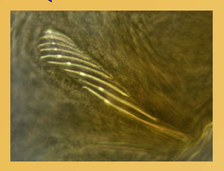
THE DISTRIBUTION AND FUNCTION OF METABOTROBIC GABA$_B$ RECEPTORS IN THE SPIDER PERIPHERAL MECHANOSENSILLA

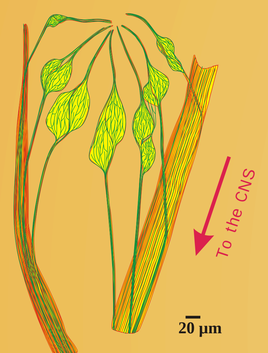
Izabela Panek[1,2], Shannon Meisner[1] and Päivi H. Torkkeli[1]. [1]Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia and [2]Department of Biophysics, N. Copernicus University, Torun, Poland

Male Cupiennius Salei



Outside view of the lyriform slit sensilla VS-3 on the spider patella

The VS-3 sensory neurons and efferent neurons



Seven pairs of sensory neurons innervating the slits in the spider patella (*yellow-red*). The fine efferent fibers (*green*) branch on top of the sensory neurons and form synapses with the sensory neurons, the glial cells and with each other.

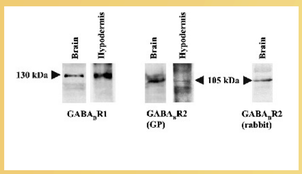
INTRODUCTION

A dense network of fine efferent fibers innervates the peripheral parts of the mechanosensilla of the tropical wandering spider (*Cupiennius salei*, Keys.). They form synapses with the axons, somata and dendrites of the mechanosensory neurons and they also synapse with each other and with glial cells surrounding the sensory neurons (Fabian-Fine et al. 1999a,b). The majority of efferent neurons are immunoreactive to an antibody against the inhibitory neurotransmitter GABA (Fabian-Fine et al. 1999b) and the sensory neurons are inhibited by agonists of ionotropic GABA receptors (Panek et al. 2002).

In addition to relatively fast inhibition via ionotropic receptors, GABA can modulate neuronal activity by activating metabotropic G-protein coupled GABA$_B$ receptors that play critical roles in long-term modulation of synaptic transmission. Two different GABA$_B$ receptor proteins, GABA$_B$R1 and R2, have been cloned from mammalian and *Drosophila* nervous systems, and both exist in several alternatively spliced forms. The functional receptor comprises a heterodimer with these subunits (Jones et al. 1998; Filippov et al. 2000; Mezler et al. 2001). Specific antibodies against both of these receptor subunits are commercially available and we tested them using Western blot analysis and immunocytochemistry in spider brain and peripheral nerve tissues. To learn how the GABA$_B$ receptor immunoreactivity correlated with the synaptic sites we performed double labeling experiments using a monoclonal antibody against synapsin that labels the synaptic vesicles on the fine efferent fibers on the spider sensilla (Fabian-Fine et al. 1999a,b). We also tested if specific GABA$_B$ receptor agonists modulate the voltage-activated currents and the excitability of identified peripheral mechanosensory neurons.

