Molecular and functional characterization of GABA receptor subunits GRD and LCCH3 from human louse *Pediculus humanus humanus*®

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ABBREVIATIONS: Coding sequence (CDS), Glutamate chloride (GluCl), Glycin like receptor of drosophila (GRD), Ligand gated chloride channel homologue 3 (LCCH3), Ligand gated ion channels (LGIC), Open reading frame (ORF), Polymerase chain reaction (PCR), Quantitative PCR (qPCR), Rapid amplification of cDNA ends (RACE-PCR), Resistance to diel drin (RDL), Reverse transcription PCR (RT-PCR), Two-electrode voltage-clamp (TEVC), Transcription starting site (TSS).
ABSTRACT

Human louse *Pediculus humanus* is a cosmopolitan obligatory blood-feeding ectoparasite causing pediculosis and transmitting many bacterial pathogens. Control of infestation is difficult due to the developed resistance to insecticides that mainly target GABA (γ-amino butyric acid) receptors. Previous work showed that *Pediculus humanus humanus* (Phh) GABA receptor subunit RDL is the target of lotilaner, a synthetic molecule of the isoxazoline chemical class. To enhance our understanding of how insecticides act on GABA receptors, two other GABA receptors subunits were cloned and characterized: three variants of Phh-grd and one variant of Phh-lcch3. Relative mRNA expression levels of Phh-rdl, Phh-grd and Phh-lcch3 revealed that they were expressed throughout the developmental stages (eggs, larvae, adults) and in the different parts of adult lice (head, thorax and abdomen). When expressed individually in the *Xenopus* oocyte heterologous expression system, Phh-GRD1, Phh-GRD2, Phh-GRD3 and Phh-LCCH3 were unable to reconstitute functional channels, whereas the subunit combinations Phh-GRD1/Phh-LCCH3, Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL responded to GABA in a concentration-dependent manner. The three heteromeric receptors were similarly sensitive to the antagonistic effect of picrotoxin and fipronil, while Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL were respectively about 2.5-fold and 5-fold more sensitive to ivermectin than Phh-GRD1/Phh-LCCH3. Moreover, the heteropentameric receptor constituted by Phh-GRD1/Phh-LCCH3 was found to be permeable and highly sensitive to the extracellular sodium concentration. These findings provided valuable additions to our knowledge of the complex nature of GABA receptors in human louse that could help in understanding the resistance pattern to commonly used pediculicides.
SIGNIFICANCE STATEMENT

Human louse is an ectoparasite that causes pediculosis and transmits several bacterial pathogens. Emerging strains developed resistance to the commonly used insecticides, especially those targeting GABA receptors. To understand the molecular mechanisms underlying this resistance, two subunits of GABA receptors were cloned and described: Phh-grd and Phh-lcch3. The heteromeric receptor reconstituted with the two subunits was functional in Xenopus oocytes and sensitive to commercially available insecticides. Moreover, both subunits were transcribed throughout the parasite lifecycle.
Introduction

Human louse (\textit{Pediculus humanus}, order \textit{Phthiraptera}) is a cosmopolitan obligatory blood feeding ectoparasite. There are two main ecotypes of human louse with near identical genomes (Olds et al., 2012): the head louse \textit{Pediculus humanus capitis} causing pediculosis, a major public health concern, and the body louse \textit{Pediculus humanus humanus}, transmitting \textit{Rickettsia prowazekii} responsible for epidemic typhus, \textit{Borellia recurrentis} responsible for relapsing fever and \textit{Bartonella quintana} that causes trench fever (Badiaga and Brouqui, 2012; Amanzouga ghene et al., 2020). Historically, massive epidemics of relapsing fever and of typhus have affected Africa and Eurasia and these diseases have recently re-emerged in Europe by travellers and increasing numbers of refugees from endemic regions (Bechah et al., 2008; Hoch et al., 2015; Wilting et al., 2015; Antinori et al., 2016; Osthoff et al., 2016; Hytönen et al., 2017; DE LA Filia et al., 2018).

