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Dorsal Unpaired Median Neurons of *Locusta migratoria* express ivermectin- and fipronil-sensitive glutamate-gated chloride channels

Running head: "GluCl in DUM neurons of *L. migratoria*"

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Abstract

Together with type A GABA and strychnine-sensitive glycine receptors, glutamate-gated chloride channels (GluCl) are members of the Cys-loop family of ionotropic receptors, which mediate fast inhibitory neurotransmission. To date, GluCls are found in invertebrates only, and therefore represent potential specific targets for insecticides, such as ivermectin and fipronil.

In this study, we identified the functional expression of GluCls in dorsal unpaired median (DUM) neurons of the metathoracic ganglion of *Locusta migratoria* using electrophysiological and molecular biological techniques. In whole-cell patch-clamped DUM neurons, glutamate-induced changes in both their membrane potentials (current-clamp) and currents (voltage-clamp) were dependent upon the chloride equilibrium potential. Upon continuous application of glutamate, the glutamate-elicited current response became rapidly and completely desensitized. Application of glutamate in the presence of 10 μM fipronil or 100 μM picrotoxin reversibly decreased GluCl-mediated currents by 87% and 39%, respectively. Furthermore, 1 μM ivermectin induced a persistent chloride current, suggesting the expression of ivermectin-sensitive GluCl α subunits. A degenerate PCR/RACE strategy was used to clone the full-length *Locusta migratoria* LmGlClα subunit. Finally, RT-PCR experiments demonstrated the presence of LmGluClα transcripts in locust DUM neurons. Our results provide the first direct evidence of a functional ivermectin-sensitive GluCl channel on the cell surface of DUM neurons of *L. migratoria*.

Introduction

Glutamate is the main excitatory amino acid neurotransmitter in the central nervous system of vertebrates, where it interacts both with ionotropic receptors, *i.e.*, ligand-gated ion channels, and metabotropic or G protein-coupled receptors (Hogner et al. 2002). In vertebrates, ionotropic glutamate receptors are permeable to cations and can be subdivided into three groups according to their respective main agonist: *N*-methyl-*D*-aspartate (NMDA), AMPA and kainate receptors.

In invertebrates, L-glutamate can also act as an excitatory transmitter at the neuromuscular junction (Wafford and Sattelle 1989; Ikeda et al. 2003). However, a biphasic response to L-glutamate is usually observed, consisting of a depolarization followed by a hyperpolarisation. A pure hyperpolarizing effect can be obtained by applying ibotenate, a conformationally restricted structural analog of glutamate (Wafford and Sattelle 1989). These responses are mediated by another class of glutamate ionotropic receptors. These are involved in inhibitory synaptic transmission, and consist of glutamate-gated chloride channels (GluCl). They were first identified in arthropods as extrajunctional glutamate receptors ('H' receptors) that cause hyperpolarisation of the locust leg muscle (Dudel et al. 1989; Cully et al. 1996). The inhibitory ionotropic glutamate receptor gates a chloride-selective ion channel and belongs to the Cys-loop ligand-gated channel superfamily, together with the nicotinic acetylcholine, type A GABA, type C GABA, glycine, type 3 5-HT receptors (Cull-Candy 1976; Cleland 1996) and the histamine gated chloride channel (Zheng et al. 2002).

Up to now, GluCl channels have only been found in invertebrate nerve and muscle cells (Cleland 1996). GluCl receptors thus form a potentially important target

for the development of insecticides having highly selective toxic activity against insects, which have become resistant to other insecticides (Raymond and Satelle 2002; Zhao et al. 2004b). Chemical compounds that affect GluCl receptors include members of the avermectine/milbemycin (A/M) class of anthelmintics such as ivermectin, as well as the phenylpyrazole insecticides, including fipronil. These products are in common use, and form effective prophylactic and curative treatments against endo- and/or ectoparasitic infestations (Horoszok 2001; Bloomquist 2003). Ivermectin, a macrocyclic lactone derived chemically from the parent macrocyclic lactone avermectin B_{1A}, appears directly to activate GluCl or, at lower concentrations, to potentiate the effect of glutamate (Yates and Wolstenholme 2004). However, due to their close homology with GluCl, it is likely that GABA-gated channels in invertebrates are also sites of ivermectin toxicity (Kane et al. 2000). Fipronil has been designated as a new GABA-gated chloride channel blocker, and introduced into pest control, for instance, against the Colorado potato beetle and against some cotton pests (Moffat 1993; Smith and Lockwood 2003). The blocking action of fipronil on GluCl channels has been demonstrated in oocytes transfected with GluCls (Horoszok et al. 2001), and a similar degree of block was observed on chloride currents induced by glutamate and ibotenate in dorsal unpaired median (DUM) neurons of the cockroach *Periplaneta americana* and the grasshopper *Melanoplus sanguinipes* (Smith et al. 1999; Raymond et al. 2000; Ikeda et al. 2003).

At the molecular and functional level, GluCls remain poorly characterized. Up to now, cloning studies have led to the identification of a small family of six GluCl genes in the nematode. In *Haemonchus contortus*, three genes encoding four subunits have been identified. In *Drosophila melanogaster*, a cDNA clone (DmGluClα) was obtained by hybridization screening (Cully et al. 1996). Electrophysiological studies

of *D. melanogaster* DmGluClα, expressed in oocytes, revealed direct activation by the acaricide nodulosporic acid and by ivermectin, as well as by the endogenous ligand glutamate (Kane et al. 2000).

