Cys-loop ligand-gated chloride channels in Dorsal Unpaired Median neurons of *Locusta migratoria*

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In insects, inhibitory neurotransmission is generally associated with members of the cys-loop ligand-gated anion channels, such as the glutamate-gated chloride channel (GluCl), the \( \gamma \)-amino butyric acid-gated chloride channels (GABACl) and the histamine-gated chloride channels (HisCl). These ionotropic receptors are considered being established target sites for the development of insecticides and therefore, it is necessary to obtain a better insight in their distribution, structure and functional properties. Here, by combining electrophysiology and molecular biology techniques, we have identified and characterized GluCl, GABACl and HisCl in DUM neurons of *Locust migratoria*. In whole-cell patch-clamp recordings, application of glutamate, GABA or histamine induced rapidly activating ionic currents. GluCls were sensitive to ibotenic acid and blocked by picrotoxin and fipronil. The pharmacological profile of the *L. migratoria* GABACl fitted neither the vertebrate GABA\( _A \) nor GABA\( _C \) receptor and was similar to the properties of the cloned *Drosophila melanogaster* GABA receptor subunit (Rdl). The expression of Rdl-like subunit-containing GABA receptors was demonstrated at the molecular level using RT-PCR. Sequencing analysis indicated that the orthologous GABACl of *D. melanogaster* CG10357-A is expressed in DUM neurons of *L. migratoria*. Histamine-induced currents exhibited a fast onset and desensitized completely on continuous application of histamine.

In conclusion, within the DUM neurons of *L. migratoria*, we identified three different cys-loop ligand-gated anion channels that use GABA, glutamate or histamine as their neurotransmitter.
Introduction

Due to the economic and agricultural importance of the migratory locusts, the neurobiology of the *Locusta migratoria* has been extensively investigated. As in other insect nervous systems, fast excitatory and inhibitory synaptic neurotransmission in *L. migratoria* is thought to be associated with the cys-loop ligand-gated ion channel (LGIC) superfamily, which includes excitatory cation-permeable nicotinic acetylcholine receptors (nAChRs), and inhibitory γ-amino butyric acid (GABA)-gated anion channels, glutamate-gated chloride channels (GluCls) and histamine-gated chloride channels (Jones and Sattelle 2007; Raymond and Sattelle 2002; Zheng et al. 2002). Concerning these latter ones, relatively little information is available in *L. migratoria*. As in other insect species, Dorsal Unpaired Median (DUM) neurons have been proven to be a good choice for examination of the neuron repertoire of electrophysiological equipment in order to investigate intrinsic properties underlying behaviour (Grolleau and Lapied, 2000). Thus, given the wide range of functions that they mediate (Grolleau and Lapied, 2000), a better understanding of the distribution of cys-loop ligand-gated chloride channels on the somata of locust DUM neurons, might aid in a better knowledge of the functioning of the locust nervous system, what could moreover lead to the exploitation of these receptors as potential target sites for the development of selective insecticides.

Like all members of the cys-loop LGICs, the invertebrate GluCl channels are pentameric transmembrane receptors (Sunesen et al. 2006). They can be found as extra-junctional muscle receptors or as postsynaptic neuronal receptors (Cleland 1996; Cull-Candy 1976). Up to now, cloning studies have led to the identification of a small family of six GluCl genes in the nematode. In *Drosophila melanogaster*, a cDNA clone (DrosGluClα) was identified and in *L. migratoria*, the expression of α subunit-containing GluCls was demonstrated (Janssen et al. 2007). L-glutamate-induced currents are blocked by fipronil, a widely used phenylpyrazole insecticide (Zhao et al. 2005). The blocking action of fipronil on GluCl channels has been demonstrated in oocytes transfected with GluCls (Horoszok et al. 2001) and on chloride currents induced by glutamate in dorsal unpaired median (DUM) neurons of the cockroach *Periplaneta americana*, grasshopper *Melanoplus sanguinipes* and the locust *L. migratoria* (Ikeda et al. 2003; Janssen et al. 2007; Smith 1999).
In insects, GABA-gated chloride channels are present in high density on nerve and muscle cells and are homologous with GluCl. They also form potentially important target sites of several classes of insecticides (Bloomquist 2003; Hosie et al. 1995; Kane et al. 2000). Insect ionotropic GABA receptors closely resemble those of vertebrates in their picrotoxin (PTX) sensitivity, but do not readily fit the vertebrate GABA<sub>A</sub> / GABA<sub>C</sub> receptor categories (Buckingham et al. 2005; Narusuye et al. 2007; Sattelle et al. 2000). The cloning of putative GABA receptor subunits from *D. melanogaster* led to the identification of the gene Rdl that accounts for the resistance against dieldrin (ffrench-Constant 1993). Injection of Rdl into *Xenopus laevis* oocytes resulted in the expression of homo-oligomeric GABA-gated chloride channels that can be blocked by PTX, are insensitive to bicuculline (Hosie et al. 1997) and can be activated by the GABA<sub>C</sub> agonists TACA and CACA (Buckingham et al. 1994). Highly conserved Rdl-like receptor genes have been identified in diptera, lepidoptera and hemiptera insects. Other GABA receptor subunits have also been characterized from *D. melanogaster*, the GABA and glycine-like receptor, GRD, the ligand-gated chloride channel homolog 3 β subunit, LCCH3 and an additional candidate AAF48539 (Ffrench-Constant et al. 1991; Gisselmann et al. 2004; Witte et al. 2002). Although the GABA receptor-like sequences of GRD and LCCH3 imply anion channel activity, co-expression of these subunits in *X. laevis* oocytes forms heteromultimeric GABA-gated cation channels that are insensitive to picrotoxinin and sensitive to bicuculline (Buckingham et al. 2005; Gisselmann et al. 2004; Zhang et al. 1995). Therefore, it seems that only the Rdl homo-oligomer is known to form an anion channel which closely mimics the pharmacology of most insect native neuronal GABA-gated ion channels (Buckingham et al. 2005; Narusuye et al. 2007).

