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# Cys-Loop Ligand-Gated Chloride Channels in Dorsal Unpaired Median Neurons of *Locusta migratoria*

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<sup>1</sup>Centre of Environmental Sciences, Department of Physiology, and <sup>3</sup>Biomedical Research Institute, Hasselt University and Transnationale Universiteit Limburg, Agoralaan, Diepenbeek, Belgium; and <sup>2</sup>Institute for Integrative Neuro-anatomy, AG Prof. Veh, Berlin, Germany

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**Janssen D, Derst C, Rigo J-M, Van Kerkhov E.** Cys-loop ligand-gated chloride channels in dorsal unpaired median neurons of *Locusta migratoria*. *J Neurophysiol* 103: 2587–2598, 2010. First published March 3, 2010; doi:10.1152/jn.00466.2009. 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 GABA-gated chloride channels (GABA<sub>Cl</sub>), and the histamine-gated chloride channels (HisCl). These ionotropic receptors are considered 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 identified and characterized GluCl, GABA<sub>Cl</sub>, and HisCl in dorsal unpaired median (DUM) neurons of *Locusta migratoria*. In whole cell patch-clamp recordings, application of glutamate, GABA, or histamine induced rapidly activating ionic currents. GluCl channels were sensitive to ibotenic acid and blocked by picrotoxin and fipronil. The pharmacological profile of the *L. migratoria* GABA<sub>Cl</sub> fitted neither the vertebrate GABA<sub>A</sub> nor GABA<sub>C</sub> 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 shown at the molecular level using RT-PCR. Sequencing analysis indicated that the orthologous GABA<sub>Cl</sub> 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

Because of the economic and agricultural importance of migratory locusts, the neurobiology of the *Locusta migratoria* has been extensively studied. 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 GABA-gated anion channels, glutamate-gated chloride channels (GluCl), and histamine-gated chloride channels (HisCl) (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 to study intrinsic properties underlying behavior (Grolleau and Lapiéd 2000). Thus given the wide range of functions that they mediate (Grolleau and Lapiéd 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, which could 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 extrajunctional muscle receptors or as postsynaptic neuronal receptors (Cleveland 1996; Cull-Candy 1976). Until 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 (DrosGluCl $\alpha$ ) was identified, and in *L. migratoria*, the expression of  $\alpha$  subunit-containing GluCl was shown (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 shown in oocytes transfected with GluCl (Horoszok et al. 2001) and on chloride currents induced by glutamate in 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 (French-Constant 1993). Injection of Rdl into *Xenopus laevis* oocytes resulted in the expression of homooligomeric 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

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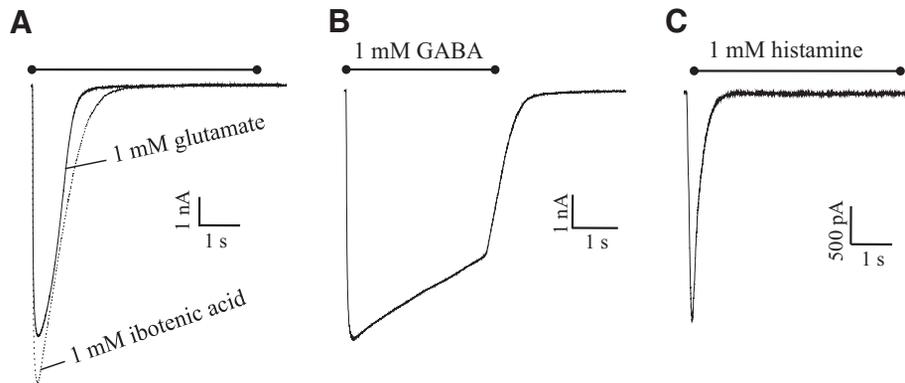


FIG. 1. Effect of different agonists of cys-loop ligand-gated chloride channels on the membrane currents of locust metathoracic ganglion dorsal unpaired median (DUM) neurons, recorded with the whole cell patch-clamp technique in voltage-clamp conditions. Typical experiments, performed in the presence 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 (C), glutamate (A), or ibotenic acid (A) onto the cell surface of the DUM neuron evoked a transient inward current that desensitized completely during drug application. Application of 1 mM GABA (B) elicited a fast activating but slow desensitizing inward current.

been characterized from *D. melanogaster*, the GABA and glycine-like receptor, GRD, the ligand-gated chloride channel homolog  $3\beta$  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, hista-

