GABA and glutamate receptors have different effects on excitability and are differentially regulated by calcium in spider mechanosensory neurons

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Abstract
GABA and glutamate receptors belonging to the ligand-gated chloride-channel family are primary targets of insecticides and antiparasitics, so their molecular structure, pharmacology and biophysical properties have attracted significant attention. However, little is known about the physiological roles of these channels or how they regulate neuronal excitability and animal behavior. Mechanosensory neurons of VS-3 slit sensilla in the patella of the tropical wandering spider, Cupiennius salei, react to the GABA$_A$-receptor agonists, GABA and muscimol, with depolarization and an increase in intracellular [Ca$^{2+}$] and, during random noise stimulation, with a mixed inhibitory–excitatory response. We established that the GABA$_A$-receptors in all VS-3 neurons are identical, but there are at least two types of glutamate receptors and some neurons do not respond to glutamate at all. Immunohistochemistry with antibodies against Drosophila inhibitory glutamate receptor (GluCls) $\alpha$-subunit suggests that in addition to VS-3 neurons, these receptors may also be present in the efferent neurons surrounding the sensory neurons. Most VS-3 neurons were inhibited but not depolarized by glutamate during random stimulation, but some depolarized and had a similar excitatory–inhibitory response to glutamate as to muscimol. The membrane-permeable Ca$^{2+}$-chelator BAPTA-AM abolished muscimol effects but potentiated glutamate effects, indicating that GABA and glutamate receptors are differentially modulated by Ca$^{2+}$, leading to diverse regulation of neuronal excitability. We hypothesize that this could be achieved by different Ca$^{2+}$-triggered phosphorylation processes at each receptor type. These findings are important for understanding the significance of Ca$^{2+}$-mediated regulation of transmitter receptor molecules and its role in controlling excitability.

Introduction
Peripheral mechanosensory neurons of the tropical wandering spider, Cupiennius salei, are innervated by efferent neurons that contain GABA (3-aminobutyric acid), glutamate, ACh (acetylcholine) and octopamine (Fabian-Fine et al., 2002; Widmer et al., 2005). Mechanosensory neurons of the lyriform VS-3 slit sensilla on the spider patella respond to each of these transmitters with an inhibitory or excitatory change in sensitivity (Panek et al., 2002; Panek & Torkkeli, 2005; Widmer et al., 2005; Torkkeli et al., 2011). When VS-3 neurons were stimulated with step stimuli, activation of GABA$_A$-receptors led to depolarization and inhibition, but with random noise stimulation it elicited mixed inhibitory–excitatory responses (Panek et al., 2002; Pfeiffer et al., 2009). GABA and muscimol also elevated intracellular [Ca$^{2+}$], which may have contributed to changes in sensitivity, as Ca$^{2+}$ modulates mechanotransduction channels and Ca$^{2+}$-activated and voltage-activated ion channels in these neurons (Panek et al., 2008; Höger et al., 2010; Torkkeli et al., 2011).

Glutamate also inhibited firing when VS-3 neurons were stimulated by step stimuli but, in contrast to GABA$_A$-agonists, did not cause significant depolarization (Panek & Torkkeli, 2005). However, glutamate in the bath solution amplified muscimol-induced depolarization. These types of interactions between invertebrate inhibitory GABA and glutamate receptors have been reported in other preparations (e.g. Kehoe & Vulfius, 2000), suggesting that some receptors may respond to both transmitters. Invertebrate inhibitory glutamate receptors (GluCls) and ionotropic GABA receptors are structurally related Cl-channels and some GluCls may even include a GABA$_A$-receptor subunit (e.g. Kehoe & Vulfius, 2000; Ludmerer et al., 2002; Eguchi et al., 2006; Knipple & Soderlund, 2010; Wolstenholme, 2010). These pentameric channels are the main targets of insecticides and antiparasitics that either block the channels (e.g. fibronil and dieldrin effects on GABA- and glutamate-gated Cl-channels) or stabilize them in the open configuration (e.g. ivermectin effect on GluClz subunit), leading to irreversible activation of the channel (Cully et al., 1994; Brodie & Maricq, 2006; McCavera et al., 2009; Knipple & Soderlund, 2010; Hibbs & Gouaux, 2011). GluCls are also related to vertebrate ligand-gated Cl-channels, particularly to glycine receptors that are also sensitive to ivermectin (Wolstenholme, 2010). Spider VS-3 neurons were irreversibly inhibited and depolarized by ivermectin, suggesting that they have GluCls (Panek & Torkkeli, 2005).

Here, we investigated the physiological significance of this coexistence of GluCls and GABA$_A$-receptors in VS-3 mechanosen-
sory neurons. We found that almost all neurons had an inhibitory–excitatory response to the GABA\textsubscript{A} agonist muscimol, but most neurons were only inhibited by glutamate and many did not respond to glutamate at all. Correspondingly, only 73% of the neurons were immunoreactive to polyclonal antibodies against Drosophila GluCl subunit (Ladmerer et al., 2002). These antibodies also labeled the surrounding efferent neurons, suggesting that glutamate may inhibit transmitter release to the sensory neurons. Muscimol response was abolished by the membrane-permeable Ca\textsuperscript{2+}-chelator BAPTA-AM, while the same treatment enhanced the glutamate response. This latter finding indicates that Ca\textsuperscript{2+} can differentially modulate two types of receptors that are both likely to be ligand-gated Cl-channels, leading to differences in the ways they control excitability.

