2010; Ohta

and Ozoe 2014). Therefore, my aim was to test specific antagonists that are known to have different potencies to different types of OA and TA receptors to learn if the VS-3 neuron responses to TA can be inhibited by these drugs.

Chapter 2. Materials and Methods

Chapter 2.1. Experimental Preparation

Central American wandering spiders (*Cupiennius salei,* Keyserling) were raised in a laboratory colony at room temperature (22 ± 2°C) and a 13:11 hour light:dark cycle. I used both male and female spiders that were 11 - 14 months of age. I autotomized the spider legs by applying pressure at the coxa-trochanteral joint following a protocol approved by Dalhousie University Committee on Laboratory Animals.

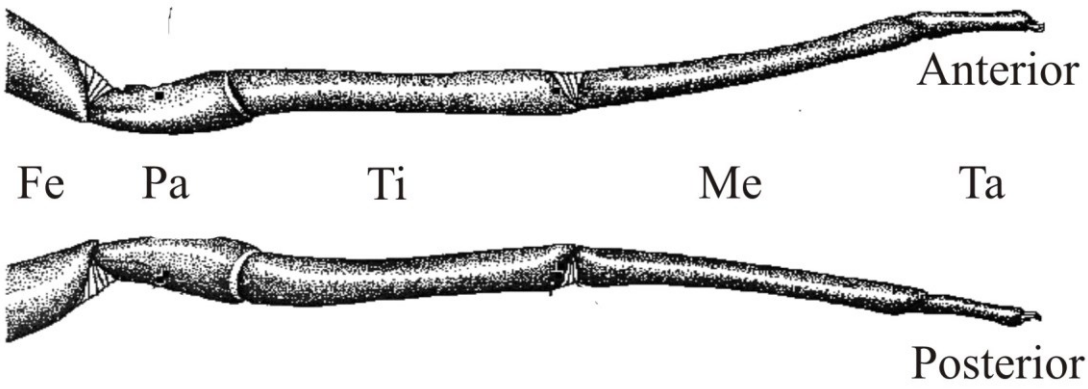

Figure 2.1. Shaved *Cupiennius salei* leg. Anterior and posterior view. Fe = Femur; Pa = Patella; Ti = Tibia; Me = Metatarsus; Ta = Tarsus. The anterior part of the patella contains the VS-3 slit sense organ. Image adapted from Seyfarth et al. 1978.

I dissected the anterior part of the patella (Figure 2.1.), which contains the VS-3 slit sensilla, while the leg was immersed in spider saline. I detached the hypodermis that contains the sensory neurons from the cuticle and attached the preparation onto a 12 mm glass coverslip (Fisher, Nepean, ON) following the protocol developed by Sekizawa et al. (1999). The coverslips were washed with concentrated nitric acid for 4-16 h, rinsed several times with distilled water and once with 100% ethanol. The preparation was allowed to rest for one hour in spider saline, to facilitate adherence to the coverslip. After this, the hypodermis preparation was transferred to a plexiglass recording chamber.

The preparation was grounded using agar salt bridges: A mixture of 2-5% agar in 1 M KCl was dissolved by heating and loaded into polyethylene tubing. AgCl coated silver wire connected to the amplifier headstage, was inserted into the agar-filled tubing. The preparation was superfused continuously by gravity with spider saline via polyethylene tubing (Figure 2.2.A). The saline was removed by a pipet pump (Drummond Scientific, Broomall, PA) creating a flow rate of about 1 ml/min. The recording chamber volume was maintained at about 400 µl at all times. 100 µl of tyramine (TA) at different concentrations was injected into the recording chamber via a separate tubing. The final TA concentration was approximately $1/4^{th}$ of the original concentration. The antagonists were added to the superfusion and gravity fed to the recording chamber for at least 15 min.

Chapter 2.2. Electrophysiology

Chapter 2.2.1. Intracellular Recording and Stimulation

I observed the VS-3 neurons using an upright microscope via 10x objective (Axioskop 2FS Plus; Carl Zeiss, Oberkochen, Germany). The preparation chamber was mounted on a Gibraltar stand (Burleigh, Mississauga, ON) that was placed on a vibration isolation table and surrounded by a Faraday cage (Technical Manufacturing, Peabody, MA) to prevent external noise.

Sharp intracellular microelectrodes were prepared from borosilicate glass (1.0 mm outer diameter, 0.5 mm inner diameter) (World Precision Instruments, Sarasota, FL) using a P-2000 laser puller (Sutter Instrument, Novato, CA). The electrodes were filled with 3 M KCl and their resistances were 40 to 80 MΩ in solution.

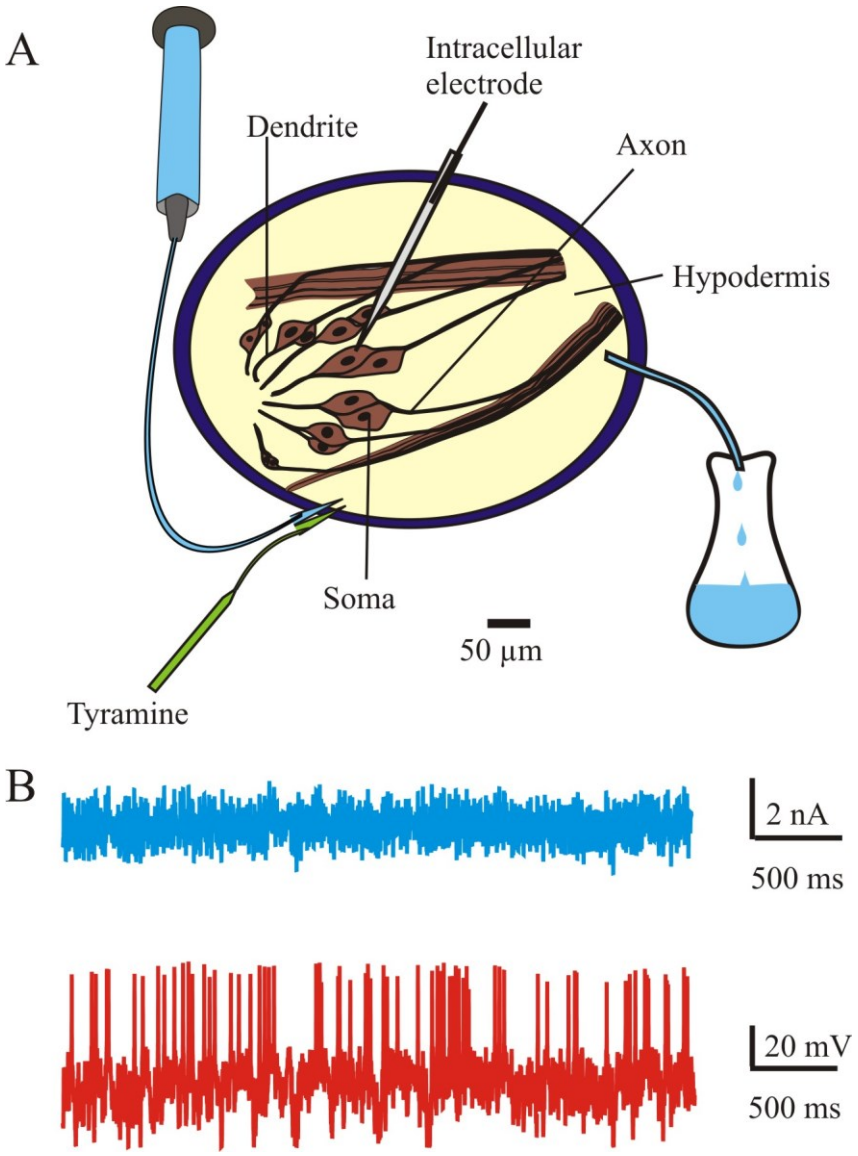

Figure 2.2. Experimental setup. A. shows the recording chamber with the VS-3 sense organ. The intracellular recording electrode is inside the soma of a VS-3 neuron. Current stimulation was applied via the same electrode. Spider saline was fed from a 10 ml syringe connected to polyethylene tubing. A separate 1 ml syringe and tubing was used for agonist (tyramine) application. **B**. A portion of the raw data during pseudorandom noise stimulation with the current input in blue and the recorded action potentials in red. Image adapted from Torkkeli et al. 2011.

The discontinuous single-electrode current-clamp method (Finkel and Redman 1984) was used in all experiments with a SEC-10L amplifier (NPI Electronic, Tamm, Germany). A switching frequency of 20 kHz was used and the duty cycle was 1/2 (current passing/voltage recording). The voltage was low-pass filtered at 33.3 kHz and the current signal at 3.3 kHz by the amplifier. Somata of the VS-3 neurons were impaled with the microelectrodes using a PatchStar micromanipulator (Scientifica, Uckfield, UK). The microelectrode was cleaned and cell penetration achieved by brief capacitance overcompensation using the amplifier "buzz" potentiometer. Only neurons that, after a 15 min stabilization period, had a stable resting potential of at least -60 mV and were firing action potentials with a step stimulus of 1 nA or lower were used for experiments.

VS-3 neurons were stimulated electrically via the intracellular recording electrode. An IBM-compatible personal computer and a data acquisition board (DT9812, Data Translation, Marlboro, MA) were used for all data recording and stimulation using custom-written software. The stimulus consisted of pseudorandom Gaussian white noise generated by the computer via a 33-bit maximum length binary sequence algorithm. This stimulus signal was low-pass filtered at 300 Hz, using a custom-built nine-pole active filter. An example of an experiment with pseudorandom current stimulus and resulting action potentials is shown in Figure 2.2.B.

The stimulus input signal and action potential output signal were initially sampled and stored at 10 kHz resolution to retain the action potential profiles. A software package then detected the action potentials offline using a threshold-detection algorithm and recorded them as Dirac delta functions by times of occurrence (French et al. 2001). The action potential signals were digitally filtered by convolution with a sin (x)/x function and

resampled at 1 kHz, to band-limit the frequency range to 0–500 Hz (French and Holden 1971).