The neurobiology of the locust has been extensively investigated, and the action of L-glutamate upon muscles of in this insect has been well studied (Grolleau and Lapied 2000; Wicher et al. 2001; Heidel and Pflüger 2006). However, in the nervous system of *L. migratoria*, relatively little is known about the effect of glutamate on efferent DUM neurons. In the locust, efferent DUM neurons extend symmetrical pairs of axons to flight and leg (tibia) muscle, where they exert myo- and/or neuromodulatory effects. In other arthropod species, such as *P. americana*, GluCls seems to have a modulatory effect on the excitability of the DUM neurons and could play a substantial role in muscle and flight control (Dubas 1991; Washio 2002). It is thus tempting to postulate that GluCls fulfill the same role in DUM neurons of *L. migratoria* and that they might well therefore also represent a useful target for insecticide pest control in this species too.

In the present study, we looked for the presence of a glutamate-gated chloride channel in an *ex vivo* preparation of DUM neurons of the metathoracic ganglion of *L. migratoria* using the whole-cell patch-clamp technique. Under current-clamp conditions, glutamate-induced changes in membrane potential were measured, while under voltage-clamp conditions, glutamate-evoked currents were pharmacologically and kinetically characterized. Finally, using molecular biology techniques, we looked for the expression of GluCl transcripts that could account for the observed glutamate responses.

Material and Methods

Isolation of metathoracic DUM neurons of the locust

Adult migratory locusts (*L. migratoria*) were used in all experiments. They were taken from a crowded laboratory colony maintained at ~32°C on a 14:10 hour light/dark cycle and on a diet of grass and oatmeal. Isolated DUM neuronal cell bodies were prepared as described previously (Brône et al. 2003). Briefly, the dorsal median region containing the neuromeres T3, A1-A3 of the metathoraric ganglion were mechanically removed and subjected to collagenase/dispase (2mg/ml) treatment. The cells were centrifuged, and subsequently washed three times with culture medium consisting of equal parts of Basal Medium Eagle with Hank's salts (BME) and Grace's Insect Medium (GIM). In the first wash, a 1% penicillin/streptomycin mixture was added to the BME/GIM culture medium. The cells were dissociated by repetitive up and down aspirations through a pipette tip, then plated on Nunc petri dishes and incubated at 28°C in a 5% CO_2 atmosphere. All products were GibcoBRL (obtained from Invitrogen, Belgium), except for the Boehringer collagenase/dispase mixture (from Roche Diagnostics, Belgium). Healthy efferent DUM neurons of diameter of approximately 40 to 60 μm were recognized by their morphological characteristics under phase contrast microscopy (Nikon Diaphot, Japan) – see, e.g., Brône et al. 2003.

Electrophysiology

Changes in the membrane potential of and current in DUM neurons of *L. migratoria* were recorded at room temperature using the whole-cell configuration of the patch-

clamp technique both in current-clamp and voltage-clamp conditions. Locust DUM neurons were transferred onto the stage of an inverted phase contrast microscope (Nikon Diaphot, Japan) in a recording chamber that was continuously perfused with solutions further described in Table 1. Electrodes made from 1.5 mm (o.d.) borosilicate glass capillary tubes, having a resistance between 800 kΩ and 1.5 MΩ, were also appropriately filled with the solutions listed in Table 1. In order to reduce contaminating Na^+ and K^+ currents through the voltage-gated ion channels, some experiments were conducted using modified intracellular and extracellular solutions (Table 1). The osmolality of ~380 mOsm/kg of these solutions, corresponding to that of *L. migratoria* hemolymph (Brône et al. 2003), was attained by addition of glucose. Liquid junction (LJ) potentials were calibrated using the Junction Potential Calculator for Windows (JPCalcW, Peter H. Barry, Dept. of Physiology & Pharmacology, Australia and Axon Instruments, Inc., California, USA), and were taken into account at the start of each experiment. The LJ potential between the standard intracellular solution (SIS) and standard extracellular solution (SES) was –18 mV, between highCl$^-$-IS and SES was –21.8 mV, between lowNa$^+$-ES and lowK$^+$highCl$^-$-IS was –6.7 mV and between lowNa$^+$-ES and lowK$^+$lowCl$^-$-IS was –19.2 mV. The experimental protocols and data acquisition were carried out using an EPC-10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) controlled by the program Pulse (HEKA Elektronik, Lambrecht, Germany) running on a personal computer (equipped with a Pentium III processor). Capacitive and leak currents were compensated, and residual capacitances and leak currents were eliminated, by means of a P/6 protocol (Moyer and Brown 2002). The series resistance was lower than or equal to 1.5 MΩ and was compensated up to 53 %. The recording of whole-cell

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current was started 5 min after rupture of the cell membrane in order to allow adequate equilibration of the cell interior with the pipette solution.

Data acquisition and analysis

Data were filtered at 2.9 kHz and sampled at 20 kHz. They were stored on a computer hard disk, and analyzed with the program PulseFit 8.77 (HEKA instruments). Further analyses, including non-linear regression, were carried out using Origin 6.0 Professional (Microcal Software, Northampton, MA).

Current-voltage relationships were fitted with the Goldman-Hodgkin-Katz (GHK) equation:

$$I_x = \frac{z^2 F^2}{RT} P_x V_m \left\{ \frac{[X_i] - [X_o] e^{-zFV_m/RT}}{1 - e^{-zFV_m/RT}} \right\}$$

Desensitization kinetics of individual glutamate-evoked currents was assessed by fitting the desensitizing phase with the following equation:

$$I = I_0 . e^{-\frac{t}{\tau}}$$

Except where stated otherwise, results are expressed as mean $\pm$ S.E.M., n being the number of experiments. Statistical comparisons were made using the Student's *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's post-tests. Differences were considered significant for $P \leq 0.05$.