Material and methods

Experimental animals and preparation

Tropical wandering spiders were maintained at room temperature (22 ± 2 °C) under a 13/11-h light–dark cycle. Legs from adult spiders were autotomized for electrophysiological and immunohistochemical experiments. Brains were removed from spiders killed via deep CO\textsubscript{2} anesthesia following a protocol approved by the Dalhousie University Committee on Laboratory Animals. A small piece of patellar cuticle containing the VS-3 slit sense organ was dissected and detached from cuticle, mounted on a coverslip and placed in an experimental chamber as a hypodermis preparation that allows ready access of pharmacological agents to the neurons (Fig. 1) (Sekizawa et al., 1999). Some experiments were performed using a cuticular preparation of the spider VS-3 organ. Hypodermis membrane was detached from the cuticle and placed on a glass coverslip in a preparation holder. The seven pairs of VS-3 neurons remained attached to the hypodermis and were impaled with intracellular sharp electrodes from above. Preparation was continuously superfused with spider saline by gravity via plastic tubing. Agonists were added to the distal part of the tubing. Suction was provided by a pump.

Electrophysiology

Neurons were observed using a compound microscope with a 10 x objective (Axioskop 2FS Plus, Zeiss, Oberkochen, Germany). The microscope was mounted on a gas-driven vibration isolation table inside a Faraday cage (Technical Manufacturing, Peabody, MA, USA). Sharp borosilicate glass microelectrodes (OD 1 mm, ID 0.5 mm, Hilgenberg, Malsfeld, Germany) were pulled with a P-2000 horizontal laser puller (Sutter Instrument, Novato, CA, USA). They were filled with 3 m KCl and had resistances of 40–80 MΩ in solution. Neurons were impaled with the microelectrodes using a PatchStar micromanipulator (Scientifica, Uckfield, UK). Recordings were made in discontinuous current-clamp mode using an SEC-10L amplifier (npi electronic, Tamm, Germany) as described previously in detail (Sekizawa et al., 1999). Switching frequency of 20 kHz and a duty cycle of ¼ were used in all experiments.

All experiments were controlled by an IBM-compatible computer using custom written software and a data acquisition board (NI6035E; National Instruments, Austin, TX, USA). Voltage stimuli were provided by the computer via a 12-bit digital/analog converter. The membrane potential recording was low-pass filtered at 33.3 kHz and the current signal at 3.3 kHz by the voltage-clamp amplifier. Current and voltage were sampled by 16-bit analog/digital converters.

Glutamate (1-glutamic acid-monosodium salt hydrate), muscimol, ibotenic acid and kainate (kainic acid monohydrate) were applied manually using a syringe and plastic tubing. To avoid turbulence, this tubing drained to the superfusion solution ∼500 μm from the VS-3 neuron somata. The total amount of the drug that was applied was 100–500 μL of BAPTA-AM [1,2-Bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)] was added to the superfusion solution for the duration of the experiment. Glutamate and ibotenic acid were aliquoted in double distilled water in high concentrations. BAPTA-AM was initially diluted in dimethylsulfoxide (DMSO), muscimol in concentrated HCl and kainate in 1 m NaOH. The DMSO concentration in final BAPTA-AM solution was 0.08%. All concentrated aliquots were kept frozen until just before each experiment.

Frequency response analysis

For frequency response recordings the VS-3 neurons were stimulated electrically via the recording electrode with a pseudorandom Gaussian white noise generated by the computer. This method has been described previously in detail (French et al., 2001; Torkkeli et al., 2011). Action potential times of occurrence were treated as Dirac delta functions, and then digitally filtered with a sin(x)/x function to restrict their bandwidth and re-sampled at 1 kHz to band-limit the frequency range to 0–500 Hz (French & Holden, 1971). The resulting signal was used as output signal in the subsequent frequency response analysis. The input signal was obtained by removing action potentials (APs) from the intracellular recording through deletion of 2 ms of samples around each AP and filling the resultant gap by linear interpolation. Input and output signals were digitally re-sampled at 1 kHz and then transferred to the frequency domain using the fast Fourier transform (Cooley & Tukey, 1965) in segments of 1024 input–output data pairs. Frequency response functions were plotted as Bode plots (phase and log gain vs. log frequency) and fitted by the power-law relationship purchased from Sigma (Oakville, ON, Canada) if not otherwise indicated.
where $f$ is frequency, $A$ is a constant describing overall sensitivity (gain at 1 Hz), $k$ is the fractional exponent, $P(f)$ is the phase lag as a function of frequency and $\Delta t$ is a time delay. Fitting was performed on the complete frequency response function in complex exponential format to obtain a minimum squared error between the experimental and fitted values. Coherence functions, $\gamma^2(f)$ (Bendat & Piersol, 1980), between the input and output were calculated and plotted against log frequency. Linear information capacity, $R$, was calculated from the coherence function using the Shannon formula (Shannon & Weaver, 1949):

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

### Western blot analysis

The spider brain tissue was rapidly frozen with liquid nitrogen and ground with a precooled mortar and pestle. The 'hypodermis' membrane carrying the peripheral nerves on proximal parts of ~20 spider legs was collected into a microtissue grinder tube (Fisher Scientific, Nepean, ON, Canada) on ice. Phosphate-buffered saline (PBS) and protease inhibitor were added to each sample and the antigen was purified using a Seize™X immunoprecipitation kit with each antibody (Pierce, Rockford, IL, USA) following the manufacturer's instructions. A standard Western blot analysis was performed as described previously in detail (Panek et al., 2002). The polyclonal primary antibodies against four different regions of the *Drosophila* GluClz subunit (Gcl1, Gcl2, Gcl3 and Gcl5) were generously donated by Merck Research Laboratories (Ludmerer et al., 2002). They were used in 1 : 200–1 : 500 dilutions. We used peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in 1 : 100 000 dilutions. Immunoreactive protein bands were visualized using an ECL plus chemiluminescent kit (Amersham Biosciences, Montreal, QC, Canada) according to the manufacturer’s instructions. For controls the primary antibody was omitted or replaced with normal rabbit serum.