Chemical insecticides were widely applied to eliminate human louse, leading to emergence of strains resistant to the most commonly used pediculicides like carbaryl (carbamates), pyrethrin (pyrethroids) and ivermectin (Clark et al., 2013; Amanzouga ghene et al., 2020; Mohammadi et al., 2021). Furthermore, the use of organochlorides (lindane) was prohibited due to high toxicity to humans and the environment (Sangaré et al., 2016). Deciphering the mechanisms of action of insecticides on human louse could help to prevent resistance. To this end, laboratory-reared human body louse is a good model, since the individuals of the colony have never been exposed to chemical products and have not developed resistance.

Pentameric transmembrane Cys-loop ligand-gated ion channels (LGIC) are the major pharmacological targets of insecticides (Tong et al., 2021). Among them, \(\gamma\)-aminobutyric acid (GABA) receptors are traditional drug targets for organochlorides (dieldrin), phenylpyrazoles (fipronil), picrotoxin and the macrocytic lactones (ivermectin). GABA receptors of insects share common molecular features: long N terminal extracellular domain constituting the GABA binding site, a Cys-loop motif of 13 amino acids and four transmembrane domains (TM1-TM4) forming the pore of the ion channel, with TM2 containing the molecular determinants of ion selectivity (Kozuska and Paulsen, 2012). In human cells, hundreds of combinations of GABA receptor subunits exist, leading to highly variable conductance for different ions, activation/desensitization times and GABA
EC$_{50}$ (concentration required to mediate 50% of the maximum current in electrophysiology assay). The activity of human GABA receptors is modulated by different agonists and antagonists like benzodiazepines or barbiturates (Sallard et al., 2021). Because of this diversity, it has been shown that ivermectin is not toxic for human (Johnson-Arbor, 2022).

Four subunits of GABA receptors have been described in insects: resistance to dieldrin (RDL), glycine-like receptor of drosophila (GRD), ligand-gated chloride channel homolog 3 (LCCH3) and CG8916 (Buckingham et al., 2005). Among them, the genes encoding for GRD and LCCH3 subunits were identified in *Drosophila melanogaster* (Harvey et al., 1994), *Laodelphax striatellus* (Wei et al., 2017), *Chilo suppressalis* (Jia et al., 2019; Huang et al., 2021), *Apis mellifera* (Henry et al., 2020) and *Blattella germanica* (Jones et al., 2021). While GRD and LCCH3 did not form functional homomeric channels, their co-expression reconstituted heteropentameric cationic selective channel in *D. melanogaster*, *A. mellifera* and *Varroa destructor* (Gisselmann et al., 2004; Ménard et al., 2018; Henry et al., 2020). In human louse, we recently characterized Phh-RDL that constituted a homopentameric anion-selective receptor with higher affinity to lotilaner (isoxazoline) and lower affinity to ivermectin (Lamassiaude et al., 2021). However, the functionality of GRD and LCCH3 have never been investigated in the order *Phtiraptera*.

In this explorative study, the molecular characterization of the two GABA receptor subunits Phh-GRD and Phh-LCCH3 is reported for the first time. The relative expression levels of *Phh-grd*, *Phh-lcch3* and *Phh-rdl* were assayed in different parts and throughout the developmental stages of human body louse. Moreover, the functional expression and pharmacological characterization of the heteropentameric receptors constituted by Phh-GRD1/Phh-RDL, Phh-LCCH3/Phh-RDL and Phh-GRD1/Phh-LCCH3 in response to GABA, picrotoxin, fipronil and ivermectin were investigated.
Materials and Methods

Reagents. All reagents are of molecular biology grade. Chemicals including GABA, acetylcholine, glutamate, glycine, aspartate, histamine, serotonin, picrotoxin, fipronil and ivermectin were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). GABA, acetylcholine, glutamate, glycine, aspartate, histamine, and serotonin were directly dissolved in the recording solution, while picrotoxin, fipronil and ivermectin were first prepared at 10 mM DMSO and then diluted in recording solution to the final concentrations in which DMSO did not exceed 0.1%.