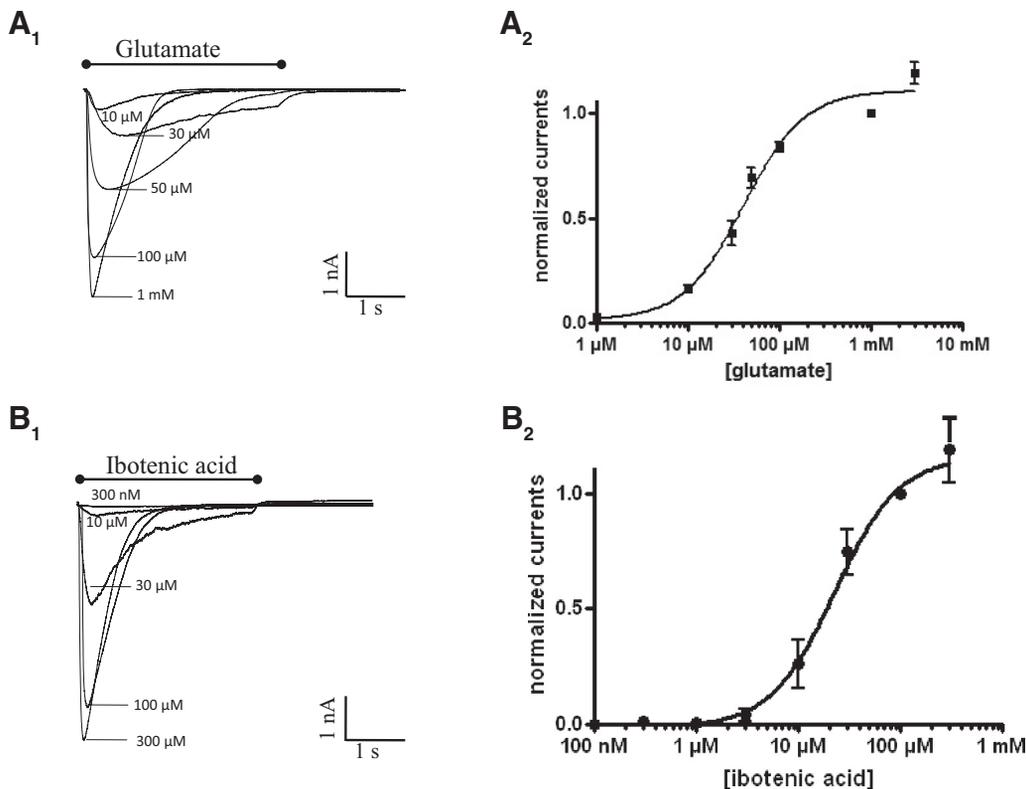


FIG. 2. Concentration-response curve of glutamate-gated currents in metathoracic ganglion DUM neurons of *L. migratoria*. Drugs were applied onto the cell surface of DUM neurons during 3 s at a  $V_H$  of  $-60$  mV. A: currents were induced by different concentrations of L-glutamate ( $A_1$ ) for which the peak amplitude at each concentration was measured and normalized to the peak current elicited by 1 mM glutamate. Mean values  $\pm$  SE ( $n = 11$ ) were plotted and fitted using the Hill equation ( $A_2$ ), which yielded an  $EC_{50}$  of  $39.7 \pm 1.3$   $\mu$ M. B: currents induced by different concentrations of ibotenic acid, a conformationally restricted structural analog of glutamate are represented ( $B_1$ ). Ibotenic acid-induced peak current amplitudes at each concentration were measured and normalized to the current evoked by 100  $\mu$ M ibotenic acid. Mean values  $\pm$  SE ( $n = 13$ ) were plotted and fitted using the Hill equation ( $B_2$ ), which showed an  $EC_{50}$  of  $22.6 \pm 1.1$   $\mu$ M.

mine 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 (DmHisCl $\alpha$ 1) and HisCl2 (DmHisCl $\alpha$ 2), were cloned thus far from *D. melanogaster* and subsequently expressed in *X. laevis* oocytes, where they can form heteromultimeric channels and 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 as yet undocumented (Jones and Sattelle 2008).

In a preceding work (Janssen et al. 2007), we reported the functional expression of GluCl $\alpha$  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 GluCl $\alpha$ . In this 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* using the whole cell patch-clamp technique and molecular biology techniques.

## 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 and maintained at  $\sim 32^\circ\text{C}$  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  $28^\circ\text{C}$  and 5%  $\text{CO}_2$ . Healthy efferent DUM neurons were recognized by their morphological characteristics under phase contrast microscopy (Bröne 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  $\text{CaCl}_2$ , 2  $\text{Mg}^+\text{ATP}$ , 10 EGTA, 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  $\text{CaCl}_2$ , 7.7  $\text{MgCl}_2$ , and 10 HEPES, pH 6.8. Glucose was added to all solutions to adjust the osmolality to  $\sim 380$  mOsm/kg, corresponding to the osmolality of the *L. migratoria* hemolymph (Bröne 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 (OD) borosilicate glass capillary tubes and had a resistance between 800 k $\Omega$  and 1.5 M $\Omega$ . Liquid junction (LJ) potentials were calibrated using the Junction Potential Calculator for Windows (JPCalc W, Peter H. Barry, Dept. of Physiology and Pharmacology, Australia and Axon Instruments) 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), and residual capacitances and leak currents were eliminated by means of a P/6 protocol. The series resistance was  $\leq 1.5$  M $\Omega$  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 (Seelze, Germany) unless specified otherwise. Monosodium glutamate, ibotenic

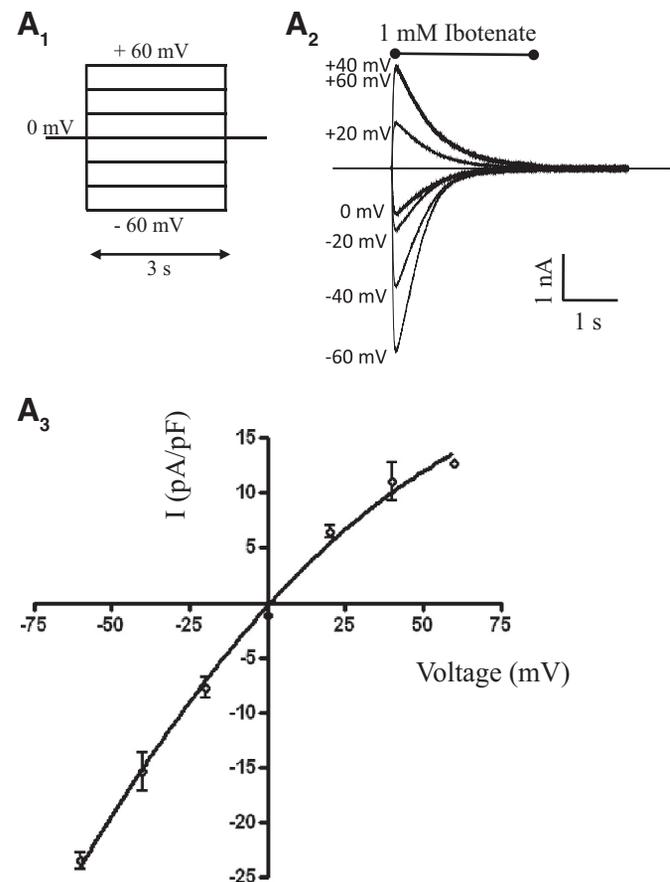


FIG. 3. Current-voltage ( $I$ - $V$ ) relationship of ibotenic acid-induced currents in locust DUM neurons recorded with the whole cell patch-clamp technique.  $A_1$ : currents ( $A_2$ ) were evoked by 1 mM ibotenic acid at holding potentials ranging from  $-60$  to  $+60$  mV ( $A_1$ ), in the presence of almost symmetrical  $\text{Cl}^-$  concentrations in the intra- and extracellular solutions. Between voltage steps,  $V_H$  was returned to  $-60$  mV, and ibotenic acid was washed out.  $A_3$ :  $I$ - $V$  relationship obtained from the ibotenic acid-induced currents described in  $A_1$ . Maximum peak currents were normalized for the cell capacitance and were plotted against holding potentials. The relation was best-fitted with the Goldman-Hodgkin-Katz (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).