### Immunohistochemistry

Cuticular patellar samples were prepared by splitting the spider patellae longitudinally and the pieces containing VS-3 slit sensillum were fixed in 4% paraformaldehyde in PBS for 20 min, followed by several rinses in PBS. Tissue was then permeabilized with 0.5% Triton X-100 in PBS for 1 h, followed by 2 h incubation in blocking solution (5% normal goat serum, 3% skimmed milk powder, 1% bovine serum albumin fraction V, and 0.1% Triton X-100 in PBS). Primary antibodies were diluted in fresh blocking solution, and the preparations were incubated overnight at 4 °C in this solution. The antibodies against *Drosophila* GluClz subunit, described above, were used in dilutions of 1 : 100–1 : 1000. A monoclonal antibody against *Drosophila* synapsin 1 (SYNORF1, 1 : 100 dilution) was a gift from Dr E. Buchner (Universität Würzburg, Germany) (Klagges et al., 1996) and has previously been tested in the spider preparation (Fabian-Fine et al., 1999). After primary antibody incubation, the preparations were washed four times in 0.1% Triton X-100 in PBS, followed by overnight incubation at 4 °C in the secondary antibodies in blocking solution. The secondary antibodies were goat anti-rabbit CY-3 (1 : 600 dilution; Jackson ImmunoResearch Laboratories) for the GluClz antibodies and goat anti-mouse Alexa Fluor 488 (10 μg/mL; Molecular Probes) for the anti-synapsin antibody. Preparations were then washed four times in 0.1% Triton X-100 in PBS, followed by five rinses in PBS. The hypodermis, carrying the sensilla, was then carefully detached from the cuticle and mounted on a microscope slide in a medium containing Mowiol dissolved in 0.3 M Tris buffer enriched with 2.5% 1,4-diazabicyclo(2,2,2)octane (DABCO) to reduce fading. For double-labeling experiments, the two primary and two secondary antibodies were used simultaneously. For controls, the primary antibodies were omitted or replaced with normal rabbit serum at the same dilutions. The preparations were examined under epifluorescence optics with an Axiosvert 100 inverted microscope (Carl Zeiss) and under a laser-scanning confocal microscope (LSM 510; Carl Zeiss) with an argon–krypton laser for Alexa Fluor 488 (488 nm) and a helium–neon laser for CY-3 (543 nm). Digital images of 0.5–1 μm optical sections were captured and analysed, and the final images were processed using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA).

### Statistical analysis

Statistical differences between means for correlated samples were determined using either a paired *t*-test or, in cases where the distribution was not normal, the nonparametric Wilcoxon signed-ranks test. For independent samples, an unpaired *t*-test or the nonparametric Mann–Whitney test were used. Results from directional tests are reported. VassarStats software (freely available at http://vassarstats.net/) was used to perform these tests.
Results

Effects of glutamate, ibotenic acid and muscimol on VS-3 neuron firing rate and membrane potential

When VS-3 neurons are stimulated by pseudorandom electrical noise they fire APs continuously. Usually the AP rate is higher at the beginning of the experiment and declines to a plateau level within 2 min (Fig. 2). To investigate how glutamate modified VS-3 neuron excitability, it was applied in the superfusion solution starting 60 s after the beginning of each experiment when the AP rate was, or close to, the plateau level. Glutamate reduced the AP rate of most VS-3 neurons at test concentrations of 100 μM, 200 μM, 500 μM and 1 mM. An example of this type of inhibitory effect is shown in Fig. 2A. In this and most other experiments membrane potential remained close to the resting level after glutamate application. However, some neurons had a quite different response to glutamate: their membrane depolarized strongly, AP firing initially stopped completely and then rose well above the plateau level. The experiment shown in Fig. 2B is an example of this type of an inhibitory–excitatory response. In addition, some VS-3 neurons were not inhibited by glutamate, but a small excitatory effect was observed, and others did not respond to glutamate at all (Table 1). These different types of responses occurred consistently at all test concen-

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Glutamate (%)</th>
<th>Ibotenic acid (%)</th>
<th>Muscimol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitory</td>
<td>36 (54)</td>
<td>9 (47)</td>
<td>12 (24)</td>
</tr>
<tr>
<td>Excitatory</td>
<td>5 (7)</td>
<td>2 (11)</td>
<td>7 (14)</td>
</tr>
<tr>
<td>Inhibitory–excitatory</td>
<td>14 (21)</td>
<td>5 (26)</td>
<td>28 (57)</td>
</tr>
<tr>
<td>No change in spike rate</td>
<td>12 (18)</td>
<td>3 (16)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Depolarization</td>
<td>33 (49)</td>
<td>16 (84)</td>
<td>47 (96)</td>
</tr>
<tr>
<td>No depolarization</td>
<td>34 (51)</td>
<td>3 (16)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Total number of neurons</td>
<td>67</td>
<td>19</td>
<td>49</td>
</tr>
</tbody>
</table>