Chemicals

Monosodium glutamate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and avermectin B_{1A} (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were

dissolved in triply distilled water. Stock solutions of fipronil (PESTANAL®, from Sigma-Aldrich GmbH, Seelze, Germany) and picrotoxin (PTX, from Sigma-Aldrich GmbH, Seelze, Germany) were made in ethanol (EtOH) and dimethylsulfoxide (DMSO), respectively. These stock solutions were diluted into the extracellular solutions immediately before the start of each experiment. The final concentrations of EtOH and DMSO in the solutions were ≤ 0.1%, at which they had no effect on glutamate-induced currents (data not shown). L-glutamate and ivermectin were applied topically through a valve-controlled tubing system positioned laterally above the cell at a distance of approximately 50 − 100 μm. The antagonists fipronil and PTX were applied to the bath.

Degenerate PCR and RACE.

To obtain the full length sequence of the *L. migratoria* GluClα channel, a Marathon RACE library was constructed using a mixture of several tissues (including muscle and neuronal tissues) as described by the manufacturer (Clontech, Hamburg, Germany). Degenerated oligonucleotides were designed and synthesized based upon the predicted amino acid sequences of the conserved regions of the glutamate-activated chloride alpha channel (GluClα) after alignment of the GluClαs from *Drosophila melanogaster* and *Caenorhabditis elegans*:

DEG-F: 5'-GT(CG)TCATGGGT(TA)TCATT(CT)TGG-3'

DEG-R: 5'-TC(CG)AG(CA)AG(TG)GC(CT)CCGAA(CT)AC(AG)AA(CT)GT-3'.

The thermocycler was programmed for 35 cycles at 94°C for 30 seconds, 41.5°C for 45 seconds, and 68°C for 45 seconds using the *L. migratoria* RACE library as a template and the Advantage Taq 2 polymerase mixture (Clontech) as polymerase.

The resulting band of 193 bp was directly sequenced, and the following primers were designed for rapid amplification of cDNA ends (RACE):

GSP-F1: 5'-GGCTCGATCAGGGTGCCGTTC-3'

GSP-F2: 5'-CCGTTCCCGCACGAGTGTCC-3'

GSP-R1: 5'-TCGAGAAGTGCCCCGAATACGA-3'

GSP-R2: 5'-ATGTTAGGCAGACGCCGGTCCA-3'.

The first RACE PCR was performed for 35 cycles at 94°C for 30 seconds and 68°C for 4 minutes using Advantage Taq 2 polymerase mixture and GSP-F1 (3'-RACE) or GSP-R1 (5'-RACE) combined with adaptor primer 1 (AP1). A second nested PCR was performed with GSP-F2 or GSP-R2 and AP2 primer. The PCR fragments (1000-2000bp) obtained were cloned into the pGEM-T vector (Promega, Karlsruhe, Germany) and sequenced until start and stop codons were identified.

RNA isolation from DUM neurons and RT-PCR.

DUM neurons of *L. migratoria* were isolated as described above with slight modifications. After the collagenase/dispase treatment, the cells were plated on a NUNC petri dish and incubated for at least 15 minutes at 28°C. The cells were picked up using a borosilicate patch pipette, then washed with ice cold PBS (pH 7.4) and transferred into an RNase-free microtube.

Total RNA was prepared from the material obtained from about 40 DUM neurons with the PicoPure® RNA Isolation KIT (Arcturus Bioscience Inc., Westburg B.V., Leusden, Netherlands) with an additional DNase treatment (Rnase-free DNase set, Qiagen) following the instructions of the suppliers. In addition, RNA from a mixture of several neuronal tissues (abdominal and thoracic ganglia, brain) was

prepared. First strand cDNA was synthesized using the Promega RT system of Promega Benelux BV, Leiden, Netherlands).

The cDNA of isolated locust DUM neurons and locust neuronal tissues was used as a template for a PCR using Taq DNA polymerase (Applied Biosystems). A fruitfly cDNA was used as a positive control. PCR primers DEG-f and DEG-R were used for amplification during 40 cycles at 94°C for 30 seconds, 41.5°C for 45 seconds, and 72°C for 45 seconds. PCR products were than separated on a 2% high-resolution agarose gel. Several distinctive bands were observed. The band of interest (~190-200 bp) was extracted from the gel using the Qiaquick gel extraction kit (Qiagen), purified and, in the case of isolated DUM neurons, subjected to a second PCR amplification using the same primers and incubation conditions. The amplified PCR fragments were either directly sequenced or cloned into pGEM-T for sequencing.

Results

Glutamate elicits changes in the membrane properties of locust DUM neurons

When recorded with solutions close to physiological conditions (SIS-SES; see Methods and Table 1), whole-cell patch- and current-clamp locust DUM neurons had a resting membrane potential of -34.4 ± 3.8 mV (n = 6). As shown in figure 1A, application of 1 mM glutamate hyperpolarized the membrane to -62.6 ± 1.81 mV (n = 6). When Cl^- concentrations were identical in intracellular and extracellular solutions, DUM neurons had a resting membrane potential of -58.8 ± 1.4 mV (n = 5), and in that case glutamate induced a depolarisation of the membrane to -7.9 ± 3.4 mV (n = 5; figure 1B).