Isolation of RNA and synthesis of first strand cDNA. Human body lice were reared in the BioMAP laboratory as previously described (Lamassiaude et al., 2021). Total RNA was extracted from 30 mg of adult lice using NucleoSpin® RNA plus extraction kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer’s instructions. For RACE-PCR, the cDNA ends were amplified from 5 µg of RNA using Gene Racer kit (Invitrogen™, Thermo Fisher Scientific, Courtaboeuf, France) according to the manufacturer’s instructions. Each 5’ and 3’ RACE-PCR was performed with gene-specific primers and nested gene-specific primers binding to a site at short distance apart (Table 1, Fig. 1). The thermal cycling conditions were 94°C for 5 min, then 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min and final extension at 72°C for 5 min. For RT-PCR, 0.5 µg of RNA was reverse-transcribed with a mixture of oligo dT and 5’ RACE-PCR gene-specific primers and the superscript III reverse transcriptase (Invitrogen™). Full-length Phh-grd and Phh-lcch3 transcripts were amplified using 1 µl of cDNA and 10 pmol of two gene-specific primers (Table 1) using GoTaq DNA Polymerase (Promega, Charbonnières-les-Bains, France) according to the manufacturer’s instructions. After migration on agarose gel, the PCR products were purified using NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel) according to the manufacturer’s instructions. All RACE-PCR, RT-PCR and PCR products were cloned in PGEM-T Easy vector (Promega) and sequenced by Eurofins genomics.

Sequence analysis and phylogeny. All cloned sequences were compared with the putative sequences deposited in Vector Base® with Geneious software (Biomatters, Auckland, New Zealand) and Basic Local Alignment Search Tool (BLAST®, U.S. National Library of Medicine, Rockville Pike, Bethesda, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Deduced amino acid sequences of full-length
Phh-GRD and Phh-LCCH3 were obtained from ExPASy translate (Swiss Institute of Bioinformatics, Lausanne, Switzerland, https://web.expasy.org/translate/), signal peptide cleavage sites were predicted using the SignalP - 5.0 server (DTU Health Tech, Lyngby, Denmark, https://services.healthtech.dtu.dk/service.php?SignalP-5.0) (Petersen et al., 2011) and transmembrane domains were identified using the TMHMM program (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). Multiple sequence alignments were done by Clustal omega algorithm (http://www.clustal.org/omega/) (Madeira et al., 2019) then viewed and annotated by Jalview software (https://www.jalview.org/). All amino acid sequences of GABA receptor subunits of *A. mellifera*: Am-GRD (NP_001292813.1), Am-LCCH3 (XP_026298403.1), Am-RDL (AJE68941), *D. melanogaster*: Dm-GRD (NP_524131.1), Dm-LCCH3 (NP_996469.1), Dm-RDL (NP_523991), *L. striatellus*: Ls-GRD (KX355313), Ls-LCCH3 (KX355312), Ls-RDL (BAF31884.1), *Nasonia vitripennis*: Nv-GRD (NP_001234887.1), Nv-LCCH3 (001234895.1), *Tribolium castaneum*: Tc-GRD (XP_015834304.1), Tc-LCCH3 (NP_001103251.1), Tc-RDL (NP_001107809), *V. destructor*: Vd-GRD (KY748054), Vd-LCCH3 (KY748055), Vd-RDL (KY748050) and Phh-RDL (MT321072) were obtained from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA7) software (https://www.megasoftware.net/) (Kumar et al., 2016) using the Neighbour-Joining method and the bootstrap values were calculated on 1050 replicates. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Phh-β1, a subunit of nAch from human louse, was used as an out-group to root the tree.

**Expression vector cloning and synthesis of cRNA.** The full-length *Phh-grd* variants and *Phh-lcch3* were amplified using GoTaq polymerase (Promega) and cloned in the pTB207 expression vector using the In-Fusion® HD Cloning kit (Takara Bio Europe SAS, Saint-Germain-en-Laye France) as described (Lamassiaude et al., 2021). Recombinant plasmids were purified using E.Z.N.A.® Plasmid DNA Mini kit (Omega Bio-Tek, Inc., Norcross, Georgia) and correct cloning was confirmed by sequencing (Eurofins Genomics, Ebersberg, Germany). cRNAs were obtained from plasmids linearized by Msc1 (Thermo Fisher Scientific) using mMmessage mMMachine® T7
transcription kit (Thermo Fisher Scientific) following the manufacturer's instructions. cRNA concentrations were measured by spectrophotometry (NanoDrop, Thermo Fisher Scientific) and integrity of cRNAs was confirmed by running 500 ng in 1% agarose gel in TAE buffer (40 mM Tris acetate-1 mM EDTA).