Table 1. Agonist effects on VS-3 neuron AP rate and membrane potential

Fig. 2. Glutamate effects on AP rate and membrane potential. (A1) The gray trace shows the membrane potential ($E_m$) of −73 mV during the experiment, the black trace includes APs elicited by pseudorandom Gaussian white noise electrical stimulus. Insets (above) show the same recording at different time scales before, during and after application of 500 μM glutamate. (A2) The original recording was converted to AP rate (AP/s) using 1-s bins. The blue trace is AP rate of the same neuron as in A1 without glutamate application. Spiking decayed to a plateau level during the first ~100 s of noise stimulation. The red trace shows the AP rate from the recording in A1. AP rate decreased after glutamate application followed by a return to the plateau level. (B1) Similar experiment as in A but from a different VS-3 neuron. This neuron depolarized from −78 to −56 mV after application of 500 μM glutamate and stopped firing during the depolarization (inset). (B2) The blue trace is the AP rate from a control recording of the same neuron as in B1. The red trace is the AP rate from the experiment in B1 showing AP rate declining rapidly to zero when glutamate was applied followed by an increased rate that continued after the membrane potential had returned to resting level.
trations. The naturally occurring glutamate analog ibotenic acid was tested at 50 and 100 μM concentration and had a similar range of effects on VS-3 neurons as glutamate. Glutamate receptor agonists were also tested in nine experiments on cuticular preparations while the neurons were stimulated mechanically and a similar range of responses was observed as during electrical stimulation.

While the purely inhibitory, non-depolarizing responses to glutamate and ibotenic acid were different to those of VS-3 neurons to the GABA\(_A\) agonists GABA and muscimol, the inhibitory–excitatory responses were very similar for all four agonists (Pfeiffer et al., 2009). To compare these effects properly, we performed several experiments with muscimol, glutamate and ibotenic acid under similar conditions (Table 1). The most common outcome with muscimol, when tested at 20, 50 or 100 μM concentration, was an inhibitory–excitatory response, while glutamate and ibotenic acid more often caused purely inhibitory responses. It is notable that 18% of neurons tested had no change in AP rate, and 51% did not depolarize at all when glutamate was applied, while only 4% – or two neurons from a total of 49 – did not respond to muscimol. Ibotenic acid had no effect on 16% of the neurons tested.

For further comparison, Fig. 3 shows the membrane potential amplitudes and firing rates before and after application of each agonist. AP rates only include those experiments where inhibition or excitation occurred, while membrane potential values are included from all experiments. All three agonists produced statistically significant changes in inhibitory and excitatory AP rates and membrane potentials (paired \(t\)-tests unless otherwise indicated: inhibitory AP rates: muscimol \(t_{26} = 11.07, P \leq 0.0001\); glutamate \(t_{26} = 10.19, P \leq 0.0001\); ibotenic acid \(t_{26} = 4.85, P = 0.0001\); excitatory AP rates: muscimol \(t_{26} = -9.14, P \leq 0.0001\); glutamate \(t_{26} = -4.16, P = 0.0003\); ibotenic acid \(W = -28, P = 0.01\), Wilcoxon rank sum test; membrane potentials: muscimol \(t_{26} = -16.2, P \leq 0.0001\); glutamate \(t_{26} = -3.99, P \leq 0.0001\); ibotenic acid \(t_{26} = -5.26, P \leq 0.0001\) ). However, when the percentage changes between agonists were compared, it was clear that, on average, muscimol produced significantly larger depolarization and stronger inhibition than glutamate or ibotenic acid (Table 2). In contrast, the magnitudes of glutamate- vs. muscimol-induced excitatory changes were not statistically significantly different and ibotenic acid had an excitatory effect on such a small number of neurons that reliable statistical comparison was not possible.

**Frequency response analysis**

The excitatory effect of muscimol is more pronounced at high than low stimulating frequencies (Pfeiffer et al., 2009). To test if glutamate effects on AP rate also depend on stimulation frequency, we performed frequency response analysis from AP rates during both the inhibitory and excitatory phases of glutamate response. For comparison, similar analysis was also performed on excitatory responses to muscimol. Inhibitory responses to muscimol were so strong at all frequencies that the neurons stopped firing almost completely, making it impossible to perform frequency response analysis. Figure 4A shows examples of fits to the data during the inhibitory period after glutamate application as well as fits to the data from the same neuron under control conditions. Glutamate induced a clear decrease in the gain and coherence at all stimulating frequencies, but the phase did not change. Changes in the frequency response parameters from 15 similar experiments are shown in Fig. 4B. When tested with a paired \(t\)-test, both the amplitude (A) and information capacity (R) decreased significantly during inhibition (A: \(t_{14} = 2.89, P = 0.006\); R: \(t_{14} = 6.91, P \leq 0.0001\)), but the fractional exponent (k), which affects the phase and the slope of the amplitude plot, was not significantly different \(t_{14} = -0.67, P = 0.26\).