These results suggested that glutamate could activate a glutamate-gated chloride channel (GluCl) in locust DUM neurons. Such GluCls in other species are often characterized by a complete desensitization under a continuous glutamate superfusion with a time constant on the order of hundreds of milliseconds to seconds. In order to examine the kinetics of the glutamate-evoked currents in DUM neurons, recordings were obtained under voltage-clamp conditions using identical chloride concentrations in the intracellular and extracellular solutions. Under those conditions, application of 1 mM glutamate at a holding potential of −70 mV elicited an inward current that reached a peak amplitude of −4.2 ± 0.7 nA (n = 7) within 150–250 ms (figure 2). As expected for GluCls, the current desensitized completely in the continuous presence of glutamate following a mono-exponential time course (τ_{decay} = 194 ms ± 39 ms, n = 7).

Glutamate-induced currents are carried by chloride ions

To assess whether or not the main ionic species carrying the glutamate-induced currents was chloride, the current-voltage relationships were obtained using two different intracellular solutions: one containing nearly identical intracellular and extracellular Cl^- concentrations (lowK$^+$highCl$^-$-IS) and a second one containing a lower Cl^- concentration in the intracellular solution (lowK$^+$lowCl$^-$-IS). These solutions were also modified (see Table 1) in order to reduce contaminating currents through the voltage-dependent channels (e.g., K_v, Na_v, Ca_v). Currents were evoked by repeatedly applying 1 mM glutamate for 3 seconds and at the same time applying a voltage step ranging from −60 mV up to +60 mV. In between applications, V_H was returned to −70 mV and glutamate was washed out (Figure 3A).

Maximum peak currents were measured at each voltage step and normalized for the cell capacitance. The reversal potential (E_{Glu}) was calculated after fitting the data with the GHK equation (see Methods; Figure 3B). Using nearly identical Cl^- concentrations in the extracellular and intracellular solutions, glutamate-induced currents reversed in polarity at a membrane potential of +2.6 mV (n = 4), which was close to the calculated chloride equilibrium potential ($E_{Cl^-} = -0.9$ mV). At lower intracellular Cl^- concentrations, E_{Glu} shifted to a more negative value (−13.6 mV; n = 3), which was in good agreement with the expected shift in the calculated Nernst equilibrium potential for chloride ions ($E_{Cl^-} = -20$ mV).

These results clearly demonstrate that the glutamate-induced currents in locust DUM neurons are mainly carried by chloride ions.

Pharmacology of glutamate-induced currents

Glutamate-induced currents in locust DUM neurons were then tested for their sensitivity to two known blockers of GluCls: fipronil and picrotoxin (PTX). As shown in Figures 4A and 4B, both drugs reversibly inhibited glutamate-gated currents. Ten micromolar fipronil inhibited to 13.1 ± 2.5 % of controls (n = 5; Figure 4A$_2$). After washout, glutamate currents recovered to 74.4 ± 4.4 % (n = 5) of the control. In the presence of PTX, glutamate-evoked currents were reduced to 38.8 ± 4.2% (n = 4; Figure 4B$_2$), whereas recovery was to 87.8% ± 8.2% (n = 4) after washout.

Finally, currents induced by ivermectin (IVM), a macrocyclic lactone which is known irreversibly to activate GluCl channels directly (Cleland, 1996), were compared to glutamate-induced currents. In this series of recordings, currents induced by 1 mM glutamate in locust DUM neurons had a mean peak amplitude of −2.7 nA ± 0.7 nA (n = 5; Figure 4C$_1$). In contrast, 1 µM IVM induced a current that did not

desensitize, and did not even fully reverse after the removal of IVM. IVM-induced currents amounted to -5.9 ± 1.5 nA (n = 6; Figure 4C$_2$).

These pharmacological data further support the presence of a glutamate-gated chloride channel in efferent DUM neurons of *L. migratoria*.

Cloning of full length LmGluClα

Since functional GluCls usually require the expression of at least GluClα subunits (Cully et al. 1996, Semenov and Pak 1999), we developed a degenerate PCR and RACE strategy in an attempt to clone the full length sequence of *L. migratoria* GluClα (LmGluClα). Within the isolated cDNA prepared as described in the Method section, a 1362 bp open reading frame was identified encoding for a 453 amino acid protein. Typical features of GluCl subunits as in other members of the Cys-loop ligand-gated ion channel superfamily were found: a large extracellular N-terminal domain containing the ligand-binding site, a conserved Cys-loop and four transmembrane regions (Figure 5). Moreover, the LmGluClα subunit showed strong homology to orthologous sequences from *Drosophila melanogaster* (CAA05260, 82.2% identity, 87.6% similarity), *Tribolium castaneum* (XP_973383, 84.8% identity, 91.4% similarity), *Anopheles gambiae* (XP_321697, 83.8% identity, 88.6% similarity), *Apis melifera* (ABG75738, Jones & Sattelle 2006, 78.3% identity, 87.1% similarity), *Musca domestica* (BAD16657, 81.3% identity, 86.7% similarity), and *Lucilia cuprina* (AAC31949, 83.7% identity, 89.1% similarity). The cDNA sequence of the LmGluClα subunit was deposited in GenBank under Acc.No. DQ643254.

DUM neurons of L. migratoria express LmGluClα messengers

The expression of LmGluClα in locust DUM neurons was assessed as follows. mRNAs of isolated DUM neurons were reverse transcribed and used as template for a two-step PCR procedure. After the first PCR, a faint band was visible (Figure 6A). This PCR band was excised, purified and re-amplified (Figure 6B). Direct DNA sequencing confirmed the LmGluClα sequence.