Chapter 2.3. Frequency Response Analysis

The digitally filtered action potential signals were used as the output signals for the frequency response analysis. For the input, action potentials were removed from the original intracellular membrane potential recording by deleting 2 ms of data centered at each action potential, and filling the resultant gap by linear interpolation. Therefore, the fluctuating analog membrane potential formed the input and the action potentials the output. Input and output signals were converted to the frequency domain by the Fast Fourier Transform in segments of 1024 input–output data pairs (Cooley and Tukey 1965). Fourier components were resampled and averaged over frequency in exponentially increasing numbers to give an approximately equally sampled logarithmic frequency scale.

Frequency response and coherence functions were obtained by direct spectral estimation (Bendat and Piersol 1980) using averaged frequency components from multiple Fourier transforms (typically >100 averages). Frequency response functions were plotted as phase and log gain against log frequency (Bode Plots). They were fitted by the power-law relationship:

Equation 1: $G = Af^k$

Equation 2: $P(f) = k\,90° - \Delta t(f)\,360°$

where G is gain, f is frequency and A describes overall sensitivity (gain at 1 Hz), k is the fractional exponent, $P(f)$ is the phase lag as a function of frequency, and Δt is a time delay that includes the rise time of the action potentials.

Coherence functions $\gamma^2(f)$ (Bendat and Piersol 1980) between the input and output were plotted against log frequency. Linear information capacity (R) was calculated from the coherence function using the Shannon formula (Shannon and Weaver 1949):

Equation 3: $$R = \int \log_2 \{1/(1-\gamma^2(f))\}\, df$$

Chapter 2.4. Chemicals

All chemicals were purchased from Sigma Aldrich (Oakville, ON). Spider saline was prepared with 223 mM NaCl, 6.8 mM KCl, 8 mM $CaCl_2$, 5.1 mM $MgCl_2$ and 10 mM HEPES (Höger et al. 1997). 1 M NaOH was used to adjust the pH to 7.8.

Tyramine hydrochloride (T2879), chlorpromazine hydrochloride (C8138), mianserin hydrochloride (M2525), metoclopramide hydrochloride (M0763) and yohimbine hydrochloride (Y3125) were aliquoted in double distilled H_2O, at 10 mM concentrations and stored at -80°C. On the day of each experiment, the required concentration were prepared in spider saline. Phentolamine was prepared fresh on the day of each experiment directly in spider saline.

Chapter 2.5. Statistical Analysis

I performed the statistical analyses on the data using paired one-tailed *t*-tests for correlated samples. The analyzed data was either normally distributed or very close to

normalcy. The tests were done online using VassarStats software (http://vassarstats.net/). Statistical significances in the figures are depicted as follows: $p \leq 0.05$ = *; $p \leq 0.01$ = **; and $p \leq 0.001$ = ***.

Chapter 3. Results

I performed 70 intracellular recordings from the mechanosensory neurons innervating the spider VS-3 slit sensilla. After each cell was impaled with an intracellular electrode, its viability was tested using brief positive and negative current pulses while the cell was allowed to stabilize for at least 15 to 20 min before the actual experiment. Only neurons that had a stable resting potential of at least -60 mV and action potential amplitude of at least 40 mV were used for experiments. All experiments started with a control current-clamp recording, where the preparation was superfused in spider saline while the neurons were stimulated for six minutes with electrical pseudorandom white noise. Depending on the stimulus intensity, the neurons responded with either a constant firing rate or with an initial higher firing rate that adapted to a plateau during the first 100 s of the recording (Figure 3.1.B.). I used white noise stimulation rather than step or sinusoidal stimuli because it contains the wide bandwidth of stimuli that these neurons detect in their natural environment (Pfeiffer et al. 2009) and allows the complete frequency response and coherence functions to be determined from a single recording.

Chapter 3.1. Tyramine (TA) Effects on Spider VS-3 Neurons

To investigate if TA has an effect on VS-3 neurons, I made six minute long recordings using the same stimulus intensity as the control experiment, while the preparation was superfused in spider saline. TA was applied between 45 to 60 seconds after the start of the recording (Figure 3.1.). Between each control and TA experiment, there was a resting period of 8-10 minutes to allow the cells to recover from adaptation. If more than

one TA application was performed on the same neuron, the preparation was washed a minimum of 15 minutes before the second application.

Chapter 3.1.1. TA Effects on Action Potential Firing Rate and Membrane Potential

TA effects were investigated by observing changes in action potential firing rate and membrane potential during the 5 min recording period following application. I tested TA concentration from 1 nM to 1 mM. TA had no effect on the VS-3 neurons below 1 μM concentration. At and above 1 μM, TA application was followed by increase in firing rate and a membrane depolarization. Figure 3.1. shows an example where application of 50 μM TA depolarized the neuron by 7 mV and induced a four-fold increase in firing rate. Both effects began simultaneously, between 20 to 120 s after TA application and lasted from 2 minutes until the end of the recording.

The initial firing rate varied in different experiments and I observed that the TA effect on firing was stronger when the initial rate was low (below 10 AP/s) than when the rate was initially high. At very high initial firing rates (over 40 AP/s), the TA effect was very small, suggesting that the neuron was firing at its maximum frequency and TA was not able to produce higher firing rates. Differences in the baseline firing rate may be caused by heterogeneity of the 14 different VS-3 neurons in each preparation, their condition during the recording and differences in the capacitance of the recording electrode. I adjusted the stimulation intensity to produce as clear TA effect and as comparable results as possible. To standardize the results in later analyses, the TA induced spike rate changes were converted to relative changes with respect to the control.

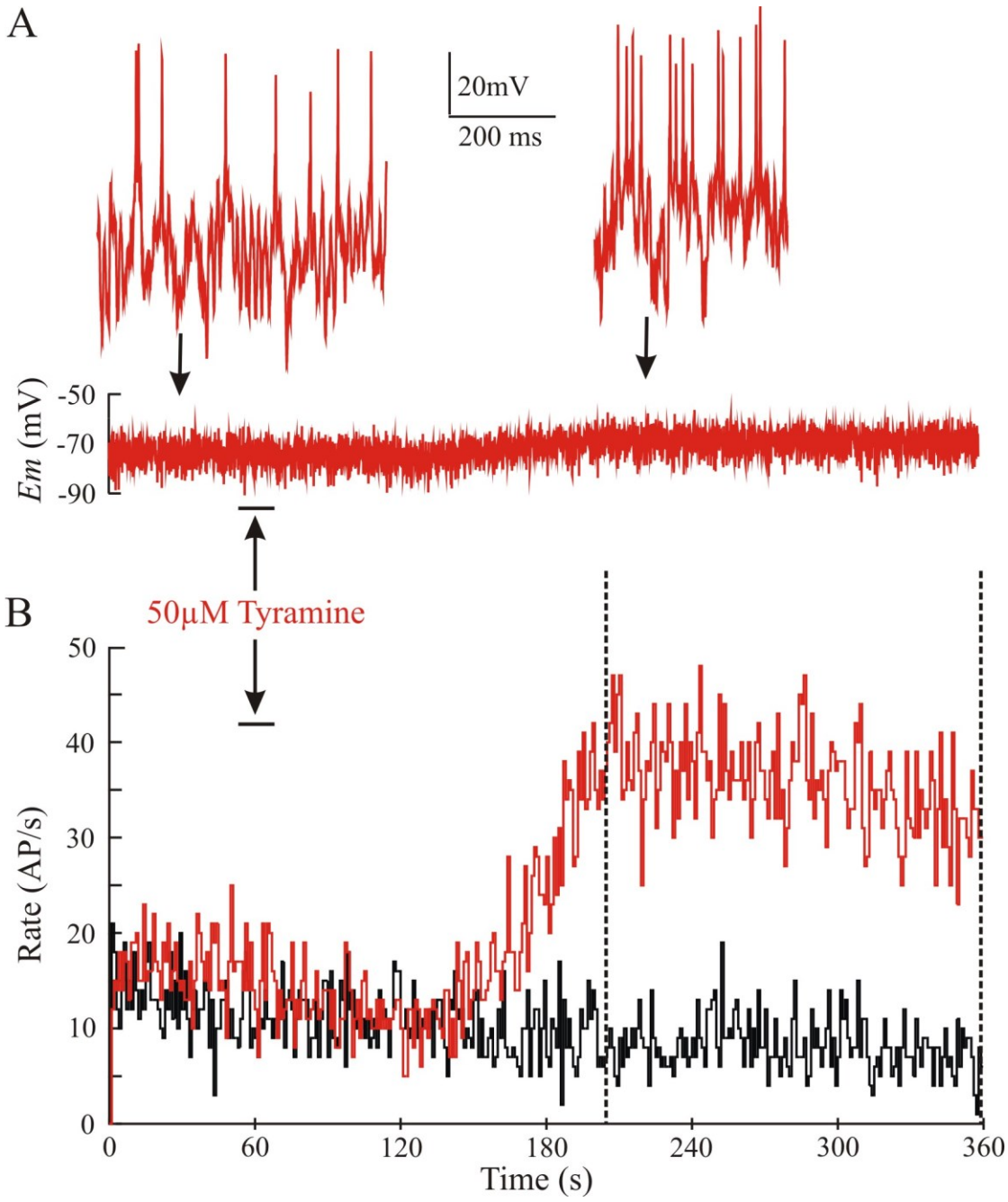

Figure 3.1. TA effect on a spider VS-3 neuron. VS-neurons were stimulated by pseudorandom white noise. TA was applied at the time indicated. **A.** Lower trace shows the membrane potential (*Em*) with the action potentials (AP) removed. TA depolarized this neuron by 7 mV. Upper traces show segments of the actual action potential recording before and after TA application at a different scale. **B**. The red trace is the firing rate from the recording shown in A and black trace is the firing rate from the same neuron without TA application. Dashed lines in **B** indicate the time period that was used for frequency response analysis.