Both glutamate and muscimol raised the gain, phase and coherence during excitatory periods (Fig. 5A and B). These changes are reflected in the fitted frequency response parameters (Fig. 5C), although the effects of glutamate on A and R were somewhat smaller than those induced by muscimol (paired \(t\)-test for muscimol: A: \(t_{26} = -3.58, P = 0.0007\), k: \(t_{26} = -2.17, P = 0.02\); R: \(t_{26} = -4.97, P \leq 0.0001\).
and for glutamate: \( t_0 = -2.79, P = 0.01, k: t_0 = -1.99, P = 0.04; R: t_0 = -2.89, P = 0.009 \). Muscimol’s effect on coherence in the experiment of Fig. 5B was stronger at high than low stimulating frequencies. Although there was some variability in the magnitude of this change in different experiments, both glutamate and muscimol induced statistically significant increases in \( k \), indicating enhanced sensitivity at high stimulating frequencies.

**BAPTA-AM modulation of glutamate and muscimol responses**

Changes in intracellular Ca\(^{2+}\) concentration have been shown to contribute to firing behavior of VS-3 neurons and mechanosensory neurons in other preparations (Höger et al., 2010). GABA and muscimol application raised intracellular [Ca\(^{2+}\)] in VS-3 neurons (Panek et al., 2008), but it is not known if and how Ca\(^{2+}\) is involved in the physiological responses caused by GABA or glutamate receptor agonists. Therefore, we added the membrane-permeable Ca\(^{2+}\) chelator BAPTA-AM to the VS-3 neuron superfusion solution and examined changes in neural responses to glutamate and muscimol. Ca\(^{2+}\) chelation alone did not have any effect on VS-3 neuron membrane potential or plateau AP rate in 18 experiments after up to 60 min in BAPTA-AM superfusion (data not shown).

BAPTA-AM effects on AP rates and membrane potential changes in two experiments where glutamate was applied are shown in Fig. 6A and B: the typical inhibitory response became somewhat larger and was followed by a higher plateau level after BAPTA-AM (Fig. 6A). Glutamate-induced depolarization was significantly enhanced by BAPTA-AM in this neuron. BAPTA-AM had also a strong effect on inhibitory–excitatory glutamate responses (Fig. 6B): following 25 min of superfusion with BAPTA-AM, both the inhibition and the excitation lasted longer than under control conditions. After 40 min, inhibition was even longer-lasting, interrupted by a brief excitation and then followed by a new inhibition. While the depolarization amplitude was not significantly affected by BAPTA-AM in this neuron, its duration became gradually longer. Similar results, indicating that the glutamate effect was progressively strengthened by BAPTA-AM, were seen in a total of eight experiments.

As seen in Fig. 6C, the BAPTA-AM effect on VS-3 neuron muscimol responses was very different from its effect on glutamate responses. In this and nine other experiments the typical inhibitory–excitatory muscimol response, as well as the depolarization, either disappeared completely or were significantly reduced after superfusion with BAPTA-AM.

**Kainate effects on VS-3 neurons**

As responses of VS-3 neurons to glutamate and its analog ibotenic acid were somewhat variable, we investigated whether some of these responses could have been caused by activation of excitatory glutamate receptors either in VS-3 neurons themselves or in the surrounding efferent neurons, which in turn would have activated some other, e.g. GABAA receptors. Of the well-known agonists of different types of excitatory glutamate receptors, NMDA and AMPA have previously been tested on this preparation (Panek & Torkkeli, 2005), but neither had any effect on the membrane potential or firing behavior. Here, we tested kainate, an agonist of the third main type of excitatory glutamate receptor. Application of 50 \( \mu \)M (\( n = 14 \)) or 250 \( \mu \)M (\( n = 7 \)) kainate had no effect on VS-3 neuron AP rate or membrane potential.

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**Table 2. Comparison of the magnitudes of agonist-induced changes in membrane potential (\( \Delta E_m \)) and AP rate during inhibitory and excitatory phases**

<table>
<thead>
<tr>
<th>Variable</th>
<th>1. Glutamate</th>
<th>2. Ibotenic acid</th>
<th>3. Muscimol</th>
<th>1 vs. 2</th>
<th>1 vs. 3</th>
<th>2 vs. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta E_m )</td>
<td>5 ± 10% (67)</td>
<td>12 ± 10% (19)</td>
<td>22 ± 10% (49)</td>
<td>0.0013*</td>
<td>( \leq 0.0001 )†</td>
<td>( \leq 0.0001 )†</td>
</tr>
<tr>
<td>( \Delta AP ) rate</td>
<td>59 ± 23% (50)</td>
<td>64 ± 37% (14)</td>
<td>93 ± 12% (40)</td>
<td>0.33†</td>
<td>( \leq 0.0001 )‡</td>
<td>0.0011**</td>
</tr>
<tr>
<td>( \Delta AP ) rate</td>
<td>72 ± 85% (19)</td>
<td>21 ± 18% (7)</td>
<td>61 ± 59% (35)</td>
<td>n/a</td>
<td>0.45†</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Values are mean percentage change ± SD with the number of experiments in parentheses. The nonparametric Mann–Whitney test was used to investigate statistical differences between the distributions. \( z = -3.01, U = 926, \ z = -7.23, U = 2935.5; \ z = 3.68, U = 195.5; \ z = -0.44, U = 377.5; \ z = -6.21 \ U = 1765.5 \); \( **z = 3.05, U = 125; \ z = 0.12, U = 325.5 \).
We tested four different antibodies generated against non-overlapping peptide sequences of the *Drosophila* GluClα subunit (Ludmerer et al., 2002). These antibodies were initially immunoprecipitated with the spider brain and peripheral tissue and the purified complex was tested in Western blots. Three antibodies detected specific bands in the brain tissue and two antibodies in the peripheral tissue (Fig. 7). The Gcl2 band was at 60 kDa while Gcl3 and Gcl5 were both at 50 kDa.