Together with glutamate and GABA, histamine has also been implicated as an inhibitory neurotransmitter in insect, where it directly activates a chloride channel and mediates rapid inhibitory neurotransmission. More specifically, histamine has been designated as the major inhibitory neurotransmitter in photoreceptors and mechanoreceptors (Cebada and Garcia 2007; Stuart 1999; Witte et al. 2002; Zheng et al. 2002). Two histamine-gated chloride channel subunits, HisCl1 (DmHisClα1) and HisCl2 (DmHisClα2), were cloned so far from *D. melanogaster* and subsequently expressed in *X. laevis* oocytes where they can form heteromultimeric channels as well as HisCl1 homomultimeric chloride channels (Zheng et al.
2002). Other ligand-gated chloride channels such as the nicotinic acetylcholine (nACh)-gated (Putrenko et al. 2005) and serotonin-gated anion channel (Ranganathan et al. 2000) were described in *C. elegans*, but their insect counterparts are up to now undocumented (Jones and Sattelle 2008).

In a preceding work (Janssen et al. 2007), we reported the functional expression of GluCls in DUM neurons of *L. migratoria*. We observed that ivermectin (IVM), a potent open channel agonist of glutamate-gated chloride channels and GABA-gated chloride channels (Tribble et al. 2007), induced significantly greater currents than glutamate itself. This led us to suggest that *L. migratoria* DUM neurons could also express GABA-gated channels, besides GluCls. In the present study, we therefore systematically explored the functional expression of members of the cys-loop ligand-gated chloride channel family, including GABA- and histamine gated channels in an *ex vivo* preparation of DUM neurons of the metathoracic ganglion of *L. migratoria* by using the whole-cell patch-clamp technique and molecular biology techniques.

**Material and Methods**

*Isolation of metathoracic DUM neurons of the locust*

Adult migratory locusts (*Locusta migratoria*) were used in all experiments. They were taken from a crowded laboratory colony, maintained at ~32°C on a 14:10 h light/dark cycle and on a diet of grass and oatmeal. Isolated DUM neuronal cell bodies were prepared as described previously (Janssen et al. 2007). Briefly, the dorsal median region of the metathoracic ganglion was mechanically removed and subjected to collagenase/dispase treatment. The cells were centrifuged, subsequently washed, dissociated by repetitive suction through a pipette tip and finally plated on Nunc petri dishes and incubated overnight at 28°C and 5% CO₂. Healthy efferent DUM neurons were recognized by their morphological characteristics under phase contrast microscopy (Brône et al., 2003).

*Electrophysiological experiments*
Changes in total membrane currents of the DUM neurons of *L. migratoria* were studied at room temperature using the whole-cell configuration of the patch-clamp technique in voltage-clamp and under continuous bath perfusion. The recording electrode was filled with an internal solution containing (in mM): 6.5 NaCl, 170 CsCl, 1 CaCl₂, 2 Mg⁺ATP, EGTA 10 and 10 HEPES, pH 6.65. For the current-voltage relationship of GABA-gated chloride channels, recording electrodes were filled with a modified internal solution, containing less chloride (100 mM CsCl instead of 170 mM CsCl). The extracellular solution used to record induced ionic currents contained (in mM): 172.5 cholineCl, 6.5 KCl, 2 CaCl₂, 7.7 MgCl₂, 10 HEPES, pH 6.8. Glucose was added to all solutions in order to adjust the osmolality to ~380 mOsm/kg, corresponding to the osmolality of the *L. migratoria* hemolymph (Brône et al. 2003). The experimental protocols and data acquisition were performed using a personal computer controlled EPC-10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Pipette electrodes were made from 1.5 mm (o.d.) borosilicate glass capillary tubes, and had a resistance between 800 kΩ and 1.5 MΩ. Liquid junction (LJ) potentials were calibrated using the Junction Potential Calculator for Windows (JPCalcW, Peter H. Barry, Dept. of Physiology & Pharmacology, Australia & Axon Instruments, Inc., California, USA) and were taken into account at the start of each experiment. The LJ potential between intracellular and extracellular solutions was +7.7 mV. Capacitive and leak currents were compensated for automatically by the Pulse program (HEKA Elektronik, Lambrecht, Germany) and residual capacitances and leak currents were eliminated by means of a P/6 protocol. The series resistance was lower than or equal to 1.5 MΩ after compensation. Data were filtered at 2.9 kHz and sampled at 20 kHz. The recording of whole-cell currents began 5 min after rupture of the cell membrane, so that the cell interior was adequately equilibrated with the pipette solution. For the current-voltage relationship, data plots were fitted with the Goldman-Hodgkin-Katz (GHK) equation and concentration– effect curves were fitted with the Hill equation. Acquired data were stored on a computer hard disk for later analysis.
**Chemicals**