**Quantitative PCR (qPCR).** Total RNA extraction and cDNA synthesis were done as described in section 2.1 from 30 mg of nits, L1, L2, L3 larvae stages, as well from heads, thoraxes and abdomens of adult lice. Applying comparative CT experiment $2^{\Delta \text{Ct}}$, 100 ng of cDNA were added to 10 µl of Sybergreen master mix (Thermo Fisher Scientific) and 10 pmol of gene-specific primers (Table 2) in a final volume of 20 µL. The qPCR was performed with primary denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min using the StepOnePlus Real-time PCR System (Applied Biosystems, Thermo Fisher Scientific) following the conditions recommended by the manufacturer. Actin was used as endogenous control and a melt curve cycle (95°C for 15 s, 60°C for 1 min and 95°C for 15 s) was added to confirm specific amplification.

**Functional expression and pharmacology of receptors.** Defolliculated oocytes of *Xenopus laevis* (Ecocyte Bioscience, Dortmund, Germany) were injected individually with 30 ng of cRNA of Phh-grd1, Phh-grd2, Phh-grd3, Phh-lcch3, Phh-rdl, Phh-grd1/Phh-lcch3, Phh-grd2/Phh-lcch3, Phh-grd3/Phh-lcch3, Phh-grd1/Phh-rdl or Phh-lcch3/Phh-rdl using a Drummond nanoject II microinjector (Dominique Dutscher SAS, Bernolsheim, France) and maintained for 3-5 days at 19°C in incubation solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 2H$_2$O, 1 mM MgCl$_2$, 6H$_2$O, 5 mM HEPES, 2.5 mM C$_3$H$_7$NaO$_3$, pH 7.5, with 100 U/mL penicillin and 100 µg/mL streptomycin). Two-electrode voltage-clamp (TEVC) was used to measure currents generated by the application of increasing concentrations of GABA (0.3, 1, 3, 10, 30, 100 and 300 µM in recording solution: 100 mM NaCl, 2.5 mM KCl, 1 mM CaCl$_2$, 2H$_2$O, 5 mM HEPES, pH 7.3). The membrane potential was clamped at -60 mV and current signals were recorded with an oocyte clamp OC-725C amplifier (Warner instrument, Holliston, Massachusetts) and analysed by pCLAMP 10.4 software (Molecular Devices, San Jose, California). Normalized peak current amplitudes were plotted against the corresponding concentrations of GABA. Acetylcholine, glutamate, glycine, aspartate, histamine and serotonin were tested at 30 µM on Phh-GRD1/Phh-LCCH3 to verify its specificity for GABA. To analyse the ion
selectivity of the channel constituted by Phh-GRD1/Phh-LCCH3, reverse potential-ion concentration relationship was obtained by applying voltage-ramps (-60 mA to +60 mA) in the presence of 100 μM GABA using recording solution with decreasing concentrations of Na⁺ (100, 75, 50, 25, 12.5 and 0 mM) by the replacement of NaCl with tetra-ethyl ammonium chloride. The change in reversal potential was plotted against the concentrations of sodium.

The effect of antagonists (10 μM picrotoxin, 1 μM fipronil or 1 μM ivermectin) was evaluated on Phh-RDL, Phh-GRD1/Phh-LCCH3, Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL by pre-incubation of each antagonist alone for 90 s, followed by their co-application with 100 μM GABA for 10 s. The observed responses were normalized to the response induced by 100 μM GABA alone performed before challenging with the antagonist.

**Statistical analysis.** Statistical comparisons were performed with Prism 7 (GraphPad software, Inc., San Diego, California) using one-way ANOVA test followed by Tukey’s multiple comparisons test. The sample size and the number of experiments were determined according to the previous results on Phh-RDL (Lamassiaude et al., 2021). A power of the test > 80% was calculated *a posteriori* with a statistical tool of Anastats (anastats.com) when differences were observed between the groups. The study is explorative and the P values are descriptive.
Results

Identification of Phh-grd and Phh-lcch3 transcripts. Using tBLASTn with Apis mellifera, Am-grd (NM_001305884.1) and Am-lcch3 (NM_001077812.1) as query against the whole genome sequence of P. humanus humanus in Vectorbase®, Phh-grd (XM_002433141.1; PHUM 616750) and Phh-lcch3 (XM_002430956.1; PHUM 507170) subunit genes located respectively in the super contig DS235886 and DS235842 were identified. The coding sequences (CDS) of 1272 bp for Phh-grd and 1404 bp for Phh-lcch3 are organized in 9 and 10 exons encoding for proteins of 423 aa and 467 aa, respectively. Confusingly, no signal peptide is annotated on the putative protein sequence of both subunits.