The predicted molecular weight of *Drosophila* GluClα subunit is 50 kDa (Ludmerer et al., 2002). The amount of peripheral tissue that we could obtain for these experiments was limited, and it is possible that the Gcl5 antibody did not produce any bands due to the lack of adequate tissue. The fourth antibody, Gcl1, did not produce any specific bands on Western blots.

All of the four GluClα antibodies were also tested by immunohistochemistry against the spider patellar hypodermis preparation that contains the VS-3 and other mechanosensory organs. The Gcl1 antibody did not produce specific labeling, but the other three antibodies were immunoreactive against the mechanosensory organs.

Brightness of the labeled structures varied in different neurons in the VS-3 organs as seen in Fig. 8, where Gcl3 and Gcl5 antibodies were used. Some neurons could barely be distinguished from the background while others were strongly labeled. The morphological arrangement of VS-3 neurons in different legs varies, making it difficult to identify each neuron. However, the number 2 neuron pair always has one small and one large neuron. In Fig. 8A the larger neuron is strongly labeled while the smaller neuron is very faint, but in Fig. 8B both neurons in this pair are brightly labeled. Similar differences are also clear in other neurons. Each of the three antibodies were tested in a minimum of ten preparations and, on average, 73% of the VS-3 neurons were strongly labeled while the others were much fainter. Immunoreactivity was present in the soma, but also in the axonal and dendritic regions. This is not clearly visible in Fig. 8,
where the confocal image stacks contain 15 1-µm optical sections mainly on the soma region while the dendrites and axons are not fully visible. No labeling was seen in control experiments where primary antibody was omitted or replaced with normal rabbit serum. Figure 8C shows a control preparation where primary antibody was omitted. Only faint lipofuscin autofluorescence is visible in the VS-3 somata, as previously reported in this preparation (Fabian & Seyfarth, 1997).

To see if the GluClα immunoreactivity was restricted to the sensory neurons or also present in the efferent fibers surrounding them, we performed double labeling with the Gcl2 antibody and an antibody against a synaptic vesicle protein, synapsin, that is only present in the efferent fibers. The same synapsin antibody has previously been tested on this preparation using Western blots, immunohistochemistry and immunoelectron microscopy and shown to specifically label synaptic vesicles in efferent fibers (Fabian-Fine et al., 1999). Figure 9 shows one VS-3 organ where all six neuron pairs that are visible were immunoreactive to the Gcl2 antibody and the synapsin labeling was restricted to the fibers surrounding the VS-3 neurons. In this figure, which contains 25 1-µm optical sections, the Gcl2 and synapsin labeling on dendritic and axonal regions is also evident. In the double exposure images (Fig. 9C and higher magnification of a single optical section in Fig. 9E) the Gcl2- and synapsin-immunoreactive punctae are close to each other in several locations. In locations with overlapping labeling (white), immunoreactivity is probably in the same cells.

In addition to the VS-3 organ, all GluClα antibodies labeled other sensory organs, such as tactile hairs and joint receptors, in the spider.
cross-sections through the axo-somatic region of a VS-3 organ. Labeling, we tested two of the antibodies on electron microscopic
sections. Granules are visible in the VS-3 neuron somata. Scale bars = 20 μm.

Figure 10 shows images from four sections. Both Gcl3 and Gcl5 antibodies produced immunoreactivity in the VS-3 neuron membranes as well as in the efferent fibers and some labeling was also present in glial cells. Most of the labeling was on synaptic sites, although some was not associated with visible synaptic structures. However, the fixation and background labeling were minimal to preserve immunoreactivity and under these conditions ultrastructural synaptic structures are not always visible.

Discussion

Glutamate and GABA receptors in VS-3 neurons

That muscimol depolarized nearly all VS-3 neurons and that the majority had inhibitory–excitatory responses suggest that they have only one type of ionotropic GABA-receptor. The properties of this receptor have previously been described (Panek et al., 2002). Smaller numbers of receptors in some neurons may have resulted in smaller, either excitatory or inhibitory, responses. VS-3 neurons also have metabotropic GABA receptors, but their activation does not cause similar rapid changes in membrane potential or firing as ionotropic receptors (Panek et al., 2003). On the other hand, both glutamate and ibotenic acid caused two very different types of responses, suggesting that at least two different types of glutamate receptors were present. One response was almost identical to the muscimol effect, with large depolarization and inhibitory–excitatory response, while the other was non-depolarizing, and purely inhibitory. Initially, we suspected that glutamate and ibotenic acid may have activated GABA_A receptors in some neurons either directly or by stimulating efferent neurons to release GABA. However, this seems unlikely as none of the excitatory glutamate receptor analogs, NMDA, AMPA or kainate, had any effect on VS-3 neurons, and the glutamate effect was unchanged when synaptic transmission was blocked by Ni²⁺ in the superfusion solution (Panek & Torkkeli, 2005). In addition, Ca²⁺ chelation by BAPTA-AM abolished the muscimol effect, while the same treatment potentiated the neurons’ responses to glutamate.