WESTERN BLOT ANALYSIS

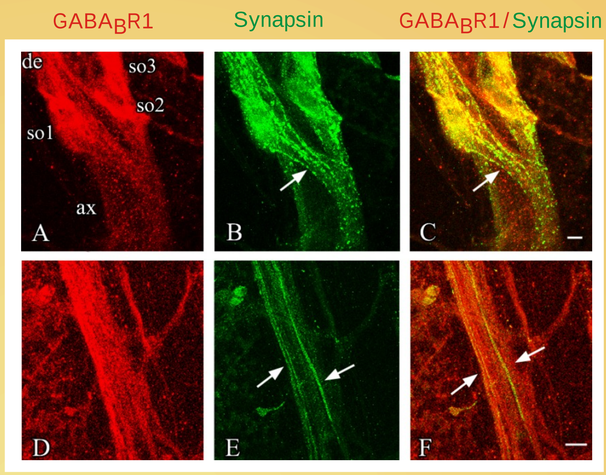
Western blot analysis was used to test the specificity of anti-GABA$_B$ receptor antibodies on spider brain and peripheral nervous tissues. The peripheral tissue was obtained from the hypodermis in the leg femur and patella. The tissue was frozen with liquid nitrogen and ground. Primary antibodies were obtained from Chemicon: guinea pig anti-GABA$_B$R1 (AB1531, 1:2,500 dilution), guinea pig anti-GABA$_B$R2 (AB5394, 1:500 dilution) and rabbit anti-GABA$_B$R2 (AB5848, 1:500 dilution). The peroxidase conjugated anti-guinea pig (1:10,000 or 1:1,500) and anti-rabbit (1:5,000) secondary antibodies were obtained from Jackson laboratories. Immunoreactive protein bands were visualized using an ECL plus chemiluminescent kit (Amersham) according to the manufacturer's instructions.



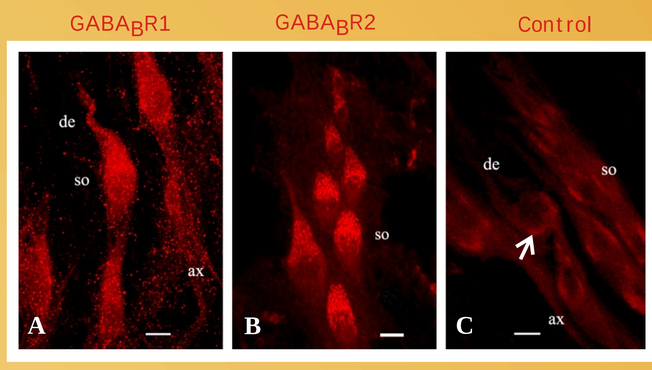
The guinea pig anti-GABA$_B$R1 antibody revealed a distinct band of approximately 130 kDa in the spider brain and leg hypodermis homogenates (*left*). This is consistent with the molecular weight of the GABA$_B$R1A splice variant. The guinea pig anti-GABA$_B$R2 (GP) antibody produced a discrete band at 105 kDa, but additional, less discrete bands were present in the hypodermis homogenate (*middle*). The rabbit anti-GABA$_B$R2 antibody only induced one band at about 105 kDa in the spider brain homogenate (*right*).

GABA$_B$ RECEPTOR IMMUNOCYTOCHEMISTRY

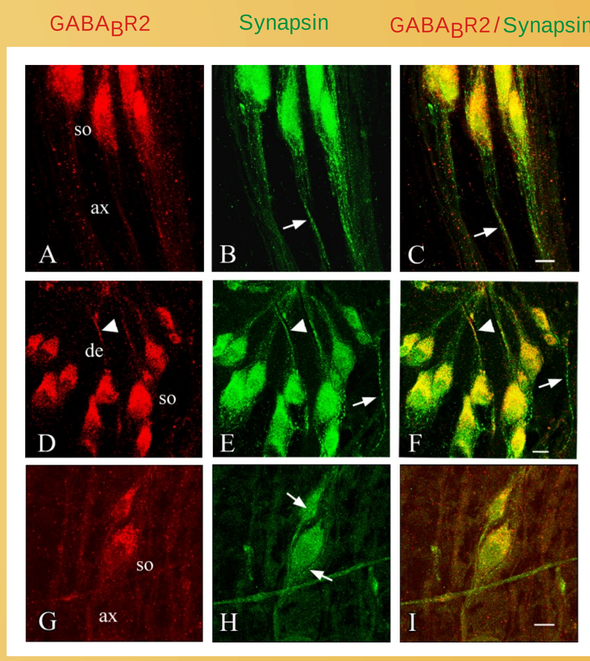
A piece of cuticle from the spider patella containing the VS-3 slit sense organ was used for whole mount immunocytochemistry. The tissue was fixed in 4% paraformaldehyde (20 min) and incubated overnight at 4°C in the primary antibody in blocking solution (5% NGS, 1% BSA, 3% milk powder, 0.6% Triton X-100 in PBS). The antibodies were used in the following concentrations: guinea pig anti-GABA$_B$R1 (1:2,500), anti-GABA$_B$R2 (1:1,000) and mouse anti-synapsin (SYNORF1, Dr. E. Buchner, Würzburg) 1:100. After careful washing the tissue was incubated overnight in the secondary antibodies in blocking solution at 4°C. The secondary antibodies were goat anti-guinea pig CY-3 (1:1,000, Jackson), and goat anti-mouse Alexa Fluor 488 10 µg/ml (Molecular Probes). The samples were mounted in Mowiol. For double labeling experiments the two primary and two secondary antibodies were used simultaneously. The preparations were examined under a laser scanning confocal microscope (LSM 510, Zeiss) with an argon-krypton laser for Alexa Fluor 488 (488 nm) and a helium-neon laser for CY-3 (543 nm).