To characterize 5' and 3' ends of Phh-grd and Phh-lcch3, RACE-PCR was done on total RNA extracted from adult lice using primers binding to the putative exons 2 (5') and 6 (3') of Phh-grd and exons 3 (5') and 8 (3') of Phh-lcch3 (Fig. 1). For Phh-grd 5' RACE, two transcription start sites (TSS) were identified at positions 766932 and 780980 (Fig. 1A). In both cases, the transcripts have the same 5' end organization: an extension of 29 bp in the 5' direction of the annotated E1 (becoming E3) and two extra 5' exons of 198 bp (E2) and 54 bp (E1). It is important to note that in the transcribed E2, 162 bp did not match with the genome of human body louse. Indeed, at the genomic level, these 162 bp are located in a stretch of undefined nucleotides suggesting a mistake in sequencing/assembly of the whole genome (Fig. 1A, N228). Results of 3' RACE of Phh-grd revealed two possible sites for the end of transcripts after and inside putative exon 9. These alternative transcripts were found in many clones and further confirmed by results of transcriptomics (unpublished data). The RACE-PCR result of Phh-lcch3 showed a modified position of E1 (Fig. 1B) including single TSS at position 430552 and single end of transcript at position 435043. Differences were observed in the lengths of putative and transcribed E4 (27 nucleotides shorter), E5 (26 nucleotides longer) and E9 (61 nucleotides longer) (Fig. 1B).

Based on the sequence analysis of RACE-PCR products, primers were designed to amplify and clone three full-length coding sequences of Phh-grd (Phh-grd1, Phh-grd2 and Phh-grd3) and one full-length coding sequence of Phh-lcch3. Phh-grd transcripts were 1869 bp including a 5' untranslated region (UTR) of 75 bp, a CDS of 1771 bp and a 3'UTR of 23 bp, encoding for 622 aa. Phh-GRD was transcribed from the entire open reading frame (ORF) composed of 10 exons and
located on contig DS235886 from position 781055 to 793502 (Fig. 1A). The cloned mRNA sequence of Phh-lcch3 had 1569 bp with a 5' UTR of 25 bp, a CDS of 1521 bp and a 3' UTR of 23 bp, encoding for 506 aa. The entire ORF of 10 exons was located on contig DS235843 from position 430552 to 435018 (Fig. 1B).

**Sequence and phylogenetic analysis of Phh-GRD and Phh-LCCH3.** The multiple sequence alignment of deduced amino acid sequences of Phh-GRD1, Phh-GRD2 and Phh-GRD3 with those well described of other insects/mite revealed that all variants possessed all the typical features of LGIC: 4 TM domains, C-C loops, and the signal peptide missing in the putative sequence (Fig. 2A). Sequence analysis showed the presence of AD at position -1', -2' (aa 306-307) and F at position 13' (aa 321) as indicators of sharp cation selectivity of resulting channel as described in many reports (Fig. 2A) (Galzi et al., 1992; Corringer et al., 1999; Keramidas et al., 2004; Henry et al., 2020). Phh-GRD2 showed the single mutation S117P in the loop D compared to Phh-GRD1 and Phh-GRD3, while Phh-GRD3 revealed mutations W295C (in TM1), K365R, Y366C and G374R, compared to Phh-GRD1 and Phh-GR2. Alignment of deduced amino acid sequences of the cloned Phh-LCCH3 sequence with those of other insects/mite showed that, as for Phh-GRD, all the characteristic features of cys-loop LGIC could be found (Fig. 2B). The amino acid D was replaced by a R at position 100 located in the GABA binding site of Phh-LCCH3. The presence of amino acids SA at position -1, -2' (aa 282-283) and T at position 13' (aa 301) rendered the Phh-LCCH3 subunit as moderate in terms of ion selectivity in contrast to the sharp anion selectivity of Phh-RDL (MT321072) (Fig. S1). Comparison with the Phh-RDL sequence also highlighted some amino acids differences in the GABA binding site of Phh-GRDs (F148Y-loop A and E206G-loop B) and Phh-LCCH3 (R100D-loop D, S163G and F193Y-loop B) (Fig. S1). Sequences of the cloned Phh-GRD1, Phh-GRD2, Phh-GRD3 and Phh-LCCH3 were deposited in NCBI gene bank under the accession numbers OM128123, OM128124, OM128125 and OM128126, respectively.