Previous electrophysiological and pharmacological work suggests that spider VS-3 neurons have ionotropic glutamate receptors that, similarly to invertebrate GluCls, are sensitive to ivermectin (Panek & Torkkeli, 2005). The immunohistochemical results here, with three antibodies directed to different regions of the Drosophila GluClz subunit, as well as the rapid responses to glutamate and ibotenic acid, also suggest that these responses are mediated by ionotropic glutamate receptors, different from excitatory glutamate receptors that would probably be sensitive to NMDA, AMPA or kainate. However, we have not excluded the possibility that metabotropic glutamate receptors may be at least partially involved in the glutamate responses.

A variety of glutamate- or GABA-sensitive ligand-gated Cl⁻channels have been identified in other invertebrate preparations and they are often present in the same cells (Cleland, 1996; Kehoe & Vullius, 2000; Jones & Satelle, 2006, 2007, 2008). For example, in addition to GABA currents, cockroach thoracic ganglion neurons have three, and their dorsal unpaired median (DUM) neurons have two, types of glutamate-gated Cl⁻ currents (Raymond et al., 2000; Zhao et al., 2004; Narahashi et al., 2010). Locust DUM neurons have GABA-, glutamate- and histamine-gated Cl⁻channels (Janssen et al., 2010) and both GABA and glutamate depolarized crab neurosecretory neurons (Duan & Cooke, 2000). As invertebrate ligand-gated Cl⁻channels are targets of insecticides and antiparasitics, their molecular, pharmacological and biophysical properties have been intensely investigated (Knipple & Soderlund, 2010; Hibbs & Gouaux, 2011). Several subunits have been cloned and

leg. Figure 9D shows a stack of three 1-μm optical sections through a tactile hair cell. Gcl2 labeling is visible in all parts of the neuron and very close to the fibers labeled by synapsin in several locations.

To better determine the ultrastructural location of the GluClz labeling, we tested two of the antibodies on electron microscopic cross-sections through the axo-somatic region of a VS-3 organ.
they form functional homo- or heteromeric channels in expression systems, but native receptors are probably formed from heterologous subunits (Ludmerer et al., 2002; Eguchi et al., 2006; Knipple & Soderlund, 2010). Surprisingly little is known about the significance of these channels in regulating neuronal function. In some preparations, they cause inhibition (e.g. Dent et al., 1997; Cleland & Selverston, 1998; Raymond et al., 2000; Pemberton et al., 2001), but in most cases only transmitter-induced currents or membrane potential changes have been investigated (e.g. Duan & Cooke, 2000; Kehoe & Vulfius, 2000; Zhao et al., 2004; Janssen et al., 2007; Narahashi et al., 2010). Depending on the Cl\(^-\) equilibrium potential of each cell, activation of Cl-channels leads to either an outward current and hyperpolarization or an inward current and depolarization. Depolarization is often thought to cause excitation, but increased membrane conductance can reduce the effect of other synaptic currents, and depolarization can activate voltage-gated K-channels, inactivate Na-channels and have either effect on Ca-channels. Therefore, the actual effect on AP discharge cannot be directly assumed from the membrane potential change. Depolarization may cause an inhibitory or excitatory effect, or mixed response as seen in VS-3 neuron GABA responses. Furthermore, the inhibitory glutamate effect in VS-3 neurons occurs without significant change in membrane potential.

**Intracellular Ca\(^{2+}\)**

In most mature neurons, GABA induces Cl-influx, hyperpolarization and a decrease in Ca\(^{2+}\) levels (van den Pol et al., 1996). However, in addition to spider VS-3 neurons, GABA\(_A\) agonists raise [Ca\(^{2+}\)] in many other neurons where Cl\(^-\) efflux results in membrane depolarization (Panek et al., 2008). This is especially common in presynaptic locations, but also in mature and immature mammalian brain neurons (Kullmann et al., 2005; Yamada et al., 2004; Long et al., 2009), after neuronal trauma (van den Pol et al., 1996) and in epileptogenic
induced current was completely inhibited by the Cl-channel blocker (Sekizawa et al., 2009). This is also possible in VS-3 neurons, where muscimol-induced usually leads to Ca²⁺ entry via voltage-gated Ca-channels (Long et al., 2012). CaMK-II leading to differential modulation of cockroach neurons, an increase in intracellular Ca²⁺ led to an enhanced GABA current via phosphorylation that in cockroach DUM neurons (Kim et al., 2008). In these neurons GABAₐ activation usually leads to Ca²⁺ entry via voltage-gated Ca-channels (Long et al., 2009). This is also possible in VS-3 neurons, where muscimol-induced depolarization could open low-voltage-activated (LVA) Ca-channels (Sekizawa et al., 2000; Panek et al., 2008). However, muscimol-induced current was completely inhibited by the Cl-channel blocker picrotoxin, but not by Ni²⁺, which inhibits LVA-channels (Sekizawa et al., 2000; Panek et al., 2002, 2008). Maintenance of a long-lasting Cl-current could be achieved by a Ca²⁺ -dependent process, such as Ca-activated inward current, or phosphorylation, but it is also possible that muscimol activates other types of Ca-channels.

Ca²⁺ chelation of VS-3 neurons by BAPTA-AM removed muscimol-induced inhibition, and reduced excitation and depolarization, suggesting that these effects depend on Ca²⁺. In honeybee and cockroach neurons, an increase in intracellular [Ca²⁺] led to an enhanced GABA current via phosphorylation that in cockroach DUM neurons was mediated by Ca²⁺ /calmodulin kinase (CaMK-II) (Alix et al., 2002; Grünewald & Wersing, 2008; Dupuis et al., 2010). In mammalian cerebellar neurons, specific GABAₐ receptor subunits regulate [Ca²⁺] via CaMK-II leading to differential modulation of specific synapses (Houston et al., 2008; Long et al., 2009). Covalent modification of the receptor structure by phosphorylation can alter its biophysical properties and regulate synaptic transmission. Phosphorylation of GABAₐ receptors via a Ca²⁺ -dependent process could induce long-lasting depolarization and regulate excitability in VS-3 neurons.