acid, GABA, histamine, glycine, muscimol, 5-HT, *trans*-4-aminocrotonic acid (TACA), and gabazine were dissolved in deionized water. PTX and bicuculline were dissolved in ethanol (EtOH) and dimethylsulfoxide (DMSO), respectively, to make stock solutions and diluted with the extracellular solutions before the start of the experiment. The final concentrations of EtOH and DMSO in the solutions were  $\leq 0.1\%$ , 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) 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 analyzed with the PulseFit 8.77 software (HEKA instruments) to assess current amplitudes and decay kinetics. Further analysis and nonlinear regression analysis were carried out using Origin 6.0 professional (Micral Software, Northampton, MA) and SigmaPlot (Jandel Scientific Software, San Rafael, CA). Numerical data in the text are expressed as the means  $\pm$  SE,  $n$  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  $EC_{50}$  and  $IC_{50}$  values were statistically compared using an one-way ANOVA followed by Dunnett's post-test. 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/dispase treatment the cells were plated on a NUNC petri dish and incubated for  $\geq 15$  min at  $28^{\circ}\text{C}$ .

Approximately 30 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 RNA Isolation KIT (KIT0204, Arcturus Bioscience, Westburg B.V., Leusden, The 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 (catA1260, Promega Benelux BV, Leiden, The 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 on the predicted amino acid sequences of the conserved regions of the GABA-gated anion channel after alignment of the GABA receptor from six characteristic insect species (*D. melanogaster*, *M. domestica*, *T. castaneum*, *A. mellifera*, *S. exigua*, and *B. mori*). PCR primers DEG-F (5'-AT(CT)GA(AG)AT(CT)GA(AG)AG-(CT)TTCGG-3') and DEG-R (5'-GCCAT(AG)TA(CTG)CC(CG)-AC(AC)GT(CTG)GC(AG)TATTC-3') were used for amplification with 40 cycles at  $94^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 45 s, and  $72^{\circ}\text{C}$  for 45 s. PCR products were separated on a 2% high-resolution agarose gel. The band of interest ( $\sim 450$  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

### GluCl of locust DUM neurons

As previously reported, L-glutamate activates a GluCl in locust DUM neurons (Fig. 1A), which is characterized by a fast activating inward current with complete desensitization under

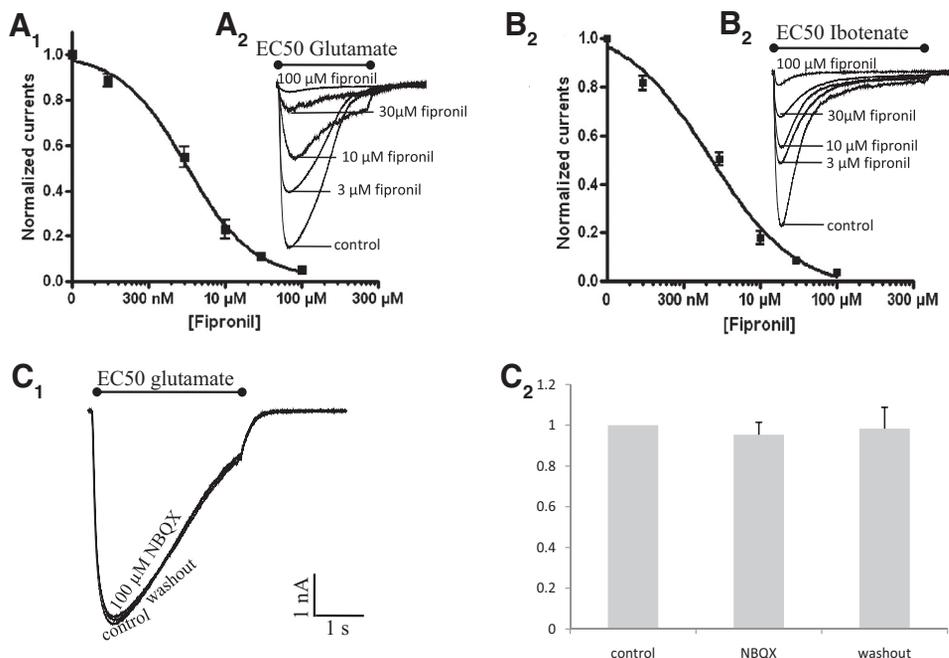


FIG. 4. A and B: blocking effects of fipronil on L-glutamate- and ibotenic acid-induced currents in locust metathoracic ganglion DUM neurons. L-glutamate or ibotenic acid at their  $EC_{50}$  was repeatedly applied for 3 s at a  $V_H$  of  $-60$  mV in almost symmetrical  $\text{Cl}^-$  concentrations in intra- and extracellular solutions, alone or in combination with different concentrations of fipronil ( $A_2$  and  $B_2$ ). A period of 60 s was left between applications to allow recovery, after which control responses to L-glutamate and ibotenic acid were again checked (washout).  $A_1$  and  $B_1$ : concentration-response curve of the inhibitory effects of fipronil on L-glutamate- and 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  $\pm$  SE were fitted using the Hill equation ( $B_1$ ), which showed an  $IC_{50}$  of fipronil for L-glutamate ( $A_1$ ) of  $3.7 \pm 1.1 \mu\text{M}$  ( $n = 6$ ) and for ibotenic acid ( $B_1$ ) of  $4.7 \pm 1.2 \mu\text{M}$  ( $n = 6$ ). C: L-glutamate at its  $EC_{50}$  was applied for 3 s at a  $V_H$  of  $-60$  mV alone and in combination with  $100 \mu\text{M}$  of 2,3-dihydroxy-6-nitro-7-sulfamoylbenzoquinoline-2,3-dione (NBQX) as described above ( $C_1$ ). NBQX had no significant effect on the glutamate-activated currents, as shown in  $C_1$  and summarized in the histogram in  $C_2$  (means  $\pm$  SE;  $n = 6$ ;  $P = 0.09$ ).

continuous glutamate perfusion (Janssen et al. 2007). In this study, ibotenic acid, a structural analog 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  $7.4 \pm 1.8$  nA;  $n = 3$  and  $9.5 \pm 1.2$  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  $\mu$ M), as described in Fig. 2,  $A_2$  and  $B_2$ . Glutamate induced a discernible current from a concentration of 10  $\mu$ M and ibotenic acid from 3  $\mu$ M. Near saturation was achieved at 1 mM for glutamate and at 300  $\mu$ M for ibotenic acid (Fig. 2,  $A_1$  and  $B_1$ ). Both concentration-response relationships were fitted with the Hill equation and yielded an  $EC_{50}$  of  $39.7 \pm 1.3$   $\mu$ M ( $n = 11$ ) and a Hill coefficient ( $n_H$ ) of  $1.3 \pm 0.4$  for glutamate-evoked current and an  $EC_{50}$  of  $22.6 \pm 1.1$   $\mu$ M ( $n = 13$ ) and an  $n_H$  of  $1.3 \pm 0.1$  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<sub>3</sub>). 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 ( $E_{Cl^-} = +2.7$  mV).