Discussion

In the work reported in this paper, we have clearly demonstrated that efferent DUM neurons of *L. migratoria* express functional glutamate-gated chloride channels (GluCls), using molecular biology and electrophysiological methods. In the presence of physiological solutions mimicking the hemolymph, glutamate hyperpolarised the membrane of DUM neurons as would be expected were GluCls to be present and active. Voltage-clamp experiments indicated that glutamate-induced currents in locust DUM neurons: 1) desensitize completely under continuous glutamate application, 2) are mainly carried by chloride ions, and 3) are blocked by fipronil and PTX, consistent with the properties of GluCls in other invertebrate species. Expression of GluClα subunit-containing GluCls was suggested by the sensitivity to IVM, which induced non-desensitizing persistent currents. Expression was confirmed by the RT-PCR detection of GluClα transcripts in locust DUM neurons. Finally, we also report the full DNA sequence of the GluClα subunit of *L. migratoria*.

In invertebrates, glutamate can interact with two classes of ionotropic receptors: excitatory channels, mainly permeable to cations as in vertebrates, and inhibitory anionic channels that are only found in invertebrates. When using intra- and

extracellular solutions with an ionic composition close to that of the hemolymph, we observed a hyperpolarizing response upon glutamate application, suggesting that locust DUM neurons only express inhibitory glutamate-activated channels (Wafford and Sattelle 1989; Ikeda et al. 2003). The chloride dependence of glutamate-induced responses was further confirmed by the shift to depolarization when E_{Cl^-} was changed from -79.6 to -4.1 mV and by the concordance of reversal potentials of glutamate-induced currents with chloride equilibrium potentials. Thus, it appears that *L. migratoria* DUM neurons express inhibitory ionotropic glutamate receptors only, and not their excitatory counterparts. This was also observed for DUM neurons of other insect species such as the cockroach *Periplaneta americana* (Washio et al. 2002), and for the stomatogastric ganglion of the lobster *Panulus interruptus* (Cleland and Selverston 1998). This is in contrast to the expression of both inhibitory and excitatory glutamate receptors by muscle cells in the locust or the crayfish (Kerry et al. 1987, 1988; Dudel and Franke 1987), or by motor neurons such as the fast coxal depressor motor neuron of the cockroach thoracic ganglion (Wafford and Satelle 1989).

In our hands, both glutamate-induced voltage and current changes were monophasic. Furthermore, under voltage-clamp conditions, glutamate-induced currents completely desensitized in about 1 second in the continuous presence of glutamate. These properties are similar with those reported for *Caenorhabditis elegans* GluCls expressed in *Xenopus* oocytes (Forrester et al. 2003). In cockroach DUM neurons, on the other hand, biphasic glutamate responses were observed, consisting of an initial transient and a subsequent prolonged phase (Raymond et al. 2000; Heckman and Dudel 1995; Zhao et al. 2004a). The desensitizing and non-desensitizing components of the glutamate-induced responses seem to be due to the

presence of pharmacologically distinct GluCls: the former sensitive and the latter insensitive to PTX or BIDN. The desensitizing glutamate-evoked current we report in locust DUM neurons is also PTX-sensitive. It therefore probably corresponds to the transient current described in cockroach CNS neurons.

In locust DUM neurons, fipronil seems to be a more powerful antagonist of GluCl-mediated responses than does PTX. At 10 μM, it blocked 86 % of glutamate-gated currents. This is in good agreement with the 75 % block by 10 μM fipronil of the transient component of glutamate responses in cockroach DUM neurons reported by Raymond et al. (2000). To the best of our knowledge, our work is the first report of an effect of fipronil on isolated DUM neurons of *L. migratoria*. The blocking effect of fipronil on GluCls had been proposed to explain part of the toxicity of this insecticide, which is widely used in pest control to eradicate dieldrin-resistant insects (Horoszok et al. 2001; Tingle et al. 2003; Zhao et al. 2004b; Smith and Lockwood 2003).

Ivermectin (IVM) at 1 μM generated an irreversible inward current under identical chloride concentrations in DUM neurons of *L. migratoria*. At concentrations in the micromolar range, avermectin-derivatives are known activators of ligand-gated chloride channels, *i.e.* strychnine-sensitive glycine receptors (Shan et al. 2001), GABA receptors (Robertson 1989; Adelsberger et al. 2000), and GluCls (Cleland 1996). Since glycine receptors are not found in invertebrates, the IVM-induced current observed in DUM neurons could be the consequence of the activation of either GABA receptors or GluCls − or even both, as already demonstrated in the fruit fly (Ludmerer et al. 2002). Accordingly, the amplitude of the current induced by 1 μM IVM was significantly higher than that induced by 1 mM glutamate, a potentially saturating concentration for GluCls. This could suggest that IVM activates both GABA receptors and GluCls in DUM neurons of the locust. On the other hand,

avermectin-sensitive chloride currents in *Xenopus* oocytes injected with *C. elegans* mRNA were GABA-insensitive, but sensitive to glutamate (Yates et al. 2003). Further characterization of IVM-induced currents is needed to solve this issue, but that is beyond the scope of this work. As far as GluCls are concerned, it is usually accepted that the ability to irreversibly bind IVM is typically diagnostic for α-like subunits of GluCls (Cully et al. 1994; Li et al. 2002). This has been demonstrated in several invertebrate species: locust (*Schistocerca americana*; Rohrer et al. 1994), barber pole worm (*Haemonthus contortus*; Forrester et al. 2001; Portillo et al. 2003), heartworm (*Dirofilaria immitis*, Yates et al. 2004), elegant worm (*Caenorhabditis elegans*; Horoszok et al. 2001; Dent et al. 1997, 2000), and strongylid worm (*Cooperia oncophora*; Njue et al. 2004). As opposed to GluClα subunits, GluClβ subunits have never been shown to respond to IVM (Cully et al. 1994; Li et al. 2002). Note, however, in some species, splice variants of GluClα subunits have been shown to be IVM resistant, e.g., DiGluClα3A from *Dirofilaria immitis* (Yates et al. 2004) or CoGluClα3 from *Cooperia oncophora* (Njue et al. 2004).