Chapter 3.1.2. Dose Response Relationship

The maximum firing rate of each neuron was measured after application of 1 nM to 1 mM TA, the control rate was subtracted from this value to provide the change in firing rate. These values were then normalized and average values used to determine the dose-response relationship. If the firing rate during the first 60 s of recording were the same in the control and TA experiments, I used the same periods in these two recordings for "control" and "tyramine" AP rates. If there was a shift in the baseline firing, then the lowest firing level before the TA effect begin was used as the "control" rate. The TA effect on firing rate reached a saturation at about 50 μM, but started to diminish significantly at concentrations above 200 μM. Therefore, only the relative values obtained below this concentration were fitted with the logistic Hill equation (Figure 3.2.A.).

Equation 4: $Y = Y_{\max}[C]^n/([C]^n + [EC_{50}]n)$,

where Y is the response, $Y_{\max}$ is maximal response, $[C]$ is TA concentration, $[EC_{50}]$ is the half-maximal effective concentration, and n is the Hill coefficient. The Hill coefficient helps us understand the degree of binding of TA to the receptor. The Hill coefficient for TA effect on firing rate was 2.3 and EC_{50} was 15.1 μM.

Membrane depolarization also depended on TA concentrations. Similarly to the effects on firing rate, the depolarization was smaller with 200 μM and 500 μM TA concentrations, but there was no saturation below this and the effect was largest, although highly variable, at 1 mM (Figure 3.2.B.). Therefore, the Hill equation was used to fit this data at all concentrations above 1 μM. The Hill coefficient for this data was 1.7 and EC_{50} was 18.0 μM.

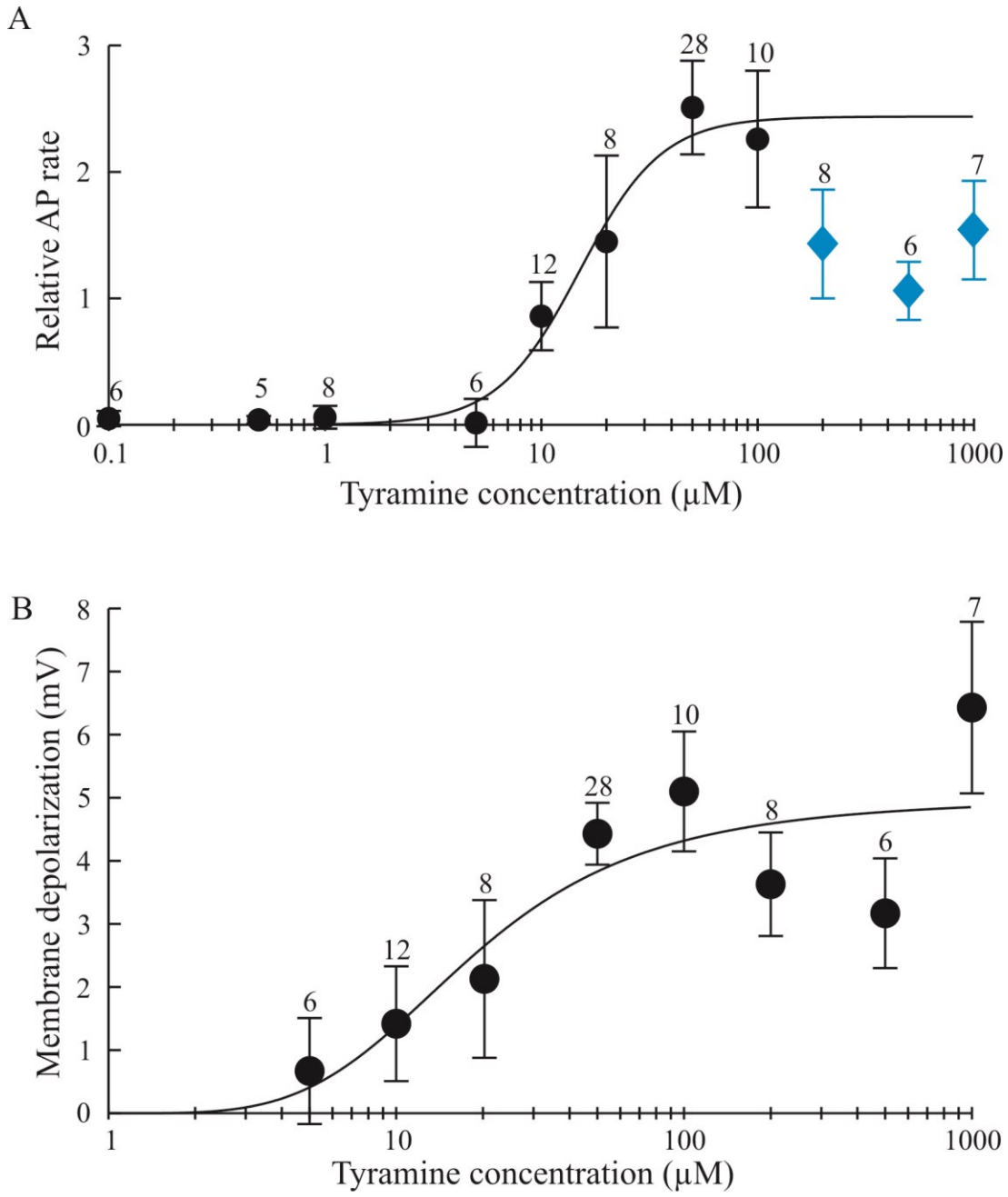

Figure 3.2. Dose response relationship of TA response. Relative change in action potential (AP) firing rate (**A)** and amplitude of membrane depolarization (**B)** plotted against TA concentration. The data was fitted with logistic Hill equation. TA effect on firing rate desensitized at high concentrations and therefore the fitting was done only on the data from concentration below 200 µM. The data points that were not fitted are indicated with blue diamonds. The results are shown as mean ± SEM with the number of experiments indicated above each error bar.

Chapter 3.2. TA Effects on VS-3 Neuron Frequency Response

To investigate whether the changes in the effects of TA on VS-3 neuron sensitivity to electrical stimulation depend on stimulation frequencies, I performed frequency response analysis. Figure 3.3. is an example that shows the fitted gain, phase and coherence functions using the data from the period of elevated firing rate after TA application and the corresponding period in the control recording (area between the dashed lines in Figure 3.1.B). This frequency response function was well fitted by the power law relationship (Equations 1 and 2, Materials and Methods), and the fitted parameters A (sensitivity) and k (fractional exponent) were significantly larger during the TA response than under control conditions. Information capacity R was obtained from the coherence function (Equation 3) and was also larger during the TA effect.

I fitted the data from twelve experiments where the results from both the control and 50 μM TA experiments could be well fitted with the power law functions to obtain values for parameters A, k and R. To quantify changes that occurred after TA application I used one tailed paired t-test. TA caused highly significant increases in the sensitivity A (t = -6.35; df = 11; $p < 0.0001$) and the information capacity R (t = -6.43; df = 11; $p < 0.0001$) and less significant change in the fractional exponent k (t = -2.25; df = 11; p = 0.0229). Increase in R occurs when firing rate increases, since faster action potentials allow more information to be encoded (Pfeiffer and French 2009). The rises in A indicated that the VS-3 neurons became more sensitive generally, and the rise in k indicated that there was also a relative increase in sensitivity to high frequency stimuli, or more rapid adaptation after TA application (French et al. 2001).