Anti-GABA$_B$R1 and anti-synapsin double staining of spider VS-3 sensilla. The VS-3 organ in *A-C* has five sensory neurons, three partially overlap each other and are labeled *so1*. The *so2* and *so3* are single cells. Orientation of neurons *so1* and *so3* is oblique and *so3* is oriented face on. *A.* shows strong immunoreactivity for anti-GABA$_B$R1 in the somata, axons and parts of dendrites of the VS-3 neurons. *B.* Anti-synapsin immunoreactive punctae in the fine fibers covering the sensory axons (*arrows*), somata and proximal parts of dendrites. *C.* shows that the immunoreactive punctae is localized close to each other in many structures but there are also areas without anti-synapsin labeling with anti-GABA$_B$R1 labeling especially in the axonal parts. *D-F* show the main leg nerve, which contains sensory axons, efferent fibers and glial cells. *D.* Anti-GABA$_B$R1 labeling was strong in the leg nerve and its branches. *E.* Strong anti-synapsin labeling in thin fibers (*arrow*). *F.* The anti-synapsin labeling overlaps with some but not nearly all of the anti-GABA$_B$R1 labeling in the main nerve. *Ax* = axon, *so* = soma and *de* = dendrite. Scale bars 20 µm in all figures.



VS-3 organ labeled with anti-GABA$_B$R1 (*A*) and R2 (*B*) using CY-3 as a fluorochrome. *A.* GABA$_B$R1 immunoreactive punctae were present in all parts of the neurons, but the cell bodies were more strongly stained. *B.* Clusters of GABA$_B$R2 immunoreactive punctae in the distal halves of the cell bodies. The dendrites are not visible in this preparation. *C.* A control preparation without primary antibody but otherwise treated as the test preparations. Bright spots that are seen on the sides of some of the cell bodies (*arrow*) are lipofuscin granules, that are common in these locations of the VS-3 neurons. *Ax* = axon, *so* = soma and *de* = dendrite. Scale bars 20 µm.