The functional expression of α subunit-containing GluCls by locust DUM neurons suggested by their IVM sensitivity was demonstrated at the molecular level. A full length LmGluClα channel was cloned, using a PCR/RACE strategy, and its messenger RNA in isolated DUM neurons was detected by RT-PCR. This does not rule out the expression of other LmGluCl subunits and, hence, does not allow any conclusion about the homo- or heteromeric nature of locust GluCls. Transfection of the cloned LmGluClα in heterologous expression systems might help to solve this question.

The functional implication of glutamate-gated chloride channels in DUM neurons of *L. migratoria* certainly deserves further attention. Since metathoracic

efferent DUM neurons innervate flight and leg muscles, their modulation by inhibitory GluCls is likely to influence flight control (Groleau and Lapied, 2000). Furthermore, and more generally, since GluCls have been described up to now in invertebrates only, a better insight into their functioning would undoubtedly contribute to the development of novel insecticides both more selective and more potent (Raymond and Satelle 2002; Zhao et al. 2004b).

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Acknowledgments

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Figure 1. Glutamate-induced membrane potential changes in locust metathoracic ganglion DUM neurons, recorded with the whole-cell patch-clamp technique in current-clamp mode. (A) In the presence of standard intracellular and extracellular solutions (SIS – SES; see inset), application of 1 mM glutamate for 1 second hyperpolarized the DUM neuron membrane potential from –35 to –61 mV. (B) When using almost identical chloride concentrations in the intracellular and extracellular solutions (highCl⁻-IS – SES; see inset), glutamate generated a depolarisation from –55 to –19 mV.

Figure 2. Glutamate-evoked currents in locust metathoracic ganglion DUM neurons, recorded with the whole-cell patch-clamp technique in voltage-clamp conditions. This figure shows a typical experiment. In the presence of almost identical chloride concentrations in the intracellular and extracellular solutions (HighCl⁻IS – SES; see inset) and at a holding potential (V_H) of –70 mV), the application of 1 mM glutamate evoked an inward current that reached a peak amplitude of 5.6 nA. Current production desensitized completely during glutamate administration, with a mono-exponential time-course ($\tau = 246$ ms).

Fig 3. Current-voltage relationship of glutamate-induced currents in locust DUM neurons recorded with the whole-cell patch-clamp technique.
(A) Currents (A_2) were evoked by 1 mM glutamate applications at holding potentials ranging from –60 to +60 mV (A_1), in the presence of almost identical Cl⁻ concentrations and intracellular (lowK⁺HighCl⁻-IS) and extracellular solutions (lowNa⁺-ES) modified to reduce contaminating voltage-dependent currents. Between voltage steps, V_H was returned to –70 mV and glutamate was washed out. B) *I-V*

relationship, obtained from glutamate induced currents described in (A) ([high Cl$^-$]$_I$; closed circles). Maximum peak currents were normalized for the cell capacitance and are plotted against the holding potentials. The relation was best-fitted with the GHK equation, which yielded a reversal potential of +2.65 mV. This was close to the calculated equilibrium potential (−0.9mV). When the intracellular chloride concentration was lowered to 83 mM ([low Cl$^-$]$_I$; open circles), the reversal potential shifted to −13.6 mV in agreement with the theoretically expected shift (−20 mV).

Figure 4. Pharmacological properties of the glutamate-gated chloride channel in DUM neurons of *L. migratoria*. 1 mM glutamate was applied for 3 seconds at a V$_H$ of −70 mV in HighCl$^-$-IS and SES conditions (see Methods), alone or in combination with 10 μM fipronil (A$_1$) or 100 μM picrotoxin (PTX, B$_1$). Recovery from inhibition (washout) was also checked. The histograms in A$_2$ and B$_2$ summarize fipronil- and PTX-induced inhibition of glutamate-activated currents (mean ± SEM; n = 5 for fipronil, 4 for PTX; ** P<0.001 and *** P<0.0001: one-way ANOVA followed by Dunnett's post-tests). (C) A 1 mM glutamate application was followed by perfusion for 3 seconds with 1μM ivermectin (IVM), which elicited an inward current that persisted on washout of IVM.

Fig.5: cDNA and protein sequence of LmGluClα. Start and stop codons are shown in bold/italics. Transmembrane regions (grey) and conserved cysteine residues of the Cys-loop (in box) are indicated. In addition, the position of the primers used for initial degenerate PCR are shown (underlined).

Fig 6. Expression of the GluClα channel in DUM neurons of *L. migratoria*. (A) Agarose electrophoresis of the first PCR using locust DUM neuron cDNA as a template (lane a2; marker = 100 bp ladder shown in lane a1). (B) Re-amplification of the corresponding 193 bp PCR product for DNA sequencing (lane b3; marker = 50 bp ladder shown in lane b1). The positive control used was DmGluClα (lane b2).

Table 1. Composition of the solutions used in patch-clamp experiments on isolated DUM neurons of *L. migratoria*.