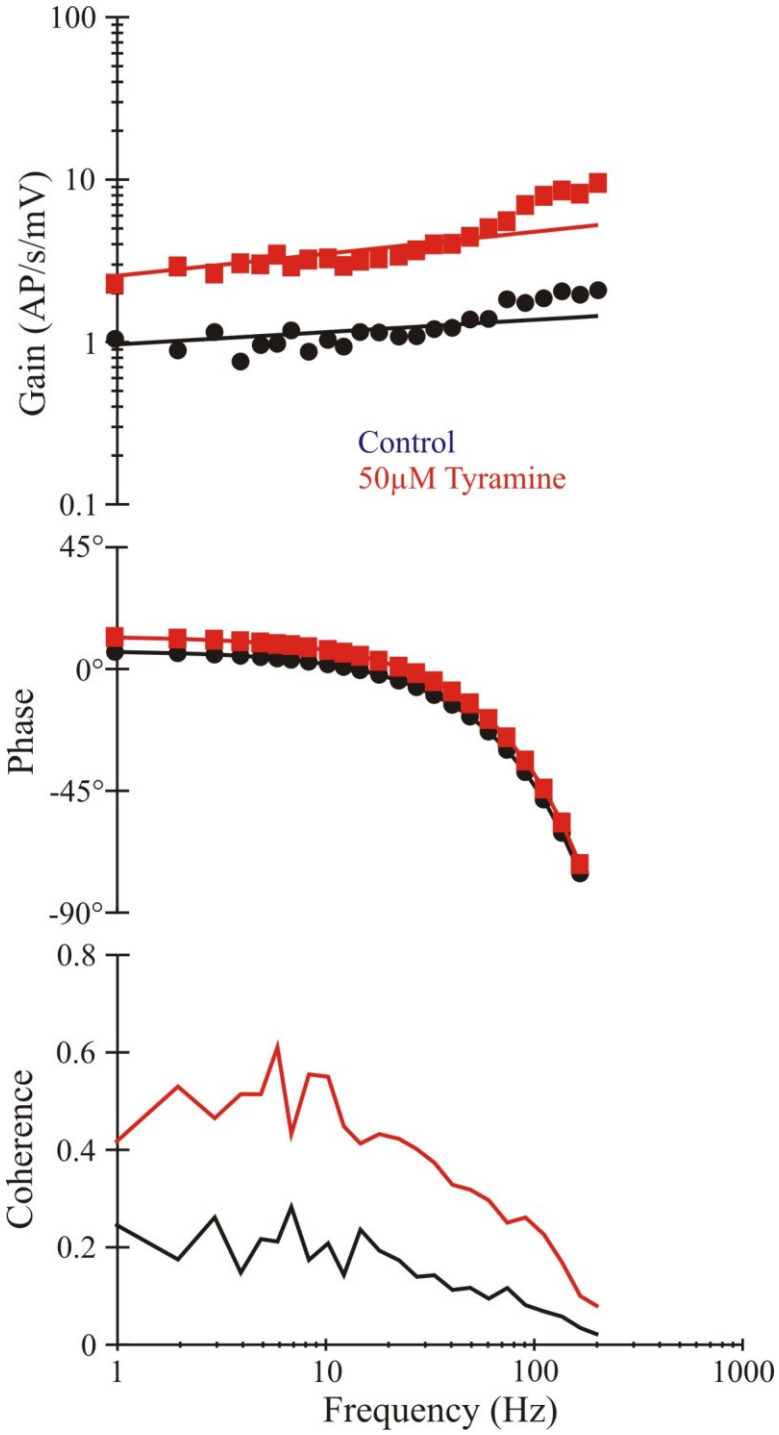

Figure 3.3. Frequency response analysis. Frequency response and coherence functions for the excitatory period of the experiment with TA application shown in Figure 3.1 (area between the dashed lines) and the corresponding period in the control recording. Solid lines show Equations 1 and 2 fitted to the gain and phase with $k = 0.06$ and 0.18, $A = 0.8$ and 1.2 AP/s/mV and the information capacities (R) from Equation 3 were 70.8 and 115.7 bits⁄s for control and TA experiments, respectively.

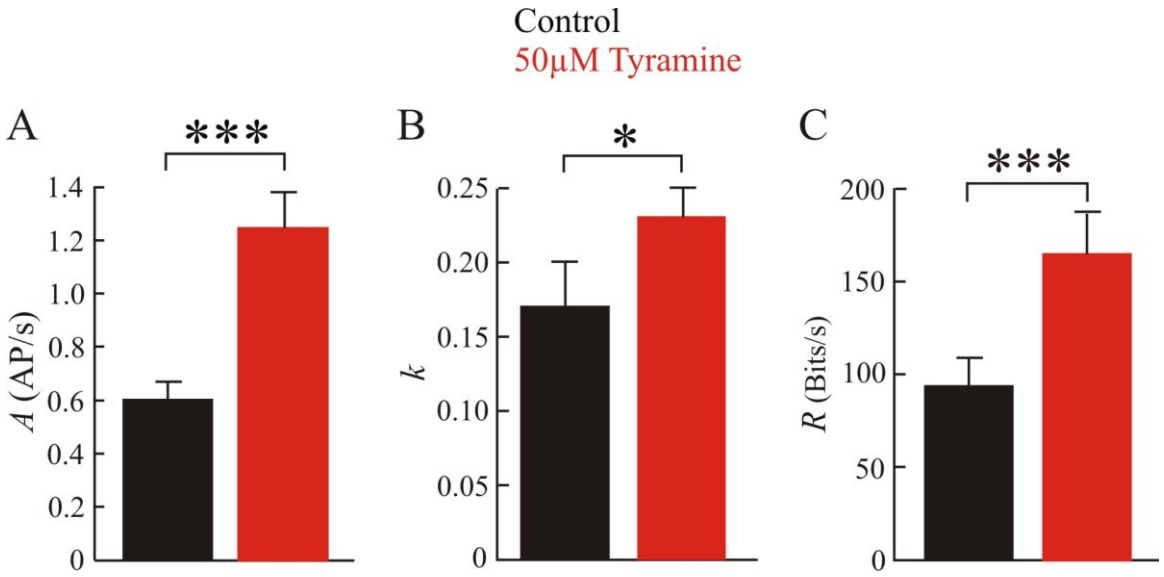

Figure 3.4. Frequency response parameters. The average (±SEM) sensitivities (*A*), fractional exponents (*k*) and information capacities (*R*) calculated from Equations 1, 2 and 3 from twelve experiments similar to those in Figure 3.3. Values of all parameters increased significantly after TA application.

Chapter 3.3. Effects of Adrenergic Antagonists on the TA Response

I attempted to characterize the types of receptors TA acts upon in VS-3 neurons by testing adrenergic antagonists that have been used to distinguish between different types of OA and TA receptors in insect tissue and/or have shown high affinity to cloned receptors expressed in cell lines (Evans 1981; Evans and Robb 1993; Ohta and Ozoe 2014). These antagonists are all also specific to different types of mammalian adrenergic receptors.

I used similar experimental protocols as described above with pseudorandom noise stimulation and intracellular recording. Once an impaled VS-3 neuron had stabilized for 15-20 min, I made 6-min long control recording while the preparation was superfused in normal spider saline without addition of any agonists or antagonists. Following an 8 to 10-min resting period, a similar recording was repeated, but TA was applied 45-60 s after the

beginning of the recording. Cells displaying a good TA response were selected for proceeding with antagonist experiments.

I added the antagonist to the superfusion for a minimum of 15 to 20 minutes. After this, I repeated the TA experiment while the antagonist was still present. At the end of this experiment, I washed the preparation with normal spider saline for 20 to 45 min. The TA experiment was repeated every 20 min during the washing period. By this stage, the cells had been exposed to at least two doses of TA and 30 minutes of antagonist. The complete experiment took at least 2.5 hours. It is very difficult to maintain a stable experiments for such a long time, and some cells died or the electrode came out after the experiment with antagonist and I did not get the final recording after the wash. I also noticed that some antagonists, appeared to cause increased deterioration of the cells.

Chapter 3.3.1. Yohimbine Effects on VS-3 Neuron TA Response and Baseline Firing

Yohimbine is an α2-adrenergic antagonist in vertebrates that has a high affinity for invertebrate α-adrenergic OA and TA receptors, but has a significantly lower affinity for β-adrenergic OA receptors (Evans and Maqueira 2005; Lange 2009; Ohta and Ozoe 2014). I tested yohimbine in two different concentrations, seven experiments with 10 μM and one experiment with 50 μM.

A typical example of the TA effects on spike rate with and without 10 μM yohimbine is shown in Figure 3.5.A. During yohimbine incubation the baseline firing decreased, TA effect was smaller than control and both of these effects reversed after wash. The baseline firing rate was higher than control after wash. When the results from seven experiments (one with 10 μM and three each 20 μM and 50 μM TA) were

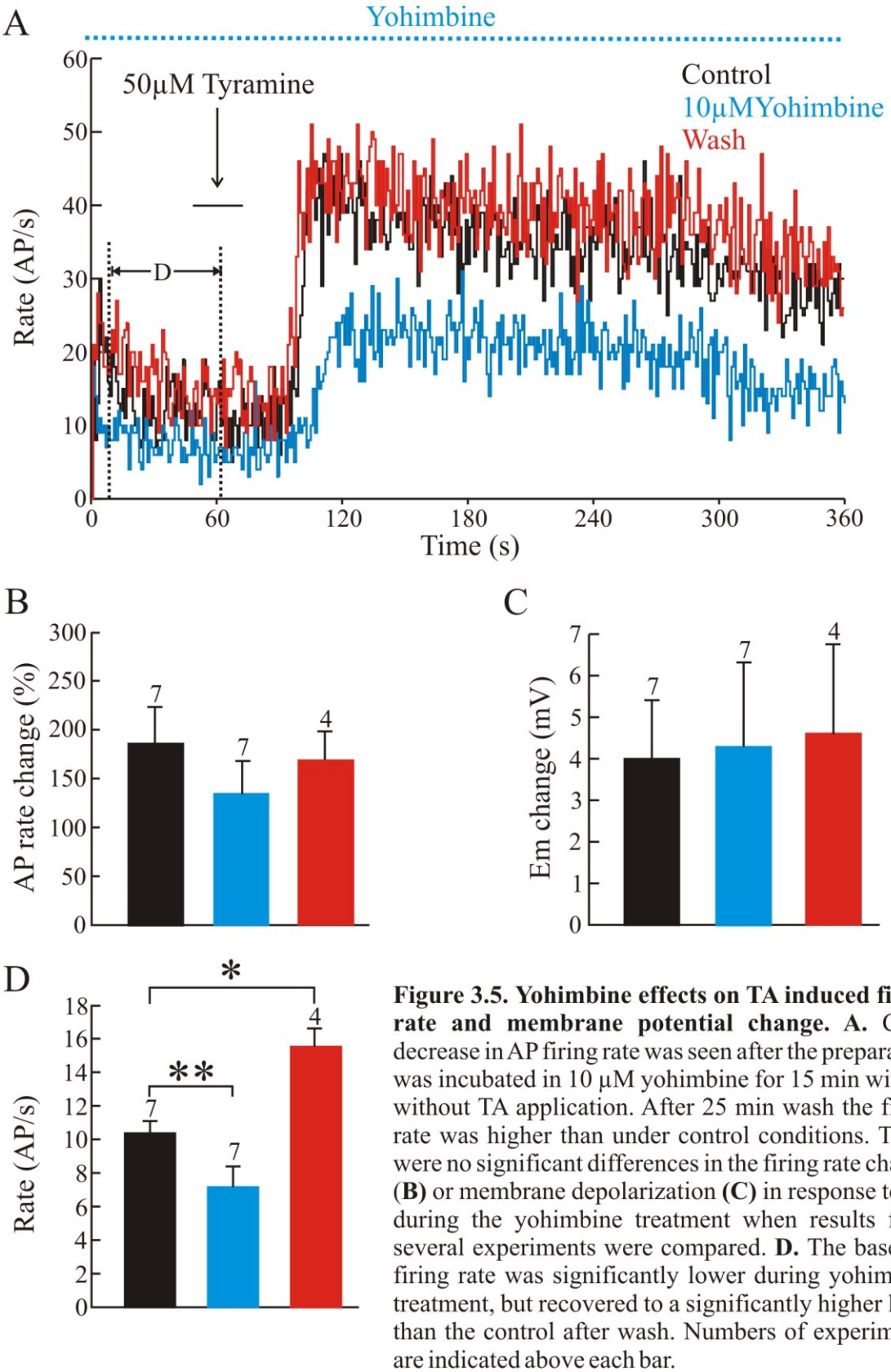

Figure 3.5. Yohimbine effects on TA induced firing rate and membrane potential change. A. Clear decrease in AP firing rate was seen after the preparation was incubated in 10 μM yohimbine for 15 min with or without TA application. After 25 min wash the firing rate was higher than under control conditions. There were no significant differences in the firing rate change **(B)** or membrane depolarization **(C)** in response to TA during the yohimbine treatment when results from several experiments were compared. **D.** The baseline firing rate was significantly lower during yohimbine treatment, but recovered to a significantly higher level than the control after wash. Numbers of experiments are indicated above each bar.