All chemicals were purchased from Sigma-Aldrich (GmbH, Seelze, Germany) unless specified otherwise. Monosodium glutamate, ibotenic acid, γ-amino butyric acid (GABA), histamine, glycine, muscimol, serotonin (5-HT), trans-4-aminocrotonic acid (TACA) and gabazine were dissolved in de-ionised water. Picrotoxin (PTX) and bicuculline were dissolved in ethanol (EtOH) and dimethylsulfoxide (DMSO) respectively to make stock solutions and then diluted with the extracellular solutions before the start of the experiment. The final concentrations of EtOH and DMSO in the solutions were ≤ 0.1%, which had no effect on recorded currents (data not shown). The chemicals were applied topically throughout a fast application perfusion system (SF-77B, Warner Instruments, Holliston, MA, USA), by rapidly moving the solution interface across the cell surface (36.9 ms exchange time).

**Data analysis**

Data obtained from whole-cell patch-clamp recordings were analysed with the PulseFit 8.77 software (HEKA instruments) to assess current amplitudes and decay kinetics. Further analysis and non-linear regression analysis were carried out using Origin 6.0 professional (Micoral Software, Northampton, MA) and SigmaPlot (Jandel Scientific Software, San Rafael, CA). Numerical data in the text are expressed as the mean ± SEM, n being the number of experiments. The significance of difference between parameters was calculated using the Student’s t test. Dose-response curves were fitted with the Hill equation and EC\textsubscript{50} and IC\textsubscript{50} values were statistically compared using an one-way ANOVA followed by Dunnett’s posttest. Differences between mean values were considered significant at \( P \leq 0.05 \).

**RNA isolation from DUM neurons and RT-PCR procedures**

DUM neurons of *L. migratoria* were isolated as described above with slight modifications. After the collagenase/dispsase treatment the cells were plated on a NUNC Petri dish and incubated for at least 15 min at 28°C. Approximately thirty cells were picked-up, and subsequently washed with ice cold PBS (pH 7.4) and transferred into an RNase-free micro tube. Total RNA was prepared from the obtained material with the PicoPure\textsuperscript{®} RNA Isolation KIT (KIT#0204, Arcturus Bioscience inc., Westburg B.V., Leusden, Netherlands) with an additional DNase
treatment (RNase free DNase set, Qiagen), following the instructions of the supplier. First Strand
cDNA was synthesized using the RT system (Promega, cat#A1260, Promega Benelux BV, Leiden, Netherlands). The cDNA of isolated locust DUM neurons was used as a template for a PCR using Taq DNA polymerase (Applied Biosystems). Degenerated primers were designed and synthesized based upon the predicted amino acid sequences of the conserved regions of the GABA-gated anion channel after alignment of the GABA receptor from 6 characteristic insect species (*D. melanogaster*, *M. domestica*, *T. castaneum*, *A. mellifera*, *S. exigua* and *B. mori*). PCR primers DEG-F (5’-AT(ACT)GA(AG)AT(ACT)GA(AG)AG(CT)TTCGG-3’) and DEG-R (5’-GCCAT(AG)TA(ACTG)CC(CG)AC(AC)GT(ACTG)GC(AG)TATTC-3’) were used for amplification with 40 cycles at 94°C for 30 sec., 50°C for 45 sec., and 72°C for 45 sec. PCR products were then separated on a 2% high-resolution agarose gel. The band of interest (~450 bp) was extracted from the gel using the DNA and Gel Band purification Kit of GE Healthcare (Buckinghamshire, UK). The amplified PCR fragments were either directly sequenced or cloned into pGEM-T for sequencing.

**Results**

The glutamate-gated chloride channel (GluCl) of locust DUM neurons

As previously reported, L-glutamate activates a glutamate-gated chloride channel (GluCl) in locust DUM neurons (Fig. 1A) which is characterized by a fast activating inward current with complete desensitization under continuous glutamate perfusion (Janssen et al. 2007). In the present study, ibotenic acid, a structural analogue of glutamate, was sequentially applied (Fig. 1A). The amplitudes and kinetics of ibotenic acid-induced currents were similar to those elicited by glutamate. The average peak amplitudes of 1 mM glutamate- and 1 mM ibotenic acid-induced currents yielded 7.4 ± 1.8 nA; n=3 and 9.5 ± 1.2 nA; n=3, respectively (*P* > 0.5).

The concentration-response relationship for glutamate and ibotenic acid to activate the GluCl receptors was determined by repeatedly applying different concentrations of glutamate (from 0.001 to 3 mM) and ibotenic acid (from 0.1 to 300 µM), as described in figures 2A₂ & B₂. Glutamate induced a discernible current from a concentration of 10 µM and ibotenic acid from 3 µM. Near saturation was achieved at 1 mM for glutamate and at 300 µM for ibotenic acid (Fig. 2A₁&B₁). Both concentration-response relationships were fitted with the Hill equation and
yielded an EC$_{50}$ of 39.7±1.3 µM (n = 11) and a Hill coefficient (n$_H$) of 1.3±0.4 for glutamate-evoked current, and an EC$_{50}$ of 22.6±1.1 µM (n = 13) and an n$_H$ of 1.3±0.1 for ibotenic acid-induced current, suggesting that ibotenic acid is a more potent agonist ($P < 0.05$).