In contrast to the muscimol experiments, VS-3 neurons depolarized more strongly and for a lot longer when glutamate was applied during BAPTA-AM treatment. The effect on spike rate was strong, causing oscillating inhibitory and excitatory phases while the membrane remained depolarized. These findings suggest that membrane repolarization was prevented because BAPTA-AM inhibited Ca-activated and perhaps voltage-activated K-currents. Continuous depolarization probably resulted in inactivation of Na-channels and inhibition of AP discharge. BAPTA-AM has previously been shown to block Ca-activated and voltage-activated K-currents in several preparations (e.g. Niesen et al., 1991; Tang et al., 2007). Interestingly, the BAPTA-AM effect on the glutamate response was very similar to the ivermectin effect on VS-3 and other invertebrate neurons (Cully et al., 1994; Panek & Torkkeli, 2005; McCavera et al., 2009). Ivermectin binds to the glutamate receptor transmembrane domain, stabilizing it in the open configuration and making it easier for glutamate to reach its binding site (Hibbs & Gouaux, 2011). It is possible that once glutamate activates GluCls in VS-3 neurons, Ca²⁺ aids in their rapid closure, and removal of this process by BAPTA-AM led to increased open probability. This mechanism could involve phosphorylation processes, different from those in GABAₐ receptors. Invertebrate ligand-gated Cl-channels have numerous potential intracellular phosphorylation sites, but very little is known about their roles in regulating the channel function (Knipple & Soderlund, 2010). Based on our findings these could be of interest for developing new species-specific insecticides.

**Location and identity of GluCls**

Three polyclonal antibodies against different regions of the *Drosophila* GluCls subunit (Ludmerer et al., 2002) strongly labeled 73% of the VS-3 neurons and other patellar sensory neurons. These results correlate well with our electrophysiological findings: in 82% of VS-3 neurons the AP-rate changed when glutamate was applied during random stimulation, and similar changes were seen in 84% of the neurons with ibotenic acid. Previously, 74% of VS-3 neurons were inhibited by glutamate and 69% by ibotenic acid during step stimulation (Panek & Torkkeli, 2005). These numbers are close to the inhibitory and inhibitory-excitatory responses seen here for glutamate (75%) and ibotenic acid (73%). There were no clear differences in the biophysical properties or morphology of neurons that lacked the GluCls or glutamate responses when compared with other neurons. The strong GluClz labeling was often seen in different neurons in different legs. Hypothetically, this arrangement could provide specific control of particular sensory neurons in each organ in each leg, a useful property for an animal that can use each of its eight legs for specific functions, and gather a variety of sensory information, during highly specialized behavioral tasks (Barth, 2001). The GluClz labeling was present in all parts of the sensory neurons, including the dendrites. This finding correlates well with the previous result in which glutamate was shown to reduce the receptor current when the neurons were stimulated mechanically under voltage-clamp (Panek & Torkkeli, 2005). The GluClz labeling in efferent neurons could mean that they can be presynaptically inhibited by glutamate to regulate the release of GABA, octopamine, ACh or glutamate to the VS-3, and other, sensory neurons.

**Physiological significance**

Although invertebrate ionotropic GABA and GluCl receptors are structurally related and appear to have somewhat similar functions, our findings suggest that in the spider sensory neurons they may perform different physiological roles. Random stimulation of sensory neurons has allowed us to see that while the GABA-mediated short-term process is inhibitory its long-term effect is excitatory. On the other hand, glutamate has a mainly inhibitory role with a minority of neurons having partially excitatory responses. If both GABA and glutamate responses described here are mediated by ionotropic receptors that are Cl-selective ion channels, they would need to have

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**Fig. 10.** Electron micrographs of VS-3 organs labeled with Dm-GluClz antibodies. (A) Immunogold labeling for Gcl5 antibody (arrow) in a VS-3 neuron close to an efferent profile. (B) Labeling for Gcl3 antibody on a VS-3 neuron (arrow) and on a glial cell (arrowhead). (C, D) Gcl3 labeling on efferent fibers (arrows). All sections are from an axo-somatic region of the VS-3 neurons. Scale bars = 500 nm in all images.

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structural differences, such as phosphorylation sites that allow different dependencies on intracellular Ca\textsuperscript{2+}, and perhaps on other messengers. However, it is also possible that the glutamate responses were at least partially mediated by metabotropic receptors. Ultimately, the different transmitter and receptor mechanisms that are present in the spider mechanosensory neurons could lead to a ability for fine adjustments of neuronal excitability. This may, in turn, provide the spider with a precise control over the sensory information that it uses to control body position and movements during complex behavioral tasks such as prey capture and courtship.

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Abbreviations

ACH, acetylcholine; AP, action potential; DMSO, dimethylsulfoxide; DUM, dorsal unpaired medium; GluCl, glutamate-gated chloride receptor; GABA, \gamma\text{-}aminobutyric acid; LVA, low-voltage-activated; PBS, phosphate-buffered saline.

References