As expected, phylogenetic analysis revealed that both Phh-GRD and Phh-LCCH3 clustered in separate clades and exhibited closer relationships to their orthologs from relevant organisms rather than other GABA subunits of *P. humanus humanus* (Fig. 3). Phh-GRD showed 67%, 66%, 65%, 60% and 58% identities with Nv-GRD, Am-GRD, Tc-GRD, Ls-GRD and Dm-GRD, respectively, while...
Phh-LCCH3 was 81%, 76%, 76%, 73% and 70% identical to Tc-LCCH3, Am-LCCH3, Ls-LCCH3, Nv-LCCH3 and Dm-LCCH3, respectively. Vd-GRD and Vd-LCCH3 of the mite *V. destructor* were more distant from Phh-GRD and Phh-LCCH3 (46% and 69% identities, respectively).

**Expression of Phh-grd, Phh-lcch3 and Phh-rdl along developmental stages.** In order to determine the expression levels of *Phh-grd*, *Phh-lcch3* and *Phh-rdl* in human body louse, mRNA from nits, L1, L2 and L3 larvae stages and adult tissues was extracted and transcribed into cDNA, then submitted to qPCR by using specific primers. *P. humanus humanus β-actin* was used as reference to normalize the expression of the transcripts. It is important to note that the expression of this housekeeping gene, supposed to be stable, was highly variable in the different developmental stages. So, the levels of expression of the subunits could be compared within a stage, but not between the stages. Even the differences were not statistically different, Phh-RDL was slightly more expressed than Phh-GRD and Phh-LCCH3 in nits (Fig. 4A), whereas it was the contrary in larvae (Fig. 4B) and in the different tissues of adults, *i.e.* head, thorax and abdomen (Fig. 4C).

**Functional receptors expressed in Xenopus laevis oocytes.** To investigate the functionality of receptors constituted by Phh-GRD and Phh-LCCH3, their respective cRNAs were injected into oocytes of *Xenopus laevis* and currents in response to GABA were recorded using TEVC. In contrast to Phh-RDL (Lamassiaude et al., 2021), none of the cloned Phh-GRD and Phh-LCCH3 variants was able to constitute a GABA-responsive homomeric receptor. However, Phh-GRD1 was able to reconstitute a functional receptor when co-expressed with Phh-LCCH3, as illustrated by the current traces obtained in response to increasing concentrations of GABA, with the maximum amplitude obtained at 100 µM (Fig. 5A). The -log concentration of GABA required to obtain 50% of the maximum response (-log EC$_{50}$) was 5.226 +/- 0.012 µM (Fig. 5B). No current was recorded with the application of acetylcholine, glutamate, glycine, aspartate, histamine and serotonin (Fig. S2), indicating that the receptor reconstituted by Phh-GRD1/Phh-LCCH3 is selectively gated by GABA. On the contrary to Phh-GRD1, Phh-GRD2 and Phh-GRD3 were not able to reconstitute a functional heteromeric receptor with Phh-LCCH3 (Fig. 5C).

It was shown previously by Ménard et al. that heteromeric GABA receptors could have RDL subunits (Ménard et al., 2018). We thus tested different Phh-RDL-containing combinations. Both Phh-
GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL were functional, as current traces were recorded in the presence of GABA (Fig. 6A and B). The -log EC$_{50}$ of 5.076 +/- 0.029 µM for Phh-GRD1/Phh-RDL and 5.025 +/- 0.032 µM for Phh-LCCH3/Phh-RDL (Fig. 6C) were similar to the -log EC$_{50}$ of Phh-RDL homomers reconstituted in *Xenopus* oocytes (5.014 +/- 0.029 µM, Fig. 6C), but were slightly higher than that calculated for Phh-GRD1/Phh-LCCH3 (5.226 +/- 0.012 µM, Fig. 5B).