Our previous study indicated that the main ionic species underlying the glutamate-induced currents was chloride (Janssen et al. 2007). To corroborate the notion of chloride selectivity of the currents evoked by ibotenic acid, the reversal potential was measured using intracellular and extracellular solutions with almost identical Cl$^-$ concentrations. The reversal potential ($E_{\text{ibotenic acid}}$) was calculated after fitting the data with the GHK equation (Fig. 3A$_3$). The ibotenic acid-induced currents reversed in polarity at a membrane potential of +7.2 mV (n = 3), which was close to the calculated chloride equilibrium potential ($E_{\text{Cl}^-} = +2.7$ mV).

Glutamate- and ibotenic acid-induced currents were then tested for their sensitivity to the phenylpyrazole fipronil, a potent open channel blocker of GluCls (Fig. 4A & B). In addition, fipronil also reversibly inhibited ibotenic acid-gated currents, as shown in figure 4B. Glutamate and ibotenic acid currents were evoked by application of their EC$_{50}$ (40 µM and 25 µM respectively). After several stable control recordings, 5 different concentrations (from 0.1 to 100 µM) of fipronil were co-applied (fig. 4A$_2$ and B$_2$). The glutamate-induced currents were suppressed by 21.4±3.2 % (n=6) and 95.7±1.3 % (n=6) by fipronil at 0.1 µM and 100 µM, respectively. Currents evoked by ibotenic acid were suppressed by 18.4±7.6 % (n=6) and 96.1±2.8 % (n=6) by fipronil at 0.1 µM and 100 µM, respectively. The concentration-response relationships for fipronil block were fitted by a sigmoid curve yielding an IC$_{50}$ for fipronil on glutamate-induced currents (Fig. 4A$_1$) of 3.7±1.1 µM and a n$_H$ of 1.5±0.3 (n=6) and an IC$_{50}$ for fipronil on ibotenic acid-induced currents (Fig 4 B$_1$) of 4.7±1.2 µM and a n$_H$ of 2.4±0.3 (n=6).

Finally, the effect of the quinoxaline NBQX, a kainate (KA) / $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, on glutamate-induced currents was evaluated (Fig. 4C). In this series of recordings, neither significant inhibition nor enhancement of the current could be observed.

The GABA-gated chloride channel of locust DUM neurons
Using almost symmetrical Cl\(^{-}\) concentrations in intra- and extracellular solutions, 1 mM GABA at a \(V_H\) of -60 mV induced rapidly activating inward currents that reached a peak amplitude of 8.8 ±1.1 nA (n=15). The currents showed little desensitization, mostly remaining sustained during GABA application. A typical experiment is shown in figure 1 B.

The amplitude of the GABA-induced currents in locust metathoracic DUM neurons was concentration-dependent. This was demonstrated by repeatedly applying different concentrations of GABA (from 1 µM to 3 mM) for 3 s at a \(V_H\) of -60 mV (Fig. 5 A\(_1\) & A\(_2\)). The peak current amplitudes, normalized as described in the legend, were plotted against the GABA concentrations. The dose-response curve (Fig. 5 A\(_1\)) shows the usual S-shape, with a just-noticeable effect at 1 µM GABA, an \(EC_{50}\) of 43.6±1.2 µM (n=18) and a \(n_H\) of 1.3±0.3.

To assess whether the main ionic species carrying the GABA-induced currents was chloride, the \(I-V\) relationships were obtained using two different intracellular solutions: one containing almost symmetrical intra- and extracellular Cl\(^{-}\) concentration and a second one containing a lower (~100 mM) intracellular Cl\(^{-}\) concentration. In these solutions, NaCl and KCl were replaced by cholineCl and CsCl, respectively, in order to eliminate contaminating Na\(^{+}\) and/or K\(^{+}\) currents. Then, an \(I-V\) protocol as shown in figure 5 B\(_1\) was applied as described earlier. The reversal potential (\(E_{GABA}\)) was calculated after fitting the data with the GHK equation (Fig. 5 B\(_2\)). Using almost symmetrical Cl\(^{-}\) concentrations, GABA-induced currents reversed in polarity at a membrane potential of +5.6 mV (n=3), which corresponded to the calculated chloride equilibrium potential (\(E_{Cl^{-}}\) = +2.7 mV). At lower intracellular Cl\(^{-}\) concentration, \(E_{GABA}\) shifted to a more negative value –13.2 mV (n=3), which was in good agreement with the expected shift in the calculated Nernst equilibrium potential for chloride ions (\(E_{Cl^{-}}\) = -17.3 mV). These findings clearly demonstrate the chloride selectivity of the GABA-induced macroscopic currents.

Currents evoked by the \(EC_{50}\) of GABA in locust DUM neurons were tested for their sensitivity to several known vertebrate GABA\(_A\) receptor antagonists: bicuculline, gabazine and picrotoxin (PTX) (Fig. 6 A, B and C). Bicuculline had no effect on GABA-induced currents up to a concentration of 100 µM (n=4; Fig. 6 A\(_1\) and A\(_2\)). In contrast, gabazine and PTX reversibly inhibited GABA-gated currents as shown in figure 6, B and C. One hundred micromolar of gabazine reduced the peak current to 37.3±3.1 % of the control (n=7; Fig. 6 B\(_2\)). After washout,
the currents recovered to 99±6.8 % of the control (n=7; Fig. 6 B2). In the presence of PTX, GABA-induced currents were suppressed to 6.9±1.5 % of the control, whereas current recovery was up to 86±4.2 % (n=6; Fig 6 C2).