Glutamate- and ibotenic acid-induced currents were tested for their sensitivity to the phenylpyrazole fipronil, a potent open channel blocker of GluCl<sub>s</sub> (Fig. 4, A and B). In addition, fipronil also reversibly inhibited ibotenic acid-gated currents, as shown in Fig. 4B. Glutamate and ibotenic acid currents were evoked by application of their  $EC_{50}$  (40 and 25  $\mu$ M, respectively). After several stable control recordings, five different concentrations (from 0.1 to 100  $\mu$ M) of fipronil were co-applied (Fig. 4, A<sub>2</sub> and B<sub>2</sub>). The glutamate-induced currents were suppressed by  $21.4 \pm 3.2$  ( $n = 6$ ) and  $95.7 \pm 1.3\%$  ( $n = 6$ ) by fipronil at 0.1 and 100  $\mu$ M, respectively. Currents evoked by ibotenic acid were suppressed by  $18.4 \pm 7.6$  ( $n = 6$ ) and  $96.1 \pm 2.8\%$  ( $n = 6$ ) by fipronil at 0.1 and 100  $\mu$ M, 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<sub>1</sub>) of  $3.7 \pm 1.1$   $\mu$ M and an  $n_H$  of  $1.5 \pm 0.3$  ( $n = 6$ ) and an  $IC_{50}$  for fipronil on

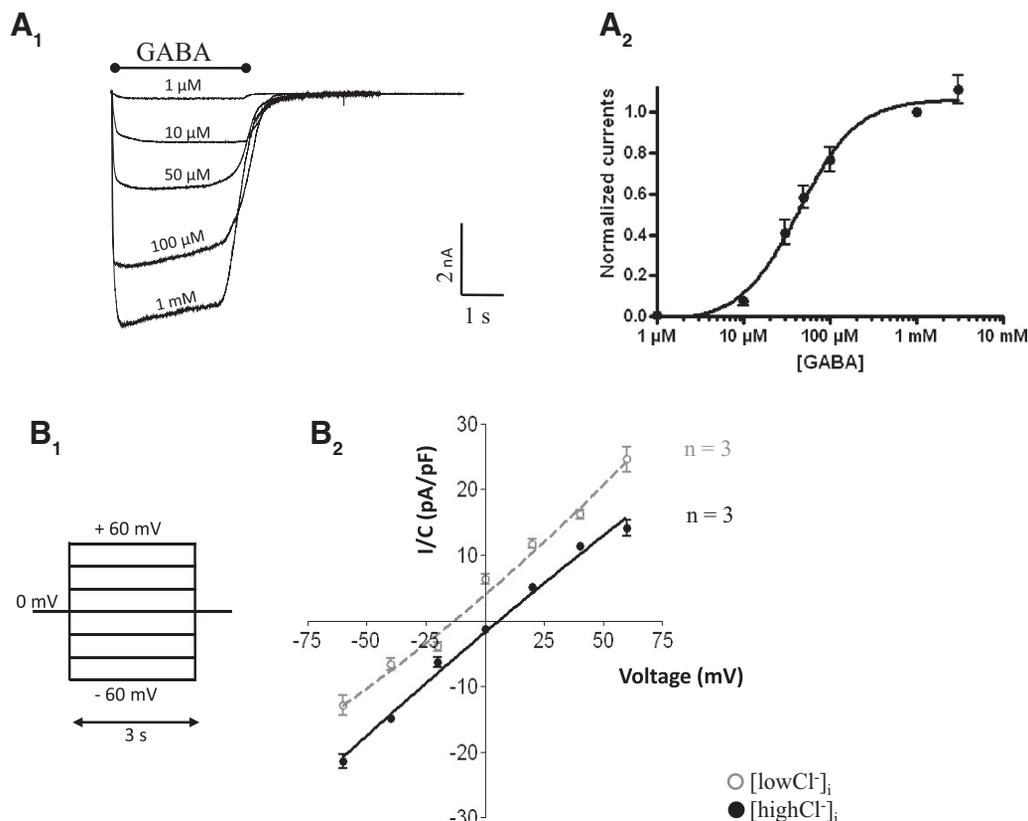


FIG. 5. Concentration-response curve of GABA-gated currents in metathoracic ganglion DUM neurons of *L. migratoria*. GABA was applied onto the cell surface of DUM neurons during 3 s at a  $V_H$  of  $-60$  mV. **A**: examples of current traces induced by different concentrations of GABA ( $A_1$ ). Peak current amplitudes at each concentration were measured and normalized to the current elicited by 1 mM GABA. Mean values  $\pm$  SE ( $n = 17$ ) were plotted and fitted with the Hill equation ( $A_2$ ), which showed an  $EC_{50}$  of  $43.6 \pm 1.5$   $\mu$ M. **B**: current-voltage ( $I$ - $V$ ) relationship of GABA-induced currents in locust DUM neurons recorded with the whole cell patch-clamp technique.  $B_1$ :  $I$ - $V$  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 ( $[lowCl]_i$ ;  $\circ$ ), the reversal potential shifted to  $-13$  mV, which was in good agreement with the theoretically expected shift in Nernst chloride equilibrium potential ( $-17.3$  mV).

ibotenic acid-induced currents (Fig. 4B<sub>1</sub>) of  $4.7 \pm 1.2 \mu\text{M}$  and an  $n_{\text{H}}$  of  $2.4 \pm 0.3$  ( $n = 6$ ).

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 was observed.