compared, there was no significant change in TA induced increase in firing rate (t = 1.41, df = 6, p = 0.1) or depolarization (t = 0.24, df = 6, p=0.41). Results from four neurons that survived long enough for an experiment after wash were similar to the control as seen in Figures 3.5.B and C.

However, the decrease in baseline firing was statistically significant. The firing rates were measured from 10-60 s after the start of each experiment (dashed vertical lines in Figure 3.5.A) and the results tested with one-tailed paired t-test (t = 0.12; df = 6, p = 0.012). As seen in Figure 3.5.A and D there was also a rebound effect after yohimbine was removed from the superfusion. The baseline firing rate was significantly higher than in the control in the four experiments that survived until the end of 15-30 min wash in normal saline (t = -3.54, df = 3, p = 0.019).

I also did one experiment using 50 μM yohimbine (Figure 3.6.). In this experiment, a similar decrease in baseline firing rate was observed as with 10 μM yohimbine and this effect was fully reversible. The TA induced change in firing rate was also clearly smaller after yohimbine treatment, recovering partially after 30 min wash in normal saline. In addition, the TA induced membrane depolarization was smaller after yohimbine treatment and partially recovered after wash (*Em* control 8 mV, yohimbine 3 mV and wash 5 mV)

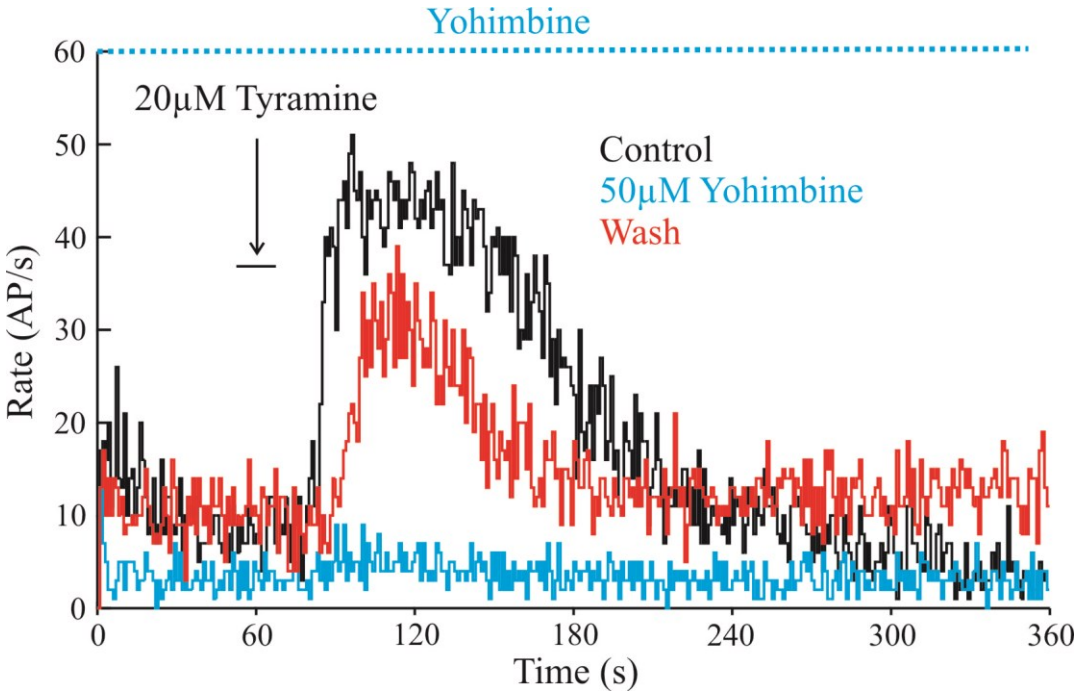

Figure 3.6. Effect of 50 μM yohimbine on VS-3 neuron firing rate and TA response. Both the baseline firing rate and the **TA** induced increase in firing rate were attenuated by 50 μM yohimbine and recovered at least partially after 30 min wash.

Chapter 3.3.2. Chlorpromazine Effects on VS-3 Neuron TA Response

Chlorpromazine is an antipsychotic drug whose main targets are dopamine 2 receptors, but it also acts on other types of mammalian G-protein coupled dopamine and 5-HT receptors. It has high affinity for some invertebrate TA receptors, but also acts on several types of OA receptors (Evans and Maquieira 2005; Ohta and Ozoe 2014).

I performed ten experiments with 10 μM chlorpromazine. Of the ten, one was done with 500 μM TA, five with 50 μM and the remaining four with 10 μM TA. Figure 3.7.A shows an example where TA induced change in firing rate was slower and smaller when chlorpromazine was in the superfusion, but this effect became even stronger after 15 min wash in spider saline. In the experiment shown in Figure 3.7.B, the firing rates were very similar whether chlorpromazine was in the superfusion or not when 50 μM TA was applied.

The majority of the neurons (7 from 10) had similar responses with and without chlorpromazine. In three experiments, chlorpromazine treated neurons had a smaller response to TA. In two of these, the cell died before washing period was completed. There was no difference in the TA induced depolarization in the ten experiments ($t = 0.56$, $df = 9$, $p = 0.29$).

There was no significant changes in the baseline firing rate when the first 10-60 s of firing in the control experiment (mean ± SD: 11.9 ± 3.1 AP/s, n=10) was compared to the experiment with chlorpromazine (12.8 ± 6.7 AP/s, n=10). However, the average firing rate was somewhat smaller during the wash (9.3 ± 5.1 AP/s, n=4). Only four neurons survived until the end of washing period and, similarly to the experiment shown in Figure 3.7.A, they did not respond to tyramine as well as during control conditions or during chlorpromazine treatment. Chlorpromazine appeared to reduce the viability of the VS-3 neurons rather than have any specific effect on their response to tyramine.

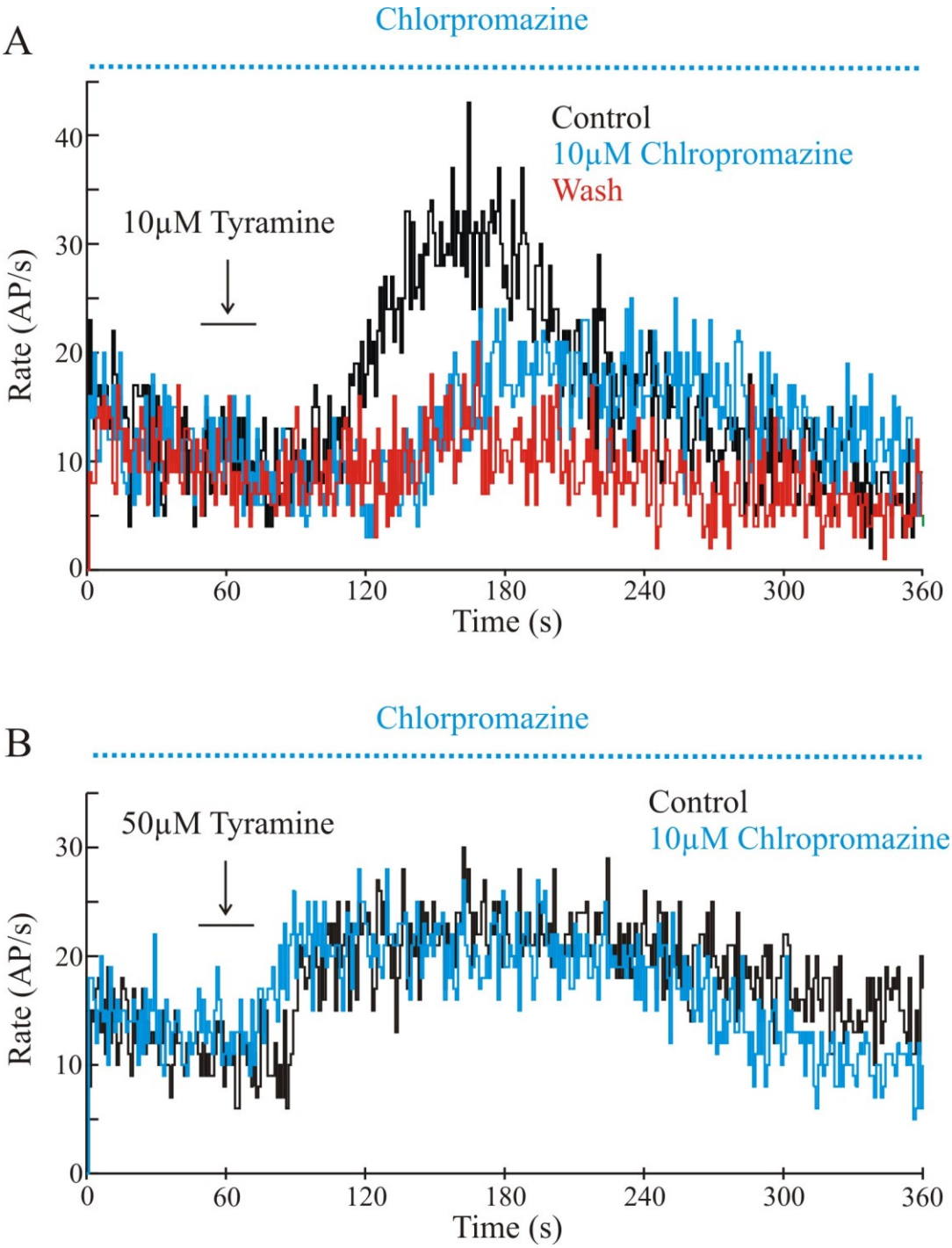

Figure 3.7. Chlorpromazine effects on TA induced change in firing rate. A. Shows an experiment where TA application induced a smaller effect on firing rate when 10 µM chlorpromazine was in the superfusion than under control conditions. After 15 min washing period, the TA effect on this cell was even smaller. **B.** An experiment where 50 µM TA caused very similar change in firing rate with and without chlorpromazine in the superfusion.