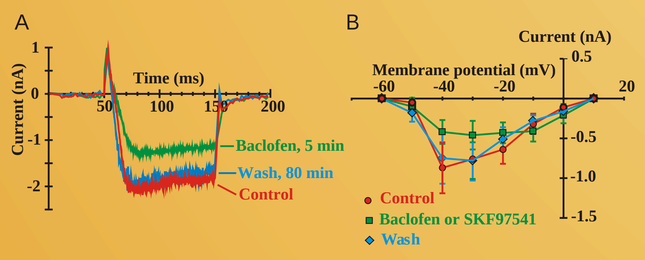


Anti-synapsin and anti-GABA$_B$R2 labeling in the sensory neurons in the spider leg. *A-C* show the cell bodies and axons of five neurons in the VS-3 organ. *A.* Anti-GABA$_B$R2 immunoreactive punctae are concentrated on the distal parts of the cell bodies with very little staining on the axons. *B.* Axons (*arrow*) and cell bodies of the sensory neurons are covered with several fine fibers densely labeled with anti-synapsin punctae. *C.* Anti-synapsin immunoreactive fibers on top of the distal parts of the sensory neurons where the anti-GABA$_B$R2 immunoreactivity is strongest. *D-F.* Distal parts of the cell bodies and the dendrites of 12 sensory neurons in a VS-3 organ. *D.* High density of anti-GABA$_B$R2 immunoreactive punctae in the distal parts of the cell bodies and some staining is present in the dendrites. *E.* Anti-synapsin immunoreactive fibers extend on top of the cell bodies and some reach to the sensory dendrites (*arrowhead*). *F.* Significant overlap of the anti-synapsin and anti-GABA$_B$R2 labeling in the distal parts of the sensory neurons and some of the sensory dendrites while most of the synapsin labeled efferent fibers that branch on the proximal parts of the cell bodies do not overlap with the anti-GABA$_B$R2 labeling. *G-I* show three sensory neurons that innervate either a tactile hair or a spine. *G.* Distal parts of the cell bodies have clusters of anti-GABA$_B$R2 immunoreactive punctae. *H.* Some anti-synapsin immunoreactive punctae (*arrows*) are seen in fine fibers that lie on top of the cell bodies and on the nerve axon that crosses the sensory axons. *Ax* = axon, *so* = soma and *de* = dendrite. Scale bars 20 µm in all figures.

Electrophysiological methods

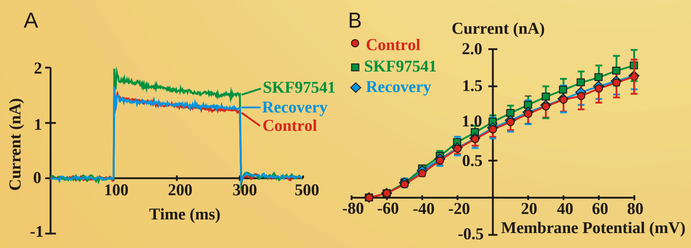
For electrophysiological recordings the hypodermis membrane with the VS-3 slit sense organ was detached from the cuticle and placed in a recording chamber. The preparation was superfused continuously with spider saline (in mM: 223 NaCl, 6.8 KCl, 8 $CaCl_2$, 5.1 $MgCl_2$, 5 sucrose, and 10 HEPES, pH 7.8). GABA$_B$ agonists were injected into the bath solution. Intracellular recordings were performed using the discontinuous single-electrode current- and voltage-clamp methods with a SEC-10 L amplifier (NPI Electronic, Germany). Microelectrodes were filled with 3 M KCl or CsCl and their resistances were 40-80 MΩ in solution. All experiments were controlled by an IBM compatible computer with custom written software (courtesy of Dr. A. S. French). The GABA$_B$ receptor agonists that were used in the experiments were: SKF97541 hydrochloride, 3-aminopropylphosphonic acid (3-APA) and (±)-Baclofen.

GABA$_B$ receptor agonist effects on voltage activated Ca^{2+} current in the spider VS-3 neurons



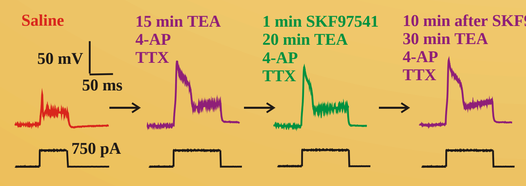
The GABA$_B$ receptor agonists baclofen and SKF97541 reversibly reduced LVA-I_{Ca} in the VS-3 neurons. *A.* An example of currents elicited from -100 mV holding potential to -30 mV test potential under control conditions, 5 min after 400 µM baclofen application and 80 min after washing are shown. *B.* Peak currents (± s.e.) from 7 similar recordings plotted against membrane potentials .

GABA$_B$ receptor agonist effects on voltage-activated outward K^+ current



***A.* Currents elicited by depolarizing the cell from a holding potential of -70 mV to +50 mV are shown under control conditions, 5 min after application of 200 µM SKF97541 and 15 min after wash. *B.* Steady-state current-voltage curves (± s.e.) from three similar recordings. The current values represent the data at the end of the 200 ms pulse at potentials from -70 mV to 80 mV at 10 mV intervals.**

GABA$_B$ receptor agonist effect on Ca^{2+} spikes.