The vertebrate GABA_A receptor agonist muscimol and the vertebrate GABA_C receptor agonist trans-4-aminocrotonic acid (TACA) elicited currents with amplitudes that increased in a concentration-dependent manner (Fig. 7 A and B). The concentration-response relationships of muscimol and of TACA for activating the GABA-gated chloride channel were assessed by repeatedly administrating different concentrations (from 100 nM to 300 µM) of muscimol and TACA (Fig. 7 A2 and B2). The peak current amplitudes, normalized as described in the legend, were plotted against the agonist concentrations and fitted to the Hill equation, yielding an EC_{50} of 18.8±1.27 µM (n=11) and a n_H of 1.46±0.11 for muscimol-evoked currents, and an EC_{50} of 15.5±1.04 µM (n=9) and a n_H of 1.78±0.16 for TACA-induced current (Fig. 7 A1 and B1).

The ability of the GABA_A receptor blocker gabazine to antagonise the responses evoked by muscimol and by TACA was investigated. Ten micromolar gabazine suppressed muscimol-induced currents to 46±6.6 % of the control (n=4; Fig. 8 A1 and A2). However, gabazine failed to antagonize TACA-induced currents as shown in figure 8 (B1 and B2).

Finally, a two-step PCR procedure using degenerated primers was used to amplify part of the GABA-gated chloride channel from L. migratoria DUM neuron cDNA. After the first step, a faint band of ~460 bp was visible (Fig. 9Aa2). After re-PCR (Fig. 9Aa3) the resulting PCR product was excised, purified and sequenced to confirm the sequence (Fig9B). At the cDNA level, the resulting sequence covered exon 2-4 (compared to genomic D. melanogaster sequence NM_079267) of the Rdl-family of GABA-gated chloride channels. The L. migratoria sequence was 75-80% identical to other orthologous insect sequences at the cDNA level, resulting in an almost completely conserved protein sequence. In comparison with the different splice variants described in D. melanogaster, the amplified PCR product was nearly identical (99%) with CG10537-A, whereas sequence identity to two other D. melanogaster splice variants was significantly lower (~92%), indicating that in L. migratoria the orthologous GABA-gated chloride channel of D. melanogaster CG10357-A is expressed. Please note, that the used PCR primers are located on different exons; therefore amplification from co-purified genomic DNA
can be excluded. The partial sequence of the *L. migratoria* Rdl-type GABA-gated chloride channel was submitted to GenBank under acc.no. FJ436416.

**The histamine-gated chloride channel of locust DUM neurons**

One millimolar histamine sporadically elicited a fast activating inward current with complete desensitization under a continuous histamine superfusion, as shown in figure 1 C. However, the response could be detected in 2 out of 21 (~9.5 %) tested neurons only. Moreover, the histamine positive neurons could not be activated by glutamate. In contrast, the other 19 DUM neurons (~90.5 %) were responsive to glutamate, as well to GABA.

**Discussion**

In the present study, the functional expression of members of the cys-loop LGIC family was investigated in efferent DUM neurons of the metathoracic ganglion of *L. migratoria*, using RT-PCR and patch-clamp techniques. To our knowledge, our work is the first report of the expression of three distinctive types of cys-loop ligand-gated chloride channels in DUM neurons of *L. migratoria*: the glutamate-gated chloride channel (GluCl), the GABA-gated chloride channel (GABACl) and the histamine-gated chloride channel.

To date, no evidence has been presented that insects utilize glycine as a neurotransmitter (Jones and Sattelle 2008), nor that, like nematodes, insects possess other ligand-gated chloride channels such as the nicotinic acetylcholine (nACh)-gated (Putrenko et al. 2005) and serotonin-gated anion channel (Ranganathan et al. 2000). Indeed, in our hands, neither application of glycine nor of serotonin induced responses in DUM neurons of *L. migratoria* (data not shown).

**The glutamate-gated chloride channel (GluCl) of locust DUM neurons**

In cockroach DUM neurons, both desensitizing and non-desensitizing glutamate-induced currents were observed, which seemed to be attributable to the presence of pharmacologically distinct GluCls. (Heckmann and Dudel 1995; Raymond et al. 2000). These glutamate-induced currents are both sensitive to ibotenic acid, and the desensitizing GluCl is weakly blocked by fipronil and PTX whilst the non-desensitizing GluCl is highly sensitive to these compounds
(Zhao et al. 2004a). The existence of GluCl channels in efferent DUM neurons was already demonstrated in our previous work (Janssen et al. 2007). In the present study, the pharmacological properties of the locust GluCl were further characterized. Both glutamate and ibotenic acid evoked a fast-activated current in a concentration-dependent manner that rapidly and completely desensitized in the continuous presence of high concentrations of the agonists. However, at lower concentrations (< 100 µM), the currents were sustained during the application of agonists, suggesting either a non-desensitizing current component as described in cockroaches (Zhao et al. 2004a) or a concentration-dependent desensitization. Although ibotenic acid is generally considered to be roughly equipotent to glutamate, or even less powerful (Cleland 1996; Forrester et al. 2003), it was considerably more effective (1.75x) in gating the glutamate-gated chloride channel in L. migratoria.