Chapter 3.3.3. Mianserin Effects on VS-3 Neuron TA Response

Mianserin is a tetracyclic antidepressant whose main targets are presynaptic α2 and 5-HT2 receptors. In insects, it has a very high affinity for β-adrenergic type OA receptors (Evans and Maquiera 2005; Balfanz et al. 2014).

I performed five experiments where the VS-3 neurons were superfused with 10 μM mianserin. In four of these experiments, I used 100 μM TA and in one 200 μM TA. In all experiments both the TA induced firing rate change and the depolarization disappeared (Figure 3.8). When tested with a paired one-tailed t-test, both mianserin effects were statistically significant (Spike rate change: $t = 4.12$, $df = 4$, $p = 0.0073$. Membrane depolarization: $t = -6.04$, $df = 4$, $p = 0.0018$).

The spike rate response did not recover even after washing for up to 30 min, but the depolarization effect recovered partially, as can be seen in Figures 3.7.B and C. All but one of these neurons survived at least 15 min washing period and two were washed for 30 min. These results indicate that mianserin is a very potent inhibitor of the VS-3 neuron TA response.

The baseline firing measured between 10-60 s from the beginning of each experiment did not change during mianserin superfusion. It was 10.2±3.0 AP/s (mean ± SD) in the five control experiments, 9.7±2.8 during mianserin superfusion and 9.3±3.9 in the four experiments after wash.

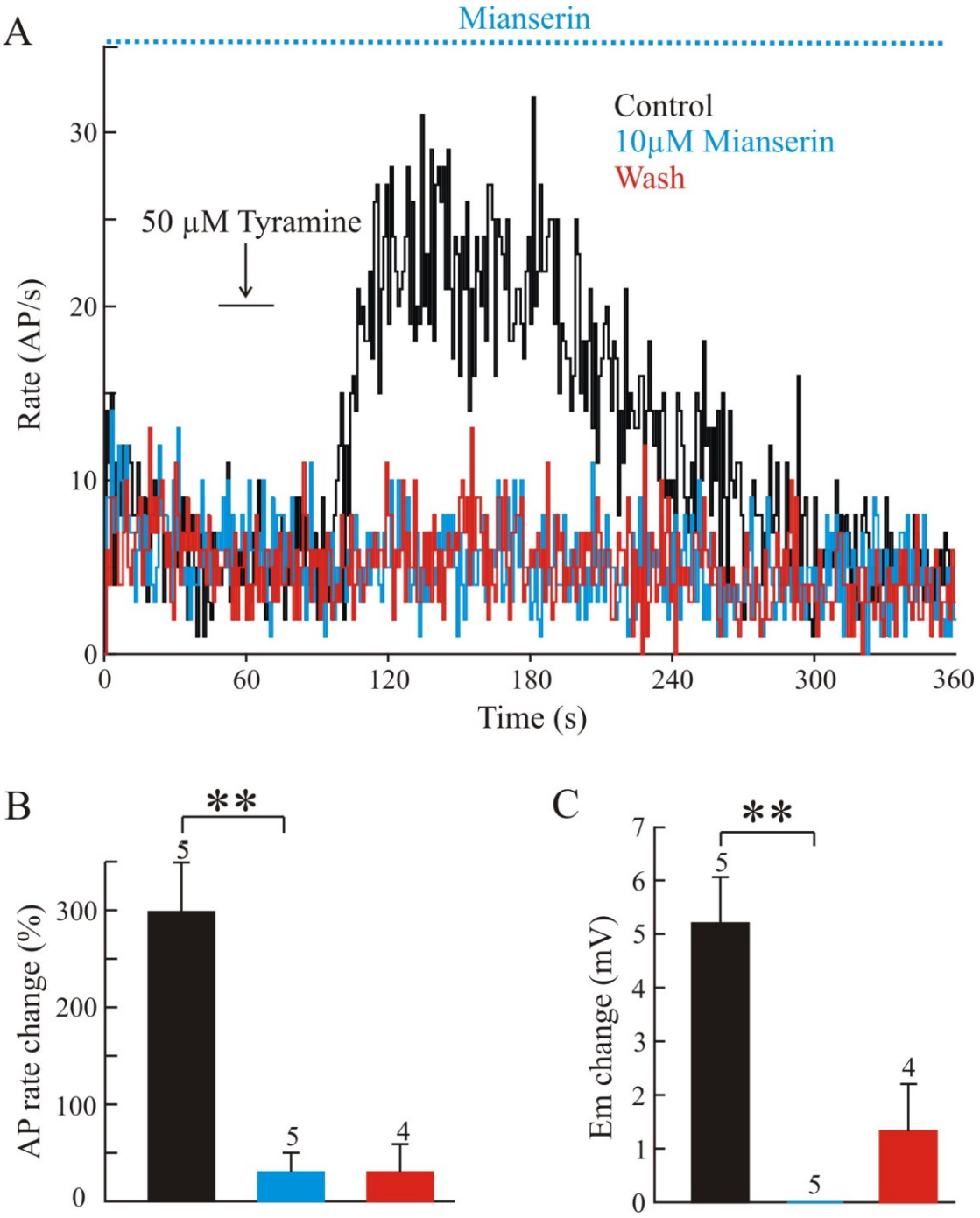

Figure 3.8. Mianserin effects on TA response. A. shows a typical experiment where mianserin completely removed the TA induced rise in action potential firing rate. The change in firing rate was significantly reduced in all experiments with mianserin in the superfusion (**B)** and this change was not reversible. **C.** VS-3 neurons did not depolarize in response to TA when mianserin was in the bath and this effect reversed partially. The numbers above the error bars represent the numbers of experiments.

I also tested 5 μM mianserin in one experiment. In this case the baseline firing rate during mianserin superfusion was higher than in the control, but the peak change in firing was at the same level as in the control. This suggests that the effect of previously applied tyramine was still ongoing and clearly not inhibited by 5 μM mianserin.

Chapter 3.3.4. Metoclopramide Effects on VS-3 Neuron TA Response

Metoclopramide is a specific dopamine 2 receptor antagonist that is commonly used antiemetic drug. In invertebrates, it has been shown to act more specifically on the β-adrenergic like octopamine receptors than other types of receptors (Evans and Robb 1993; Evans and Maquieira 2005).

I tested the effect of metoclopramide on VS-3 neuron TA response at 10 μM concentration. I made eight experiments using 50 μM TA and two experiments with 200 μM TA. As seen in Figure 3.9. metoclopramide caused a significant attenuation, but not complete inhibition, of the TA induced change in spike rate ($t = 3.92$, $df = 9$, $p = 0.0017$) and depolarization ($t = -1.86$, $df = 9$, $p = 0.048$). Seven neurons survived until the end of washing period and in most cases the metoclopramide effects were reversed (Figure 3.8.).

I also measured the baseline firing rate in all experiments as described above. It was very similar in the ten control and metoclopramide experiments; 11.2±4.3 and 11.9±5.4 (mean ± SD), but higher after wash (14.2±5.1, $n = 7$). This difference was statistically significant ($t = -4.41$, $df = 6$, $p = 0.0022$).