In normal spider saline this VS-3 neuron elicited a transient action potential (46 mV, 7.5 ms) in response to a current pulse. When K^+- and Na^+ channel blockers (25 mM TEA, 25 mM 4-AP and 1 µM TTX) were added to the bath solution the neuron fired Ca^{2+} spikes in response to similar stimulus (99 mV, 42 ms). When 80 µM SKF97541 was added to the bath solution, a transient reduction of the Ca^{2+} spike duration and amplitude was seen (90 mV, 27 ms). The Ca^{2+} spike then gradually returned to its original shape (100 mV, 42 ms).

References

Fabian-Fine R, Volknandt W and Seyfarth E-A. Peripheral synapses at identifiable mechanosensory neurons in the spider *Cupiennius salei*: synapsin-like immunoreactivity. *Cell Tissue Res* 295: 13-19, 1999a

Fabian-Fine R, Höger U, Seyfarth E-A and Meinertzhagen IA. Peripheral synapses at identified mechanosensory neurons in spiders: Three-dimensional reconstruction and GABA-immunoreactivity. *J Neurosci* 19: 298-310, 1999b

Filippov AK, Couve A, Pangalos MN, Walsh FS, Brown DA and Moss SJ. Heteromeric assembly of GABA$_B$R1 and GABA$_B$R2 receptor subunits inhibits Ca^{2+} current in sympathetic neurons. *J Neurosci* 20: 2867-2874, 2000.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA and Gerald C. GABA$_B$ receptors function as a heteromeric assembly of the subunits GABA$_B$R1 and GABA$_B$R2. *Nature* 396: 674-679, 1998.

Mezler M, Muller T and Raming K. Cloning and functional expression of GABA$_B$ receptors from *Drosophila*. *Eur J Neurosci* 13: 477-486, 2001.

Panek I, French AS, Seyfarth E-A, Sekizawa S-i and Torkkeli PH. Peripheral GABAergic inhibition of spider mechanosensory afferents. *Eur J Neurosci* 16: 96-104, 2002.

Summary and Conclusions

- **The spider brain and peripheral tissue have both of the subunits needed to form functional GABA$_B$ receptors: The 130 kDa R1A and the 105 kDa R2.**
- **In the peripheral tissue anti-GABA$_B$R1 immunoreactivity was more widespread than R2 labeling. This was similar to the findings with the same antibodies in human and rodent CNS**
- **Significant amount of the anti-GABA$_B$R1 staining did not coincide with the anti-synapsin staining suggesting that this subtype is expressed extrasynaptically as well as synaptically.**
- **The GABA$_B$ receptor agonists reduced the amplitude of LVA-I_{Ca} in the spider VS-3 neurons. The GABA$_B$ receptor agonists induced a transient small increase in the amplitude of the outwardly rectifying I_K in the VS-3 neurons.**

GABA$_B$ receptor mediated modulation can provide fine control of a cell's excitability via multiple mechanisms. Those mechanisms that regulate the voltage-activated conductances in VS-3 neurons can cause decreased excitability and reduction of neurotransmitter release: Reduction of I_{Ca} decreases Ca^{2+} entry during action potentials and increased outwardly rectifying I_K may shorten the action potential duration. In addition, several other GABA$_B$ receptor-mediated mechanism may be involved. Our findings suggest that mechanosensory input in spider peripheral sensilla may be finely tuned by GABA$_B$ receptor activation on the most distal parts of the sensory afferents. This would probably change the neuron's ability to detect different stimulus frequencies and amplitudes, and could cause a slow, sustained inhibition when the neurons are subjected to a repeated stimulus or when a change is required by behavioral circumstances.

Canadian Institutes of Health Research / Instituts de recherche en santé du Canada

Canada Foundation for Innovation / Fondation canadienne pour l'innovation