#### GABA-induced chloride channel of locust DUM neurons

Using almost symmetrical  $\text{Cl}^-$  concentrations in intra- and extracellular solutions, 1 mM GABA at a  $V_{\text{H}}$  of  $-60$  mV induced rapidly activating inward currents that reached a peak amplitude of  $8.8 \pm 1.1$  nA ( $n = 15$ ). The currents showed little desensitization, mostly remaining sustained during GABA application. A typical experiment is shown in Fig. 1B.

The amplitude of the GABA-induced currents in locust metathoracic DUM neurons was concentration dependent. This was shown by repeatedly applying different concentrations of

GABA (from 1  $\mu\text{M}$  to 3 mM) for 3 s at a  $V_{\text{H}}$  of  $-60$  mV (Fig. 5, A<sub>1</sub> and A<sub>2</sub>). The peak current amplitudes, normalized as described in the legend, were plotted against the GABA concentrations. The dose-response curve (Fig. 5A<sub>1</sub>) shows the usual S-shape, with a just-noticeable effect at 1  $\mu\text{M}$  GABA, an  $\text{EC}_{50}$  of  $43.6 \pm 1.2 \mu\text{M}$  ( $n = 18$ ), and an  $n_{\text{H}}$  of  $1.3 \pm 0.3$ .

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  $\text{Cl}^-$  concentration and a second one containing a lower ( $\sim 100$  mM) intracellular  $\text{Cl}^-$  concentration. In these solutions, NaCl and KCl were replaced by choline Cl and CsCl, respectively, to eliminate contaminating  $\text{Na}^+$  and/or  $\text{K}^+$  currents. An  $I$ - $V$  protocol as shown in Fig. 5B<sub>1</sub> was applied as described earlier. The reversal potential ( $E_{\text{GABA}}$ ) was calculated after fitting the data with the GHK equation (Fig. 5B<sub>2</sub>). Using almost symmetrical  $\text{Cl}^-$  concentrations, GABA-induced currents reversed in

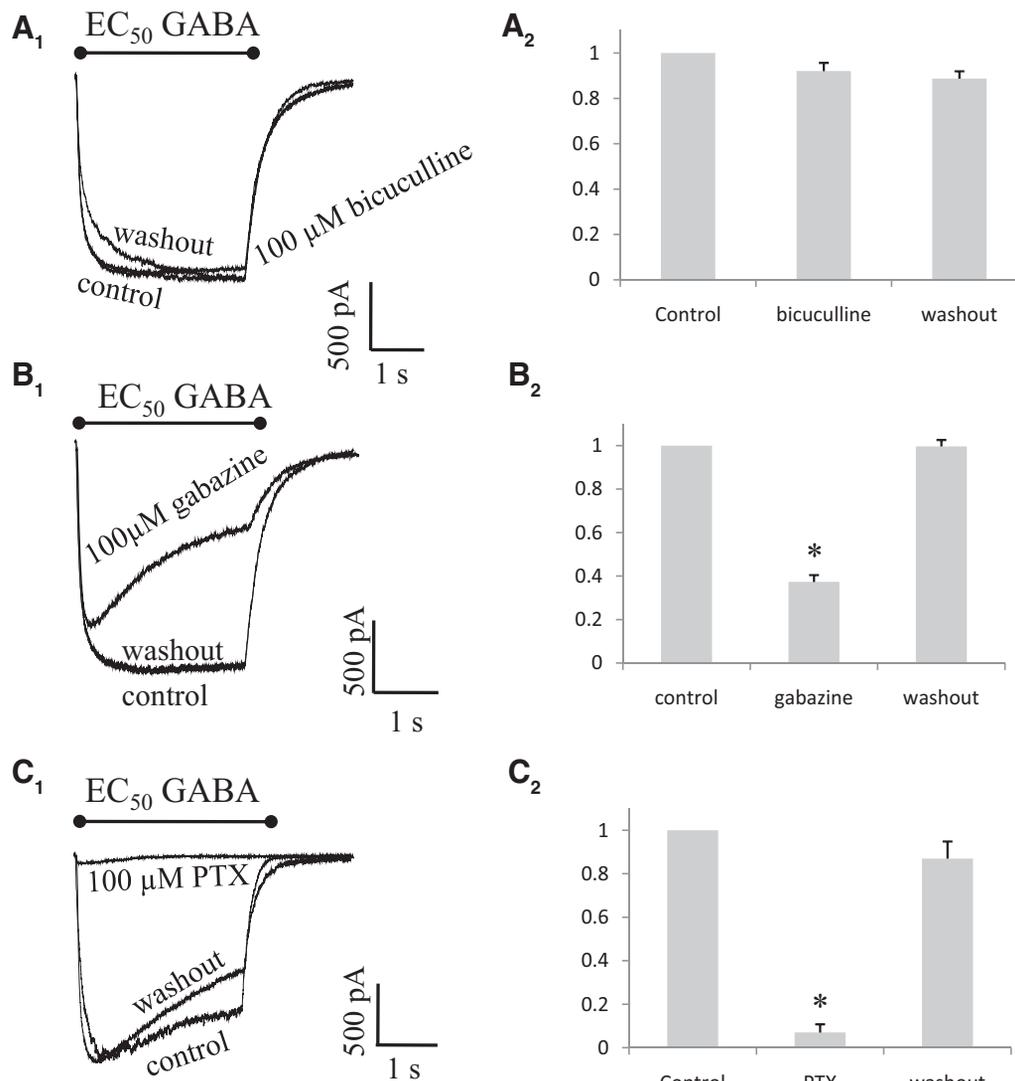


FIG. 6. Pharmacological properties of the GABA-gated chloride channels in DUM neurons of *L. migratoria*. GABA at its  $\text{EC}_{50}$  ( $45 \mu\text{M}$ ) was applied for 3 s at a  $V_{\text{H}}$  of  $-60$  mV in almost symmetrical  $\text{Cl}^-$  concentrations in intra- and extracellular solutions, alone or in combination with 100  $\mu\text{M}$  bicuculline (A<sub>1</sub>), 100  $\mu\text{M}$  gabazine (B<sub>1</sub>), or 100  $\mu\text{M}$  picrotoxin (PTX) (C<sub>1</sub>). Recovery from blockage (washout) was also evaluated. Histograms in A<sub>2</sub>, B<sub>2</sub>, and C<sub>2</sub> summarize bicuculline-, gabazine-, and PTX-induced inhibition of GABA-activated peak currents (mean  $\pm$  SE,  $n = 6$  for bicuculline,  $n = 7$  for gabazine, and  $n = 6$  for PTX;  $*P < 0.05$ ).