In cockroach neurons, fipronil is an open channel blocker of GluCls which blocks desensitizing currents in a concentration-dependent manner with an IC$_{50}$ of 730 nM (Ikeda et al. 2003; Zhao et al. 2004b). A study on native locust neuron receptors has demonstrated that fipronil blocks glutamate-gated chloride channels with an IC$_{50}$ of 30 nM (Smith 1999). In the present study, the blockage by fipronil of glutamate- and ibotenic induced currents (IC$_{50}$ of 3.71 µM and 4.69 µM, respectively) was generally less potent than in the studies described above. Whether this is due to species differences or a difference in receptor subtypes has still to be elucidated. Although, good evidence has been presented that glutamate can also act as an excitatory transmitter in many invertebrate species (Brockie and Maricq 2006; Dudel and Franke 1987; Kerry et al. 1987; Shinozaki 1988; Wafford and Satelle 1989), millimolar concentrations of kainate, AMPA or NMDA each failed to evoke any response in the lobster (Cleland and Selverston 1995). In the present study, glutamate-induced currents could not significantly be blocked by the quinoxaline NBQX, a KA / AMPA receptor antagonist, strongly suggesting the lack of these receptors.

*The GABA-gated chloride channel of locust DUM neurons*

GABACl and GluCl belong to the same superfamily of cys-loop ligand gated chloride channels and share many properties (Horoszok et al. 2001; Wafford and Sattelle 1986). Nevertheless, a GABACl receptor and a GluCl channel can exist separately or co-exist on the somata of neurons,
function independently and differ in concentration-responses, voltage dependencies, and kinetics (Buckingham et al. 1994; Duan and Cooke 2000; King and Carpenter 1989; Washio 2002; Zhao et al. 2004b).

In this work, we have accumulated several lines of evidence to demonstrate that efferent DUM neurons of *L. migratoria* express functional GABA-gated chloride channels (GABACls). By means of patch-clamp experiments we indeed clearly show that GABA-induced currents in locust DUM neurons: 1) exhibit slow, if any desensitization under continuous GABA application, 2) are mainly carried by chloride ions, 3) can be blocked by gabazine and PTX, but not by bicuculline, 4) are activated by the GABA\textsubscript{A} agonist muscimol and the GABA\textsubscript{C} agonist TACA. Furthermore, we confirmed the expression of GABACls by RT-PCR detection of GABACl transcripts in locust DUM neurons. The absence of or very faint desensitization that we observed for GABA-gated currents is in agreement with results obtained from cockroach DUM neurons (Aydar and Beadle 1999; Zhao et al. 2003), as well as with results obtained in other arthropods, such as in the lobster olfactory projection neurons (Zhai and Cooke 2000). The EC\textsubscript{50} obtained from concentration-response relationship of GABA-gated currents in DUM neuron of *L. migratoria* was also in accordance with values described in cockroach neurons, (Zhao et al. 2003), in *Xenopus* oocytes expressing the *Musca domestica* GABA-gated chloride channels (MdRdl) (Eguchi et al. 2006), and in *Drosophila* cell lines expressing the *Laodelphax striatella* GABA receptor (Narasuye et al. 2007). The reversal potential of the GABA-induced currents in locust DUM neurons indicated that the GABA-receptor predominantly conducts chloride ions. Interestingly, GABA-gated cation channels have also been identified in the crab *Cancer borealis* (Swensen et al. 2000). In addition, *D. melanogaster* GRD and LCCH3 subunits form heteromultimeric GABA-gated cation channels when co-expressed in *Xenopus* oocytes (Gisselmann et al. 2004). However, up to date, no native GABA-gated cation channel has yet been reported for any insect neuron (Buckingham et al. 2005; Gisselmann et al. 2004).

The pharmacological profile of GABA-gated currents in *L. migratoria* DUM neurons fits neither the vertebrate GABA\textsubscript{A} nor the GABA\textsubscript{C} receptor. Indeed, locust DUM neuron GABACls could be gated by the GABA\textsubscript{A} receptor agonist muscimol but also by the GABA\textsubscript{C} receptor agonist TACA.
They were insensitive to bicuculline, a GABA<sub>A</sub> receptor antagonist, but still partially inhibited by gabazine, another GABA<sub>A</sub> receptor antagonist.

These pharmacological characteristics of the *L. migratoria* GABA receptor were close to those described for the cloned *D. melanogaster* GABA receptor subunit Rdl (Ffrench-Constant et al. 1991, Hosie and Sattelle 1996; Millar et al. 1994; Zhang et al. 1995), suggesting the expression of Rdl-like subunit-containing GABA receptors. Although the cloning of putative GABA receptor subunits from *D. melanogaster* has lead to the discovery of several other GABA receptor subunits, it seems that only the Rdl homo-oligomer is able to form an anion channel which closely mimics the pharmacology of most native insect neuronal GABA-gated ion channels (Buckingham et al. 2005; Narusuye et al. 2007).

Finally, the sequencing analysis of the GABA-gated chloride channels expressed by locust DUM neurons demonstrated their orthologous nature with the *D. melanogaster* CG10357-A GABA receptors.