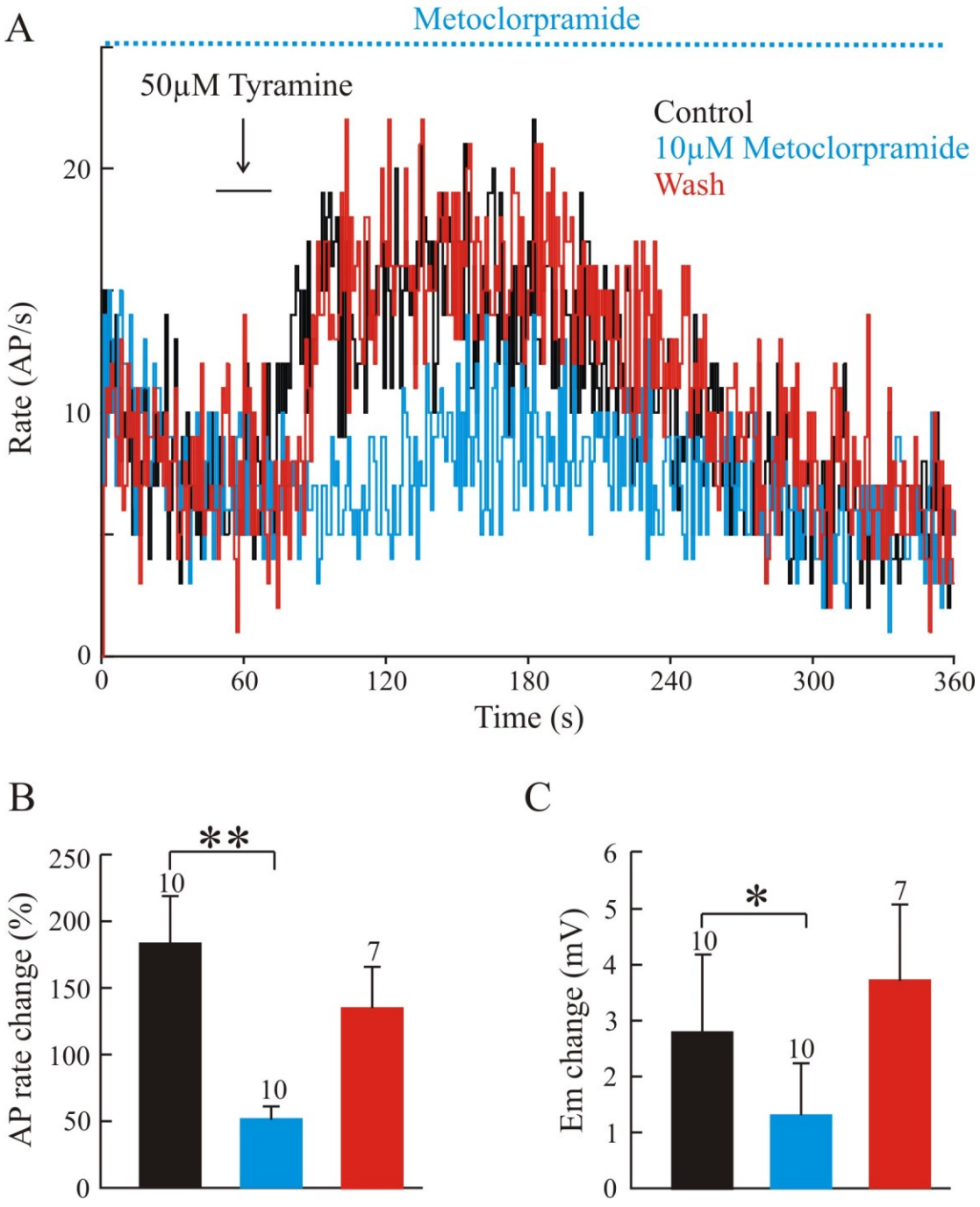

Figure 3.9. Metoclopramide effects on VS-3 neuron TA response. A. shows an example of an experiment where the firing rate decreased when metoclopramide was in the superfusion. **B.** The TA induced change in AP rate decreased significantly and **C** the depolarization was also significantly smaller when metoclopramide was in superfusion. Both effects were reversible. The numbers above error bars represent the numbers of experiments.

Chapter 3.3.5. Phentolamine Effects on VS-3 Neuron TA Response

Phentolamine is a nonselective α-adrenergic antagonist in humans but acts on both α- and β-adrenergic OA and TA receptors in insects (Evans and Maquiera 2005; Balfanz et al 2014; Ohta and Ozoe 2014)

I performed three experiments with 10 μM phentolamine and the firing rate change after application of 50 μM TA decreased clearly in all of them as seen in the two examples in Figure 3.10. The TA induced membrane depolarization was also smaller in all three experiments. Both effects recovered either partially or completely during the wash, but required a long washing period. In two experiments, the baseline firing decreased during phentolamine superfusion, which is shown in the experiment in Figure 3.10.B. In this experiment the baseline firing did not recover after 45 min wash. However, there was a significant increase in the baseline firing after 1 hour wash in both of the other experiments including the experiment in Figure 3.10.A. This figure also shows a larger response to TA than under control conditions.

Although phentolamine inhibited the TA response in all of these three experiments, the effects on baseline firing are somewhat mixed and more experiments would be needed to reach a clear conclusion.

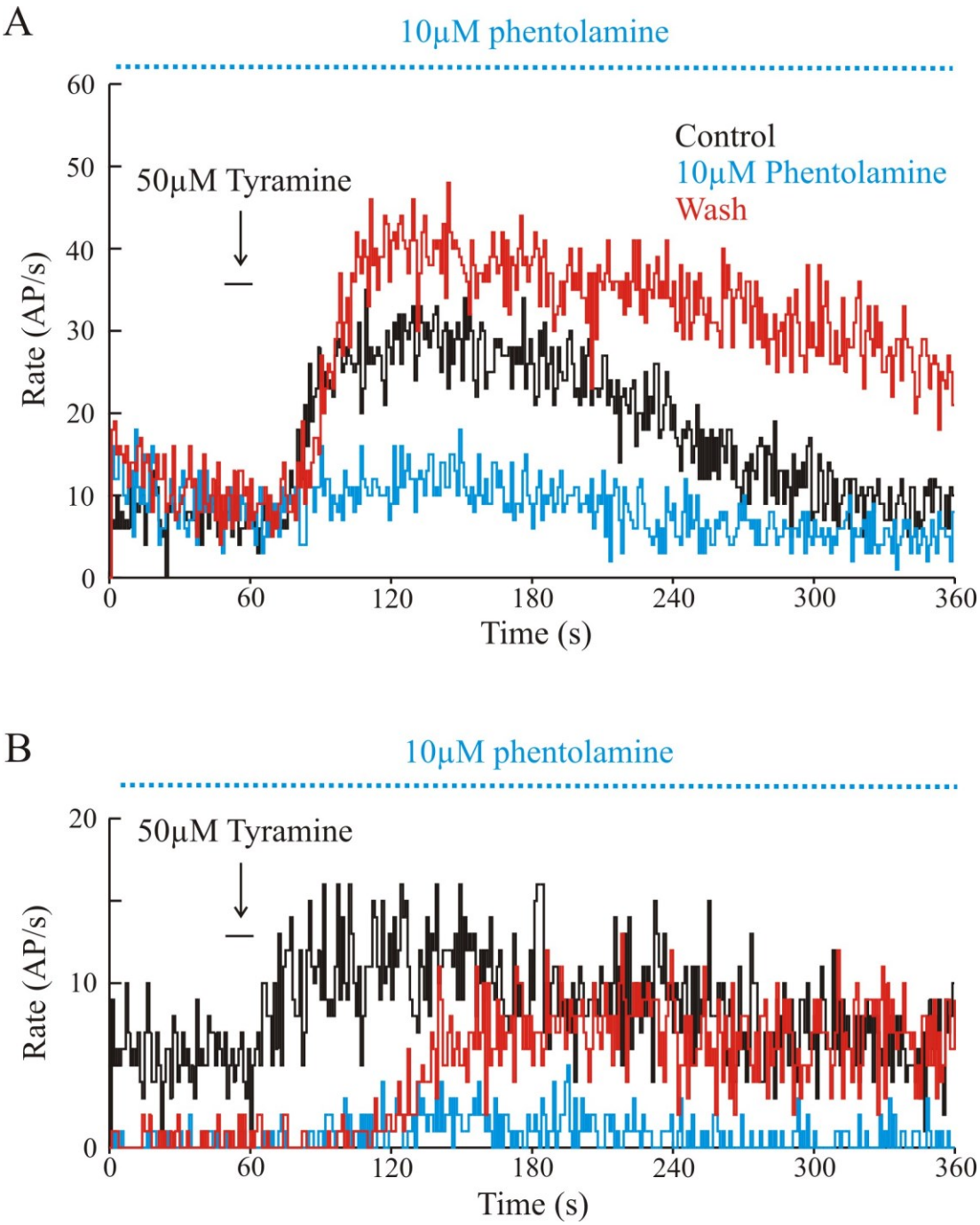

Figure 3.10. Phentolamine effects on firing rate. Phentolamine almost completely blocked the TA effect on firing rate in both experiments. This effect was completely reversed in **A** and the TA effect was larger than control after 70 min washing period. In **B** the baseline firing was also decreased during phentolamine treatment, and this effect did not recover, but there was a partial recovery in the TA induced change in firing rate after 45 min wash.

Chapter 4. Discussion

My results indicate that the spider VS-3 neurons have at least two different types of adrenergic-like receptors: One type responds to TA application by membrane depolarization and increase in action potential firing rate and the other type maintains the baseline firing. I will discuss below, how these results relate to previous research on spider mechanosensory neurons and research on other invertebrate adrenergic-like receptors.

Chapter 4.1. TA Effects on VS-3 Neurons

The effects of TA on VS-3 neuron membrane potential and firing rate are similar to previously reported OA effects on the same neurons, but the agonist concentrations needed for such effects are different (Torkkeli et al. 2011). At saturating concentrations TA depolarized the VS-3 neurons from their resting potential of about -70 mV by 4.8 mV, slightly more than the 3.8 mV depolarization previously reported for OA. However, OA depolarized VS-3 neurons at 1 nM concentration while a minimum concentration of 1 μM was needed for the TA to produce any effect. In addition, the OA effect saturated at 10-20 μM, while the TA effect required 50-100 μM concentration for saturation.