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 show 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 PTX (Fig. 6, A–C). Bicuculline had no effect on GABA-induced currents up to a concentration of  $100 \mu M$  ( $n = 4$ ; Fig. 6,  $A_1$  and  $A_2$ ). In contrast, gabazine and PTX reversibly inhibited GABA-gated currents as shown in Fig. 6, B and C. Gabazine ( $100 \mu M$ ) reduced the peak current to  $37.3 \pm 3.1\%$  of the control ( $n = 7$ ; Fig.  $6B_2$ ). After washout, the currents recovered to  $99 \pm 6.8\%$  of the control ( $n = 7$ ; Fig.  $6B_2$ ). In the presence of PTX, GABA-induced currents were suppressed to  $6.9 \pm 1.5\%$  of the control, whereas current recovery was  $\leq 86 \pm 4.2\%$  ( $n = 6$ ; Fig.  $6C_2$ ).

The vertebrate  $GABA_A$  receptor agonist muscimol and the vertebrate  $GABA_C$  receptor agonist TACA elicited currents with amplitudes that increased in a concentration-dependent manner (Fig. 7, A and B). The concentration-response relationships of muscimol and TACA for activating the GABA-gated chloride channel (GABACl) were assessed by repeatedly administering different concentrations (from  $100$  nM to  $300 \mu M$ )

of muscimol and TACA (Fig. 7,  $A_2$  and  $B_2$ ). 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  $18.8 \pm 1.27 \mu M$  ( $n = 11$ ) and an  $n_H$  of  $1.46 \pm 0.11$  for muscimol-evoked currents and an  $EC_{50}$  of  $15.5 \pm 1.04 \mu M$  ( $n = 9$ ) and an  $n_H$  of  $1.78 \pm 0.16$  for TACA-induced current (Fig. 7,  $A_1$  and  $B_1$ ).

The ability of the  $GABA_A$  receptor blocker gabazine to antagonize the responses evoked by muscimol and by TACA was studied. Gabazine ( $10 \mu M$ ) suppressed muscimol-induced currents to  $46 \pm 6.6\%$  of control ( $n = 4$ ; Fig. 8,  $A_1$  and  $A_2$ ). However, gabazine failed to antagonize TACA-induced currents as shown in Fig. 8,  $B_1$  and  $B_2$ .

Finally, a two-step PCR procedure using degenerated primers was used to amplify part of the GABACl from *L. migratoria* DUM neuron cDNA. After the first step, a faint band of  $\sim 460$  bp was visible (Fig.  $9A_2$ ). After re-PCR (Fig.  $9A_3$ ), the resulting PCR product was excised, purified, and sequenced to confirm the sequence (Fig.  $9B$ ). At the cDNA level, the resulting sequence covered exons 2–4 (compared with genomic *D. melanogaster* sequence NM\_079267) of the Rdl-family of GABACl. 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 ( $\sim 92\%$ ), indicating

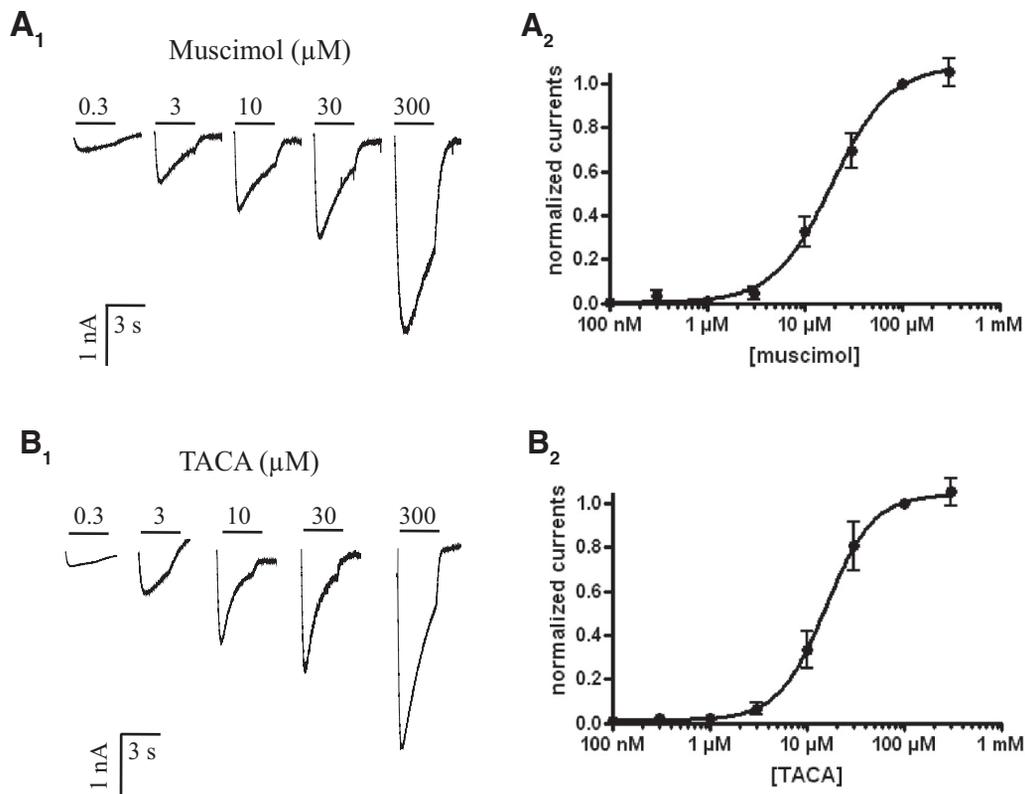


FIG. 7. Effects of  $GABA_A$  (muscimol) and  $GABA_C$  (TACA) receptor agonists on metathoracic DUM neuron of *L. migratoria*. Agonists were applied onto the cell surface of DUM neurons during 3 s at a  $V_H$  of  $-60$  mV as shown in  $A_1$  and  $B_1$ .  $A_2$  and  $B_2$ : concentration-response curves of GABA receptor agonists applied onto the surface of locust metathoracic DUM neurons. Current responses were induced by different concentrations of muscimol ( $A_1$ ) and TACA ( $B_1$ ), and the peak current amplitudes at each concentrations were measured and normalized to the current elicited by respectively  $100 \mu M$  muscimol and  $100 \mu M$  TACA. Mean values  $\pm$  SE ( $n = 11$  for muscimol and  $n = 9$  for TACA) were plotted and fitted using the Hill equation, which showed a muscimol  $EC_{50}$  of  $18.8 \pm 1.3 \mu M$  ( $A_2$ ) and a TACA  $EC_{50}$  of  $15.5 \pm 1 \mu M$  ( $B_2$ ).