It is well known that homopentameric receptors constituted by RDL are anion-selective, but few reports categorized the heteropentameric receptors GRD/LCCH3 as cation-selective (Gisselmann et al., 2004; Henry et al., 2020). Here, we tested the sodium permeability of the heteropentameric receptor Phh-GRD1/Phh-LCCH3. As expected, this receptor was found to be permeable and highly sensitive to the change in the extracellular sodium concentration. Indeed, a shift of 38 mV in reversal potential resulted from changing sodium concentration from 100 to 0 mM (Fig. 6D).

Finally, the antagonist effect of insecticides was studied on Phh-GRD1/Phh-LCCH3. Fipronil and ivermectin were tested at 1 µM, while picrotoxin was tested at 10 µM, based on their antagonist effect on Phh-RDL (Lamassiaude et al., 2021). No current was recorded during the pre-incubation period of 10s before their co-application with GABA, while 10 µM of picrotoxin almost completely abolished the GABA-elicited currents (91.1 +/- 2.4% inhibition), and 1 µM fipronil and ivermectin inhibited 72.7 +/- 1.1% and 44.7 +/- 4.1% of the signal, respectively (Fig. 7A), demonstrating their antagonist effect. In addition, picrotoxin equally antagonized the receptors constituted by Phh-RDL, Phh-GRD1/Phh-LCCH3, Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL (Fig. 7B). Similarly, fipronil blocked all tested receptors with almost the same potency (70-80% inhibition of GABA current, Fig. 7C). In contrast, ivermectin antagonized the receptors in the following order: Phh-LCCH3/Phh-RDL > Phh-GRD1/Phh-RDL > Phh-RDL > Phh-GRD1/Phh-LCCH3 (Fig. 7D).
Discussion

In this work, we reported the first molecular, functional and pharmacological characterization of heteropentameric GABA receptors in the human body louse. By using RT-PCR and RACE-PCR, the complete cDNA of Phh-grd and Phh-lcch3 was cloned and analysis of the sequences against the putative genes annotated in the database allowed us to redefine the gene organization. Notably, results of 3’ RACE for Phh-grd exhibited retention of a part of intron 7 and the whole intron 8 located between TM3 and TM4 of the intracellular domain, as for Vd-grd (Ménard et al., 2018). Since these sequences were obtained in all clones and further confirmed by the transcriptomics project (unpublished data), we hypothesize that it would be a wrong annotation of the genome rather than intron retentions. Results of 5’ RACE of Phh-grd revealed an insertion of 54 aa at the N-terminal domain before the C-C loop. Similar insertions were described in Vd-grd (Ménard et al., 2018) and Dm-grd (Harvey et al., 1994), but these insertions are not identical and seems to be species-specific. Interestingly, two in-frame methionine residues were found at the 5’ end of all cloned variants of Phh-grd, and this is in agreement with what was described in transcripts encoding for many membrane-associated proteins in drosophila (Harvey et al., 1994).