The maximum TA induced change in VS-3 neuron firing rate was about 250% and obtained with 50 μM TA (Figure 3.2.A). This is very close to the 240% change in firing rate induced by 20 μM OA (Torkkeli et al. 2011). The dose-response relationship for OA effect on firing rate in VS-3 neurons has not been measured, but in extracellular experiments from spider trichobothria neurons, OA also increased the firing rate and had an EC_{50} value of 1.39 μM and Hill coefficient of 0.96 (Widmer et al. 2005). The TA induced change in VS-3 neuron firing rate had a significantly higher EC_{50} of 15.1 μM and

a Hill coefficient of 2.3. For the membrane potential change these values were 1.7 and 18 μM. These results suggest that TA probably acts on the same receptors as OA on the VS-3 neurons, but is a less potent agonist. The higher Hill coefficient also suggests that more than one TA molecule is required to activate these receptors, but only one molecule is needed for OA to cause the same effect (Widmer et al. 2005)

Both OA and TA have previously been shown to regulate many physiological and behavioral functions in a variety of invertebrate preparations at nanomolar or low micromolar concentration (reviewed by Lange 2009; Ohta and Ozoe 2014). Even lower concentrations have been used to stimulate functionally expressed OA and TA receptors, and these studies have led to identification of the five classes of OA and TA receptors that are described in the Introduction (Chapter 1.5.3.). Based on the similarity of the OA and TA effects and their relative potency, the TA effect is likely to occur by activating an OA receptor rather than receptors that preferentially bind TA. Since TA binding to TAR1 type receptors usually activates G_i proteins, reduces cAMP and has an inhibitory effect, it is highly unlikely that the effect on firing rate would be caused by this type of receptor. There is one report concerning a cloned *Locusta migratoria* TAR1 type receptor that has higher affinity to OA than to TA and both OA and TA induce a Ca^{2+} rise (Poels et al. 2001). However, this effect is blocked by yohimbine and both agonists also attenuate cAMP. The TAR2 receptors are exclusively activated by TA and therefore the spider receptors are unlikely to members of this group.

High OA concentrations depolarized the VS-3 neurons less than low concentrations, but the concentration effect on firing rate has not been quantified (Torkkeli et al. 2011). In the present experiments, a concentration effect of TA was clear on firing

rate, but the membrane potential change was more variable with clear reduction at concentrations of 200 and 500μM but not at 1 mM. A concentration dependent effect of invertebrate adrenergic receptors is a well-known phenomenon (Verlinden et al. 2010). For example, high TA concentrations induced smaller membrane currents in prawn TAR1 receptors expressed in oocytes than low concentrations (Jezzini et al. 2014). Low OA concentrations stimulated *Chilo suppressalis* caterpillar immune response while higher concentrations had an inhibitory effect (Huang et al. 2012). One suggested reason for these differences is desensitization of the receptor, but there is also evidence of differences in second messenger mechanisms. E.g., low concentrations of OA mobilize only intracellular Ca^{2+} in caterpillar hemocytes, but high concentrations also increase cAMP (Huang et al. 2012).

Chapter 4.2. Antagonist Effects on the TA Response

The antagonist tests revealed at least two different adrenergic like receptors in the VS-3 neurons; one responsible for the TA induced spike rate increase and depolarization and the other regulating the baseline firing rate. Mianserin was the most potent blocker of the TA effect on both membrane potential and firing rate while metoclopramide was somewhat less potent but still inhibited both effects. These two antagonists are recognized as the most effective inhibitors of OAβ receptors, although they also have affinity to other types of receptors (reviewed by Verlinden et al. 2010; Ohta and Ozoe 2014). Preliminary experiments in this laboratory also show that mianserin potently blocks the VS-3 neuron response to OA. These results, as well as the fact that OA is more potent agonist than TA, suggest that the TA effect occurs by activation of OAβ type receptors in VS-3 neurons.

At 10 μM concentration yohimbine reduced the baseline firing, had very little effect on TA induced firing rate increase, but blocked it completely at 50 μM concentration in one experiment. These results indicate that the most specific effect of yohimbine was on receptors that were not responsible for the TA effect. As reviewed in the Introduction, yohimbine has a high affinity for all α-adrenergic-like receptors and/or it inhibits the second messenger mechanisms initiated by OA or TA. The α-adrenergic-like receptors include OAα, TAR1 and TAR2 receptors that have been cloned from several insect and one crustacean species (Verlinden et al. 2010; Ohta and Ozoe 2014; Jezzini et al. 2014). Some of these receptors also bind mianserin, but usually have lower affinity and require a higher concentration than yohimbine to inhibit OA or TA responses. One exception is TAR1 type receptor from *Heliothis virescens* where 1 μM mianserin inhibited the OA effect more strongly than the same concentration of yohimbine (von Nickisch-Rosenegk et al. 1996). This is also the only TA receptor that has shown a stronger effect on cAMP decrease with OA than TA.

The fact that VS-3 neuron baseline firing was reduced by yohimbine and the release of this block produced a rebound effect, suggests that the VS-3 neurons also have α-adrenergic like receptors. There is immunohistochemical evidence for the presence of OAα-type receptors on the axo-somatic region of VS-3 neurons (Widmer et al. 2005). Although these findings would not exclude the presence of TAR receptors, it is unlikely that activation of TAR1 receptor would lead to enhanced baseline firing. They preferentially activate G_i proteins leading to decrease in cAMP and likely an inhibition.

It is not clear how activity of these "baseline receptors" is regulated. During my experiments, the VS-3 preparation was dissected from the body and the leg. Although the

efferent nerve terminals surround the sensory neurons (Figure 1.3.), their somata are not present and connections to the CNS are severed. On the other hand, the sensory neurons are heavily enwrapped by glial cells (Figure 1.2.) and several other cell types are present in the preparation. It is possible that some of these cells contain and release TA or OA.

Chapter 4.3. Potential Second Messenger Mechanisms

I can only speculate about the potential second messenger mechanism that underlies the TA effect. If the receptors responsible for this effect are the OAβ receptors, they act by activating G_s protein and increasing cAMP. Previously, the cAMP analog 8-Br-cAMP has been shown to increase the VS-3 neuron and trichobothria neuron firing rates (Widmer et al. 2005; Torkkeli et al. 2011). However, the VS-3 neuron OA effect was not inhibited by the protein kinase A (PKA) inhibitor RP-cAMPS, indicating that this pathway is unlikely to mediate the physiological effect. Although many of the effects of cAMP are mediated by PKA, there are other pathways that can be activated by cAMP and it can also activate ion channels directly (Biel and Michalakis 2009). For example, the mammalian odorant receptors have cAMP gated Na^+ and Ca^{2+} channels in the plasma membrane that depolarize the membrane and the resultant Ca^{2+} rise leads to opening of Ca^{2+}-activated Cl^--channels. It is likely that Ca^{2+} is involved in the OA and TA effects, since the Ca^{2+}-chelator BAPTA-AM partially removed the OA effect on spike rate (Torkkeli et al. 2011) and there is preliminary Ca^{2+} imaging evidence that indicates a Ca^{2+} rise in VS-3 neurons when TA is applied.

The ionic current that induces membrane depolarizes when OA or TA is applied is not yet known. OA application was shown to reduce voltage-gated K^+ currents (Torkkeli

et al. 2011) and this can have an effect on excitability, but would not produce immediate depolarization. The depolarization is more likely to be caused by activation of Na^+, Ca^{2+} or Cl^- channels in the VS-3 neuron membrane.

Yohimbine effect on the baseline firing suggests that α-adrenergic-like receptors regulate the sensitivity of these neurons. These receptors are likely to activate G_q proteins and lead to mobilization of Ca^{2+} from intracellular stores. Some of the OAα receptors have also been shown to have a smaller effect in increasing intracellular cAMP (Balfanz et al. 2005) and this may also occurs in the VS-3 neurons.

Chapter 4.4. **Tyramine Effects on VS-3 Neuron Dynamic Properties**

The frequency response analysis showed that the sensitivity parameter *A* as well as the information capacity *R* increased in the presence of TA. The fractional exponent *k* also increased indicating that the change in sensitivity was larger in the high frequency range. These results are similar to those previously shown with OA effects on VS-3 neuron frequency response (Torkkeli et al. 2011). However, OA effect on *A* was smaller while its effect on *k* was larger. This suggests that OA may have a stronger effect on sensitivity at high frequencies than TA, while TA regulates the sensitivity more broadly.

It has been documented that *Cupiennius* can sense a wide variety of frequencies for detection of different types of prey and for courtship (Barth 2002). The high frequency range in my experiments was close to the male courtship signals that occur mainly at 75-100 Hz and would be detected by the female spiders. The signals produced by a fly also occur in the range of about 100 Hz. Other prey produce significantly higher frequency

signals, but these are beyond the capabilities of my experiments. The high resistance intracellular electrodes can only pass current stimuli up to about 250 Hz.

Chapter 4.5. Conclusions

The VS-3 neurons appear to regulate their baseline sensitivity through α-adrenergic-like receptors. The level of activity of these receptors could be amplified by OA or TA release to the hemolymph at the time when the spider is under stress. This would allow a greater alertness during stressful situations. Perhaps more rapid and local increases in sensitivity could be mediated through OA or TA release from efferent terminals and activation of β-adrenergic-like receptors. OA and TA can act as neurohormones released into the circulation, but some of the efferent nerves that reach the VS-3 neurons contain OA (Widmer et al. 2005). My results also suggest that there may be another source of OA or TA close to the neurons and independent of the CNS. Another possibility is that there are α-adrenergic-like receptors that are constitutively active.

Chapter 4.6. Future Directions

There are several areas that would be interesting to learn more about in conjunction with my findings. Are there efferent neurons or other cells that contain TA? This could be tested by immunohistochemistry using an antibody against TA. Is the mechanism behind the TA effect Ca^{2+} or cAMP dependent, or both? This could be examined using a Ca^{2+} chelator like BAPTA-AM, Ca^{2+} imaging, or specific inhibitors of cAMP mediated pathways. It would be interesting to know if the antagonists that I tested here have similar effects on OA response than on the TA effects. One possibility to investigate the "baseline

receptors" would be to perform Ca^{2+} imaging when yohimbine is added to the superfusion to see if it would reduce the [Ca^{2+}]. Since several of the *C. salei* adrenergic like receptors have been found in the spider hypodermis transcriptome, it should be possible to localize in which particular cells these receptors are located using *in-situ* hybridization.

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