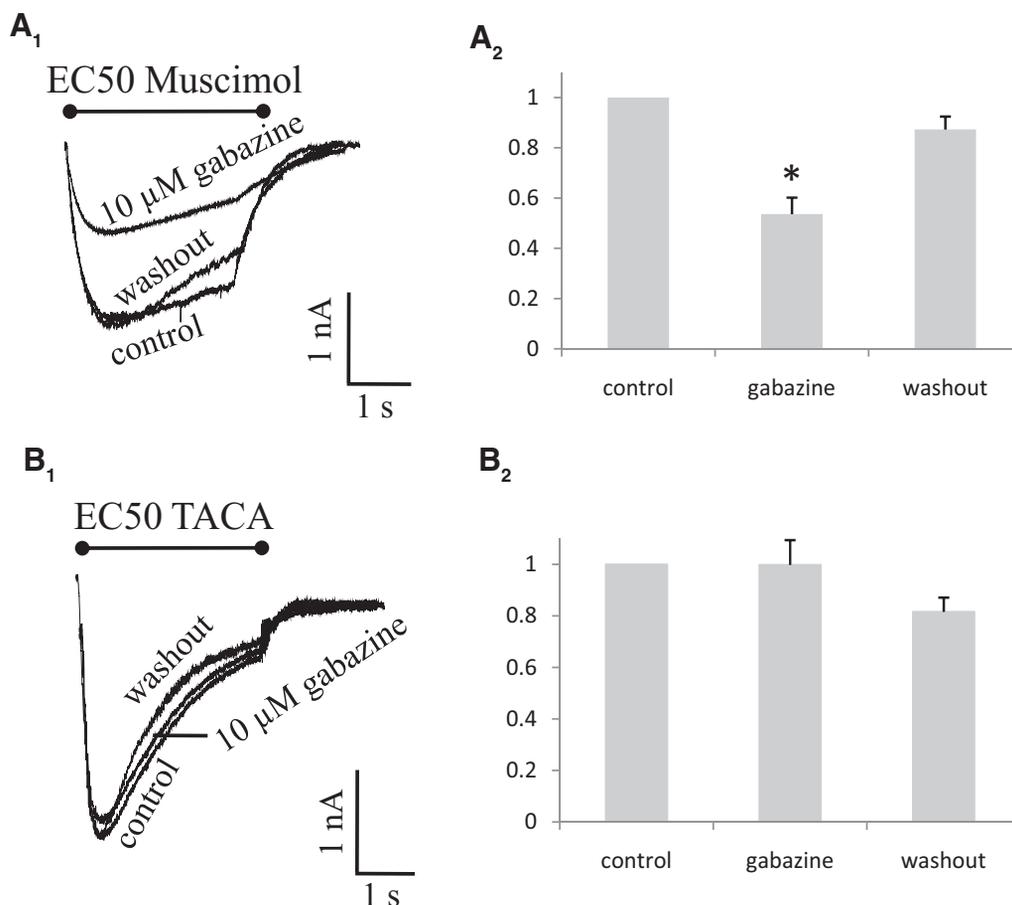


FIG. 8. Effect of gabazine on currents induced by GABA<sub>A</sub> (muscimol) and GABA<sub>C</sub> (TACA) receptor agonists in DUM neurons of *L. migratoria*. Muscimol or TACA was applied at their EC<sub>50</sub> for 3 s at a V<sub>H</sub> of -60 mV in almost symmetrical Cl<sup>-</sup> concentrations in intra- and extracellular solutions, alone and in combination with 10 μM gabazine, as shown in A<sub>1</sub> and B<sub>1</sub>. Recovery from blockage (washout) was also evaluated. Histograms in A<sub>2</sub> and B<sub>2</sub> summarize gabazine-induced inhibition of the activated currents (means ± SE, *n* = 4 for muscimol and *n* = 3 for TACA; \**P* ≤ 0.05; 1-way ANOVA followed by Dunnett's post-test).

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 copurified genomic DNA can be excluded. The partial sequence of the *L. migratoria* Rdl-type GABA<sub>C</sub> was submitted to GenBank under accession FJ436416.

#### HisCl of locust DUM neurons

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

#### DISCUSSION

In this 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*: GluCl, GABA<sub>C</sub>, and HisCl.

To date, no evidence has been presented that insects use 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 channels (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).

#### 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 GluCl<sub>s</sub> (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 while the non-desensitizing GluCl is highly sensitive to these compounds (Zhao et al. 2004a). The existence of GluCl<sub>s</sub> in efferent DUM neurons was already shown in our previous work (Janssen et al. 2007). In this study, the pharmacological properties of the locust GluCl were further characterized. Both glutamate and ibotenic acid evoked a