Extracellular solutions (ES, mM)			Intracellular solutions (IS, mM)				
	SES	lowNa$^+$-ES		SIS	highCl$^-$-IS	lowK$^+$highCl$^-$-IS	lowK$^+$lowCl$^-$-IS
NaCl	172.5	40	NaCl	6.5	6.5	6.5	6.5
KCl	6.5	6.5	K gluconate	160	-	-	-
CaCl$_2$	2	3	CaCl$_2$	1	1	1	1
MgCl$_2$	7.7	7	MgCl$_2$	-	-	-	-
HEPES	10	10	Mg ATP	2	2	2	2
choline Cl	-	90	EGTA	10	5	5	5
CdCl$_2$	-	2	HEPES	10	10	10	10
tetraethylammoniumCl	-	25	CsCl	-	-	170	75
4-aminoperidine	-	5	KCl	-	160	-	-
tetrodotoxin	-	100 nM					
			*free [Ca^{2+}]	0.2 μM	0.2 μM	0.2 μM	0.2 μM
pH	6.8	6.8	pH	6.65	6.65	6.65	6.65
osmolality	380	380	osmolality	380	380	380	380

The free Ca^{2+} concentrations of the intracellular solutions were calculated for given concentrations of Ca^{2+}, Mg^{2+} and chelators using the freeware Sliders v. 2.10, WinMAXC v.2.10 (both available at http://www.stanford.edu/~cpatton) and CaBuf (at ftp://ftp.cc.KuLeuven.ac.be/pub/droogmans).

Figure 1

A

1 mM glutamate

-35 mV

SIS
[Cl⁻]ᵢ   8.5 mM
[Na⁺]ᵢ 6.5 mM
[K⁺]ᵢ   160 mM

SES
[Cl⁻]ₒ 198 mM
[Na⁺]ₒ 172.5 mM
[K⁺]ₒ 6.5 mM

B

1 mM glutamate

-55 mV

highCl⁻-SIS
[Cl⁻]ᵢ 168 mM
[Na⁺]ᵢ 6.5 mM
[K⁺]ᵢ   160 mM

SES
[Cl⁻]ₒ 198 mM
[Na⁺]ₒ 172.5 mM
[K⁺]ₒ 6.5 mM

10 mV

250 ms

Figure 2

1 mM glutamate

$V_H = -70\ mV$

highCl⁻-IS

$[Cl^-]_i$ 168 mM
$[Na^+]_i$ 6.5 mM
$[K^+]_i$ 160 mM

SES

$[Cl^-]_o$ 198 mM
$[Na^+]_o$ 172.5 mM
$[K^+]_o$ 6.5 mM

$\tau = 246\ ms$

500 pA

1 sec

1 nA

500 ms

Figure 3

A

1 mM glutamate   1 mM glutamate

+60 mV

0 mV

-70 mV   -60 mV

3 s

1 nA

500 ms

B

I/C (pA/pF)

Voltage (mV)