**The histamine-gated chloride channel of locust DUM neurons**

In arthropods, histamine has been implicated as the major inhibitory transmitter or neuromodulator in photoreceptors and mechanoreceptors. Two ionotropic receptors, predicted from *D. melanogaster* genome coding for histamine-gated chloride channels (HisCl) were identified: Dm-His-Cl-α1 and DM-HisCl-α2 (Zheng et al. 2002). More recently, two putative HisCl (Amel-HisCl1 and Amel-HisCl2) subunits in the genome of *A. mellifera* were reported (Jones and Sattelle 2006). Although high levels of histamine can be found in the retina and midbrain of *L. migratoria* and *Schistocerca gregaria*, smaller yet significant amounts of histamine were detected in the rest of the optic lobe and in the metathoracic ganglion (Elias and Evans 1983; Gebhardt and Homberg 2004). It is nevertheless highly unlikely that efferent DUM neurons express histamine-gated chloride channels, due to their distribution in photoreceptor- and interneurons. In our hands, histamine evoked a fast activating inward current, which desensitized completely. Most interestingly, the response could be observed in 2 out of 21 (∼9.5 %) tested DUM neurons only and histamine-responsive neurons could not subsequently be activated by glutamate, or vice versa. Moreover, in 90 % of the tested neurons, GluCl<sub>i</sub>s and GABA<sub>C</sub>Cl co-localize on the cell surface. Therefore, it is plausible that our *ex vivo* preparation also includes
other types of DUM neurons which actually only differ morphologically in size i.e. local and intersegmental DUM interneurons. This being said, the 9 % activity to histamine probably accounts for the presence of either local or intersegmental DUM interneurons in our primary neuronal cell culture.

In conclusion, in this work, we have thoroughly characterized the cys-loop ligand-gated chloride channels expressed by metathoracic efferent DUM neurons which innervate flight and leg muscles of *L. migratoria*. We furthermore examined the pharmacological profile of glutamate and GABA receptors what might be a basis to the development of novel highly selective insecticides (Bloomquist 2003; Janssen et al. 2007; Williamson et al. 2007).

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**Legends**

FIG. 1. Effect of different agonists of cys-loop ligand-gated chloride channels on the membrane currents of locust metathoracic ganglion DUM neurons, recorded with the whole-cell patch-clamp technique in voltage-clamp conditions. The figure shows typical experiments, performed in the presence of almost symmetrical chloride concentrations in the intracellular and extracellular solutions at a holding potential \((V_h)\) of -60 mV. Application of 1 mM histamine (A), glutamate (B) or ibotenic acid (B) onto the cell surface of the DUM neuron evoked a transient inward current that desensitized completely during drug application. Application of 1 mM \(\gamma\)-aminobutyric acid (GABA) elicited a fast activating but slow desensitizing inward current.
FIG. 2. Concentration-response curve of glutamate-gated currents in metathoracic ganglion DUM neurons of *L. migratoria*. Drugs were applied onto the cell surface of DUM neurons during 3 seconds at a *V*<sub>H</sub> of -60 mV. A: Currents were induced by different concentrations of L-glutamate (*A*<sub>1</sub>) for which the peak amplitude at each concentration was measured and normalized to the peak current elicited by 1 mM glutamate. Mean values ± SEM (n=11) were plotted and fitted using the *Hill* equation (*A*<sub>2</sub>) which yielded an EC<sub>50</sub> of 39.7 µM ± 1.3 µM. B: Currents induced by different concentrations of ibotenic acid, a conformationally-restricted structural analogue of glutamate are represented (*B*<sub>1</sub>). Ibotenic acid-induced peak current amplitudes at each concentration were measured and normalized to the current evoked by 100 µM ibotenic acid. Mean values ± SEM (n=13) were plotted and fitted using the *Hill* equation (*B*<sub>2</sub>) which revealed an EC<sub>50</sub> of 22.6 µM ± 1.1 µM.

FIG. 3. Current-voltage (*I-V*) relationship of ibotenic acid-induced currents in locust DUM neurons recorded with the whole-cell patch-clamp technique. A: Currents (*A*<sub>2</sub>) were evoked by 1 mM ibotenic acid at holding potentials ranging from -60 to +60 mV (*A*<sub>1</sub>), in the presence of almost symmetrical Cl<sup>-</sup> concentrations in the intra- and extracellular solutions. Between voltage steps, *V*<sub>H</sub> was returned to -60 mV and ibotenic acid was washed out. *A*<sub>3</sub> *I-V* relationship, obtained from the ibotenic acid-induced currents described in A. Maximum peak currents were normalized for the cell capacitance and were plotted against holding potentials. The relation was best-fitted with the GHK equation, which yielded a reversal potential of +7.2 mV. This was close to the calculated Nernst equilibrium potential for chloride (+2.7 mV).

FIG. 4. A & B: Blocking effects of fipronil on L-glutamate- and ibotenic acid-induced currents in locust metathoracic ganglion DUM neurons. L-glutamate or ibotenic acid at their EC<sub>50</sub> were repeatedly applied for 3 s at a *V*<sub>H</sub> of -60 mV in almost symmetrical Cl<sup>-</sup> concentrations in intra- and extracellular solutions, alone or in combination with different concentrations of fipronil (*A*<sub>2</sub> & *B*<sub>2</sub>). A period of 60 s was left between applications to allow recovery after which control responses to L-glutamate and to ibotenic acid were again checked (washout). *A*<sub>1</sub> and *B*<sub>1</sub> show the concentration-response curve of the inhibitory effects of fipronil on L-glutamate- and on ibotenic acid-induced currents, respectively. The amplitudes of the responses in the presence of fipronil
were normalized to L-glutamate- and ibotenic acid-induced currents in the absence of fipronil (control) and plotted against the different fipronil concentrations. The mean values ± SEM were fitted using the Hill equation (B1) which revealed an IC$_{50}$ of fipronil for L-glutamate (A1) of 3.7 µM ± 1.1 µM (n = 6) and for ibotenic acid (B1) of 4.7 µM ± 1.2 µM (n=6).