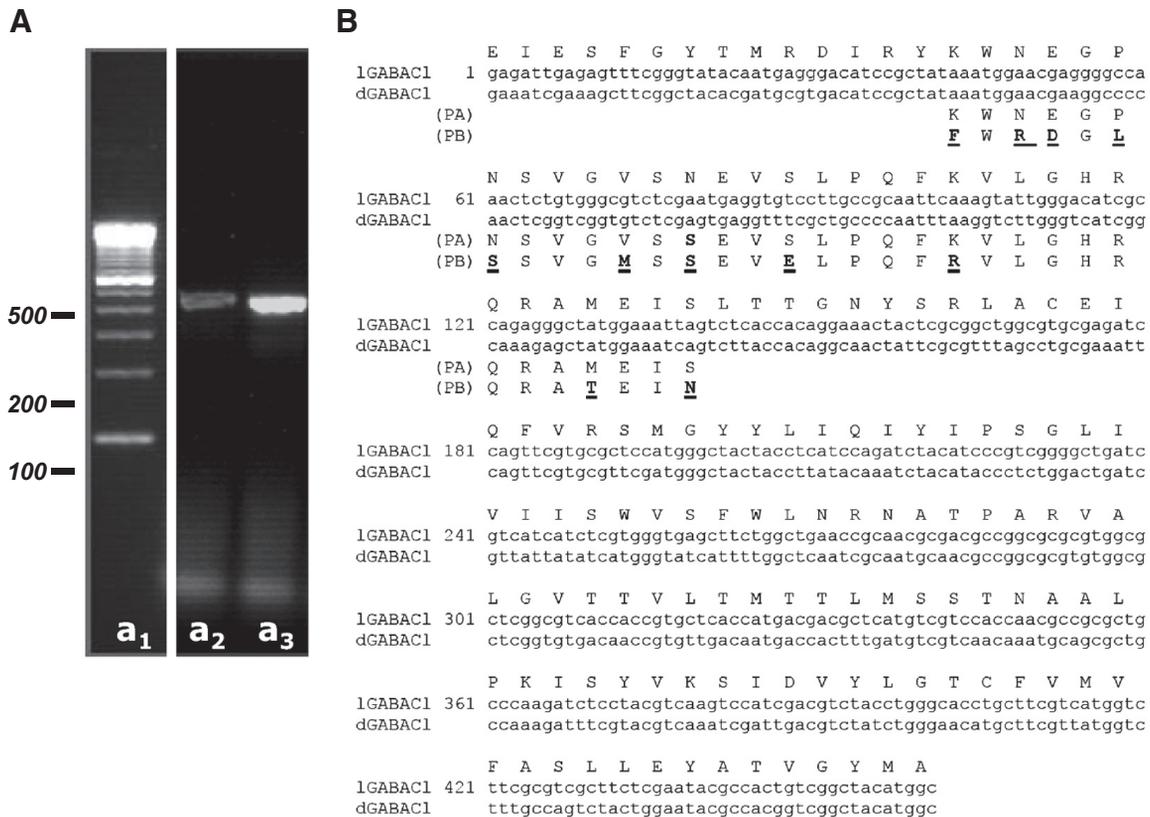


FIG. 9. A: expression of an Rdl-like GABA-gated chloride channel subunit in DUM neurons of *L. migratoria*. Agarose electrophoresis of the 1st PCR using locust DUM neuron cDNA as a template (lane a2). The faint band of ~460 bp was excised and subsequently reamplified using PCR for DNA sequencing (lane a3; marker: 100-bp ladder is shown in lane a1). B: alignment of the partial cDNA sequence of *L. migratoria* GABA-gated chloride channel (FJ436416) with its *D. melanogaster* ortholog (NM\_079267, CG10537-PA). Amino acid sequence of *L. migratoria* GABAC1 channel is also shown. In addition, *D. melanogaster* amino acid sequences are shown for an alternatively spliced region for 2 isoforms of the *D. melanogaster* GABAC1 channel (CG10537-PA, -PB), indicating that the *L. migratoria* sequence is a homolog to the *D. melanogaster* isoform CG10537-PA.

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 nondesensitizing 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 (1.75 times) in gating the glutamate-gated chloride channel in *L. migratoria*.

In cockroach neurons, fipronil is an open channel blocker of GluCl<sub>s</sub> that blocks desensitizing currents in a concentration-dependent manner with an IC<sub>50</sub> of 730 nM (Ikeda et al. 2003; Zhao et al. 2004b). A study on native locust neuron receptors has shown that fipronil blocks glutamate-gated chloride channels with an IC<sub>50</sub> of 30 nM (Smith 1999). In this study, the blockage by fipronil of glutamate- and ibotenic-induced currents (IC<sub>50</sub> of 3.71 and 4.69 μM, respectively) was generally less potent than in the studies described above. Whether this is because of species differences or a difference in receptor subtypes has yet to be elucidated. Although good evidence has been presented that glutamate can also act as an excitatory transmitter in many invertebrate species (Brookie and Maricq 2006; Dudel and Franke 1987; Kerry et al. 1987; Shinozaki 1988; Wafford and Sattelle 1989), millimolar concentrations of kainate, AMPA, and *N*-methyl-D-aspartate (NMDA) each failed

to evoke any response in the lobster (Cleland and Selverston 1995). In this 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.

#### GABAC1 of locust DUM neurons

GABAC1 and GluCl<sub>s</sub> 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 GABAC1 receptor and a GluCl<sub>s</sub> channel can exist separately or coexist 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 accumulated several lines of evidence to show that efferent DUM neurons of *L. migratoria* express functional GABAC1s. By means of patch-clamp experiments, we indeed clearly showed 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, and 4) are activated by the GABA<sub>A</sub> agonist muscimol and the GABA<sub>C</sub> agonist TACA. Furthermore, we confirmed the expression of GABAC1s 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 (Zhainazarov et al. 1997) and in crab peptide-secretory neurons (Duan and Cooke 2000). The  $EC_{50}$  obtained from the 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 (Narusuye 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 coexpressed in *Xenopus* oocytes (Gisselmann et al. 2004). However, until now, 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  $GABA_A$  nor the  $GABA_C$  receptor. Indeed, locust DUM neuron GABACl could be gated by the  $GABA_A$  receptor agonist muscimol but also by the  $GABA_C$  receptor agonist TACA. They were insensitive to bicuculline, a  $GABA_A$  receptor antagonist, but still partially inhibited by gabazine, another  $GABA_A$  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 led to the discovery of several other GABA receptor subunits, it seems that only the Rdl homo-oligomer is able to form an anion channel that 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 GABACl expressed by locust DUM neurons showed their orthologous nature with the *D. melanogaster* CG10357-A GABA receptors.

#### *HisCl* 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 HisCl were identified: Dm-HisCl- $\alpha$ 1 and DM-HisCl- $\alpha$ 2 (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 HisCl, because of 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 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 and GABACl colocalize on the cell surface. Therefore it is plausible that our *ex vivo* preparation also includes other types of DUM neurons that 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 study, we thoroughly characterized the cys-loop ligand-gated chloride channels expressed by metathoracic efferent DUM neurons that innervate flight and leg muscles of *L. migratoria*. We furthermore examined the pharmacological profile of glutamate and GABA receptors, which might be a basis for the development of novel highly selective insecticides (Bloomquist 2003; Janssen et al. 2007; Williamson et al. 2007).

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#### DISCLOSURES

No conflicts of interest are declared by the authors.

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