n = 3

n = 4

○ [lowCl⁻]ᵢ

● [highCl⁻]ᵢ

Figure 4

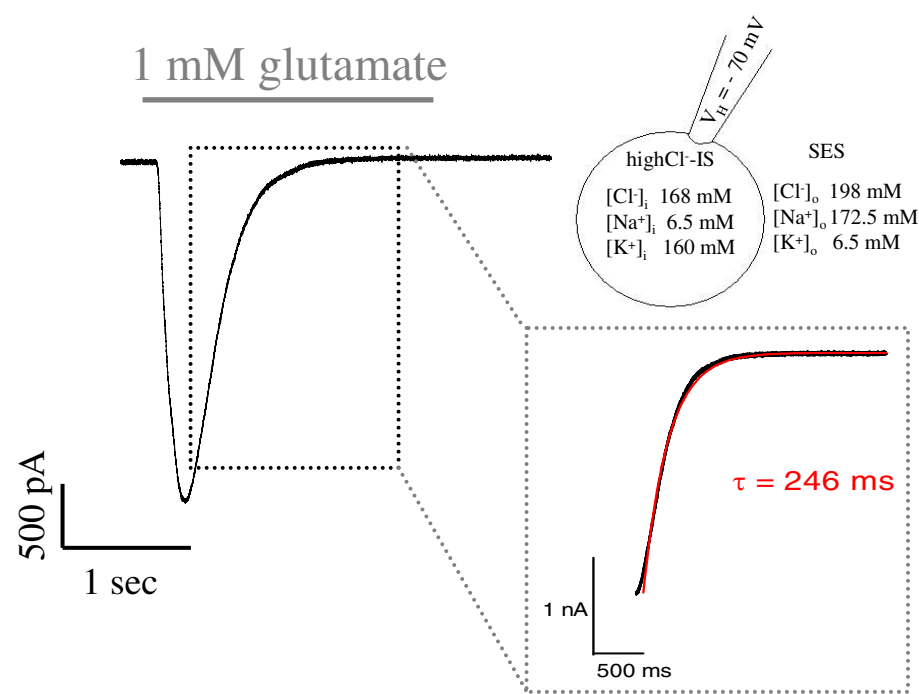**Figure 6.**

Figure 5

```
TGACGGCCGCCGGGCAGGGACTCACACCACGACCTGAGAATACATGGGCGCTCCTAACCG
                                                          *
TTCTCAGGCAGTACCATGGCCACTGTGGCCTCGTCGCTGTGGCCACCCTGTGTCCTGCTA
               M  A  T  V  A  S  S  L  W  P  P  C  V  L  L
CTCGTGCTACTGGTGCATGTGGGCTGGTGCAATCAGCCGAAAGTGAACTTCAGGGAGAAG
L  V  L  L  V  H  V  G  W  C  N  Q  P  K  V  N  F  R  E  K
GAGAAGCAGGTTCTGGATCAGATTCTGGGTCCCGGCCGCTACGACGCGAGGATCCGTCCC
E  K  Q  V  L  D  Q  I  L  G  P  G  R  Y  D  A  R  I  R  P
TCTGGAATCAACGGCACAACGGACGCACCGACATTTGTCACTGTCAACATCTTCCTTCGC
S  G  I  N  G  T  T  D  A  P  T  F  V  T  V  N  I  F  L  R
TCAATCAGCAAAATAGACGATTACAAAATGGAATACAGTGTCCAGCTTACATTCCGAGAA
S  I  S  K  I  D  D  Y  K  M  E  Y  S  V  Q  L  T  F  R  E
CAGTGGATGGACGAGAGACTCAAATTCAACGATGTTGGAGGTAAAATCAAGTACCTTACC
Q  W  M  D  E  R  L  K  F  N  D  V  G  G  K  I  K  Y  L  T
CTCACAGACGCCAACAGGGTATGGATGCCAGATTTGTTCTTCGCCAATGAGAAGGAAGGT
L  T  D  A  N  R  V  W  M  P  D  L  F  F  A  N  E  K  E  G
CACTTTCATAACATCATTATGCCCAACGTGTACATTAGAATTTTCCCATATGGTTCAGTA
H  F  H  N  I  I  M  P  N  V  Y  I  R  I  F  P  Y  G  S  V
CTGTACAGTATTCGAATCTCGCTGACGTTGGCATGCCCGATGAATTTGAAGCTGTATCCA
L  Y  S  I  R  I  S  L  T  L  A  C  P  M  N  L  K  L  Y  P
CTGGATCGTCAGGTTTGCTCACTAAGGATGGCAAGCTACGGGTGGACCACTGCCGACCTC
L  D  R  Q  V [C] S  L  R  M  A  S  Y  G  W  T  T  A  D  L
GTTTTCCTCTGGAAGGAAGGCGACCCCGTGCAGGTGGTCAAGAATCTACACTTACCACGT
V  F  L  W  K  E  G  D  P  V  Q  V  V  K  N  L  H  L  P  R
TTTACATTGGAAAAATTTCTTACAGATTATTGTAATAGCAAGACCAACACGGGAGAGTAC
F  T  L  E  K  F  L  T  D  Y [C] N  S  K  T  N  G  E  Y
AGCTGCCTGAAGGTCGACCTGCTCTTCAAGCGGGAGTTCAGCTACTACCTGATCCAGATC
S  C  L  K  V  D  L  L  F  K  R  E  F  S  Y  Y  L  I  Q  I
TACATCCCCTGCTGTATGCTGGTCATTGTGTCGTGGGTCTCCTTCTGGCTCGATCAGGGT
Y  I  P  C  C  M  L  V  I  V  S  W  V  S  F  W  L  D  Q  G
GCCGTTCCCGCACGAGTGTCCCTCGGAGTCACCACGCTCCTTACTATGGCCACCCAAACC
A  V  P  A  R  V  S  L  G  V  T  T  L  L  T  M  A  T  Q  T
TCGGGAATCAACGCCTCACTCCCGCCTGTATCCTACACTAAGGCCATCGACGTTTGGACC
S  G  I  N  A  S  L  P  P  V  S  Y  T  K  A  I  D  V  W  T
GGCGTCTGCCTAACGTTTGTGTTCGGCGCACTGCTAGAGTTCGCGTTAGTCAACTATGCG
G  V  C  L  T  F  V  F  G  A  L  L  E  F  A  L  V  N  Y  A
TCAAGATCCGATATGCACCGAGAAAACATGAAGAAACAGAGGCGGCAATGTGAGTTGGAG
S  R  S  D  M  H  R  E  N  M  K  K  Q  R  R  Q  C  E  L  E
CACGCAGCCAGCTTGGAGGCCGCTGCCGACCTTCTCGAGGACGGTGCCACCACCTTCGCC
H  A  A  S  L  E  A  A  A  D  L  L  E  D  G  A  T  T  F  A
ATGAAGCCGCTGGTCCGACATCCGGGAGACGCCCTGACGCTGGAGAAGGTGCGCCAGTGC
M  K  P  L  V  R  H  P  G  D  A  L  T  L  E  K  V  R  Q  C
GAGATCCACATGCAGCCCAAGAGGGAGAACTGCTGCCGGACCTGGCTGTCCAAATTCCCC
E  I  H  M  Q  P  K  R  E  N  C  C  R  T  W  L  S  K  F  P
ACCAGGAGCAAGAGGATCGACGTCATCTCGCGCATAACCTTCCCGCTGGTTTTTGCACTT
T  R  S  K  R  I  D  V  I  S  R  I  T  F  P  L  V  F  A  L
TTCAACCTGGTCTACTGGTCGACCTACCTGTTCCGCGAGGAGGCAGAAGACAACTGAACT
F  N  L  V  Y  W  S  T  Y  L  F  R  E  E  A  E  D  N  *
GTGCTGTGTTATATCAGAAGCAATACGGTGGTCTTGCTCAAGACACGGTGGTGTAGCTGT
TAGATTGTATAGCACCACGAAGGAAACAATCACCTCTCTTTAGCGATACGAGTGCACCTT
GTAATTATCATACATACGCCAACAAGCGTGGACAGCGGAGGTGGATTGTTAATGACGAGG
```