C: L-glutamate at its EC$_{50}$ was applied for 3 s at a V$_{H}$ of -60 mV alone and in combination with 100 µM of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline-2,3-dione (NBQX) as described above (C1). NBQX had no significant effect on the glutamate-activated currents, as shown in C1 and summarized in the histogram in C2 (means ± SE; n=6; P = 0.09).

FIG. 5. Concentration-response curve of GABA-gated currents in metathoracic ganglion DUM neurons of *L. migratoria*. γ-Aminobutyric acid (GABA) was applied onto the cell surface of DUM neurons during 3 seconds at a V$_{H}$ of -60 mV. A: Examples of current traces induced by different concentrations of GABA (A1). Peak current amplitudes at each concentration were measured and normalized to the current elicited by 1 mM GABA. Mean values ± SEM (n=17) were then plotted and fitted with the Hill equation (A1) which revealed an EC$_{50}$ of 43.6 µM ± 1.5 µM. B: Current-voltage (I-V) relationship of GABA-induced currents in locust DUM neurons recorded with the whole-cell patch-clamp technique. B2 I-V relationship, obtained from GABA induced-currents. Maximum peak currents were normalized for the cell capacitance and plotted against the holding potentials. I-V relation was best-fitted with the GHK equation, which yielded a reversal potential of +5.6 mV. This was close to the calculated Nernst equilibrium potential for chloride (+2.7 mV). When the extracellular chloride concentration was lowered to 100 mM ([low Cl$^{-}$]$_{i}$; open circles), the reversal potential shifted to -13 mV what was in good agreement with the theoretically expected shift in Nernst chloride equilibrium potential (-17.3 mV).

FIG. 6. Pharmacological properties of the GABA-gated chloride channels in DUM neurons of *L. migratoria*. GABA at its EC$_{50}$ (45 µM) was applied for 3 s at a V$_{H}$ of -60 mV in almost symmetrical Cl$^{-}$ concentrations in intra- and extracellular solutions, alone or in combination with 100 µM bicuculline (A1), 100 µM gabazine (B1) or 100 µM picrotoxin (PTX) (C1). Recovery from blockage (washout) was also evaluated. Histograms in A2, B2 and C2 summarize
bicuculline-, gabazine-, and PTX-induced inhibition of GABA-activated peak currents (mean ± SE, n=6 for bicuculline, n=7 for gabazine, and n=6 for PTX; *P < 0.05).

FIG. 7. Effects of GABA_A (muscimol) and GABA_C (TACA) receptor agonists on metathoracic DUM neuron of L. migratoria. Agonists were applied onto the cell surface of DUM neurons during 3 seconds at a V_H of -60 mV as shown in A1 and B1. A2 and B2: Concentration-response curves of GABA receptor agonists applied onto the surface of locust metathoracic DUM neurons. Current responses were induced by different concentrations of muscimol (A1) and of TACA (B1) and the peak current amplitudes at each concentrations were measured and normalized to the current elicited by respectively 100 µM muscimol and 100 µM TACA. Mean values ± SEM (n=11 for muscimol and n=9 for TACA) were plotted and fitted using the Hill equation which revealed a muscimol EC_50 of 18.8 µM ± 1.3µM (A2) and a TACA EC_50 of 15.5 µM ± 1 µM (B2).

FIG. 8. Effect of gabazine on currents induced by GABA_A (muscimol) and GABA_C (TACA) receptor agonists in DUM neurons of L. migratoria. Muscimol or TACA were applied at their EC_50 for 3 s at a V_H of -60 mV in almost symmetrical Cl^- concentrations in intra- and extracellular solutions, alone and in combination with 10 µM gabazine, as shown in A1 and B1. Recovery from blockage (washout) was also evaluated. Histograms in A2 and B2 summarize gabazine-induced inhibition of the activated currents (mean ± SEM, n=4 for muscimol and n=3 for TACA; *P ≤ 0.05: one-way ANOVA followed by Dunnett’s posttest).

FIG. 9A. Expression of an Rdl-like GABA-gated chloride channel subunit in DUM neurons of L. migratoria. Agarose electrophoresis of the first PCR using locust DUM neuron cDNA as a template (lane a2). The faint band of ~460 bp was excised and subsequently re-amplified using PCR for DNA sequencing (lane a3; Marker: 100 bp ladder is shown in lane a1).

B. Alignment of the partial cDNA sequence of Locusta migratoria GABA-gated chloride channel (FJ436416) with its Drosophila melanogaster orthologue (NM_079267, CG10537-PA). Amino acid sequence of L. migratoria GABACl channel is also shown. In addition, D. melanogaster amino acid sequences are shown for an alternatively spliced region for two
isoforms of the *D. melanogaster* GABACl channel (CG10537-PA, -PB) indicating that the *L. migratoria* sequence is homolog to the *D. melanogaster* isoform CG10537-PA.

References


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Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

A1  
GABA

- 1 μM
- 10 μM
- 50 μM
- 100 μM
- 1 mM

2 nA
1 s

A2  
Normalized currents

[BGABA]  
0.00 0.2 0.4 0.6 0.8 1.0

[BGABA]  
1 μM 10 μM 100 μM 1 mM 10 mM

B1  

0 mV
+ 60 mV
- 60 mV

3 s

B2  

[lowCl−]i

[highCl−]i

n = 3

n = 3

Voltage (mV)
Figure 6.
Figure 7.
Figure 8.
Figure 9.