None of the Phh-GRDs and Phh-LCCH3 subunits was able to reconstitute homomeric functional receptor, in agreement with the results obtained with these subunits of D. melanogaster, A. mellifera and V. destructor (Gisselmann et al., 2004; Ménard et al., 2018; Henry et al., 2020). Compared to Phh-RDL, able to form homomeric receptors functional in Xenopus oocytes (Lamassiaude et al., 2021), amino acid modifications F148Y/ E206G and R100D/S163G/F193Y (Fig. S1) were identified in the GABA binding site of Phh-GRD and Phh-LCCH3, respectively. As these amino acids belong to the seven amino acids assumed to be essential for the binding of GABA described in Dm-RDL and Am-RDL (Ashby et al., 2012; Henry et al., 2020), the lack of current upon GABA application is not surprising. When assembled together, Phh-GRD and Phh-LCCH3 respond to GABA, suggesting that the two subunits constitute a heteromeric receptor with functional GABA binding site. We can so hypothesize that the amino acid modifications in the GABA binding site of one subunit were complemented by the corresponding amino acids of the other subunit. Indeed, it has been shown that Am-GRD and Am-LCCH3 were able to respond to GABA as heteromeric receptor, in
spite of a tyrosine at the position 182 of Am-LCCH3 (Henry et al., 2020). The variant Phh-GRD2 was unable to form a functional heteromeric receptor with Phh-LCCH3. It seems that the proline at position 117 was not able to preserve the functionality of serine and was not complemented by the corresponding position of Phh-LCCH3. For Phh-GRD3, one or more of the mutations W295C, K365R, Y366C and G374R might be responsible for the non-functionality of Phh-GRD3/Phh-LCCH3. The mutation W295C is located in TM1 and the other mutations are in the highly variable intracellular area between TM3 and TM4, but data of the literature on mutations in the different TM domains of RDL give contradictory information on their importance in the functionality of GABA receptor. On one hand, Phh-RDL2, unable to form a functional receptor, differed from the functional variant Phh-RDL by only 20 amino acids, among them a mutation in TM1 (D281V) that could be responsible for its non-responsiveness to GABA in Xenopus oocytes (Lamassiaude et al., 2021). On the other hand, Cyrtorhinus lividipennis RDL with an insertion of 31 amino acids between TM3 and TM4 was much less sensitive to fipronil than the RDL without insertion (Jiang et al., 2015). Finally, no important difference in response to GABA, imidacloprid and fipronil has been shown between Am-RDL variants with variable sequences in TM3 and TM4 (Taylor-Wells et al., 2017).

Sodium permeability of the receptor Phh-GRD1/Phh-LCCH3 was confirmed by recording change in reversal potential with decreasing concentrations of sodium and high sensitivity of the resulting channel to changes in Na⁺ concentration (Fig. 6D). In A. mellifera, Am-LCCH3/Am-RDL constituted anion-selective channel, while Am-GRD/Am-LCCH3 is cation-selective (Henry et al., 2020). The existence of amino acids AD at position -1’, -2’ and F at position 13’ of Phh-GRD is a good indicator for cation selectivity of the heteromeric receptor Phh-GRD/Phh-LCH3. The same motifs were described in Dm-GRD, Vd-GRD and Am-GRD and the cation selectivity of GRD/LCCH3 receptors of the three organisms was confirmed by electrophysiology (Gisselmann et al., 2004; Ménard et al., 2018; Henry et al., 2020). This cation selectivity filter was also described in LGIC of other organisms, such as EXP-1 of C. elegans and 5HT3 and nAChRα7 receptors of rat (Corringer et al., 1999; Wotring et al., 2003). Interestingly, the introduction of three amino acids into the TM2 segment of nAChRα7 converted the cation-selective channel into an anion-selective channel gated by acetylcholine (Galzi et al., 1992). The presence of motifs SA at position -1’, -2’ and T at position 13’
(Fig. 2B) marks Phh-LCCH3 as intermediate in term of ion selectivity, meaning that the global anion selectivity of the heteromeric receptor is determined by the Phh-GRD subunit. In honey bee, Am-LCCH3/Am-RDL constituted anion-selective channel, while Am-LCCH3/Am-GRD was cation-selective (Henry et al., 2020).

The Phh-GRD1/Phh-LCCH3 heteromeric receptor responded to GABA in a concentration-dependent manner with slightly higher sensitivity to GABA than the prototype homomeric Phh-RDL, but with almost the same GABA sensitivity as Dm-GRD/Dm-LCCH3 and Am-GRD/Am-LCCH3 (Gisselmann et al., 2004; Henry et al., 2020). The EC50 calculated for the receptor Vd-GRD/Vd-LCCH3 of the mite V. destructor was much more elevated (35 µM) (Ménard et al., 2018). Absence of current in response to other potential ligands confirmed selectivity of Phh-GRD1/Phh-LCCH3 towards GABA. To our knowledge, the heteromer GRD/RDL was not investigated in insects up to now. In the present study, there was no important difference in response to GABA between the homomer Phh-RDL and the heteromers Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL, but heteromeric receptors with similar EC50 could have distinct pharmacology. Even though it is not possible to affirm that Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL are really reconstituted by both subunits and not by Phh-RDL alone. Their higher sensitivity towards ivermectin is in favour of this hypothesis, however, assays like immune co-precipitation are required to confirm the existence of heteromers. It has been shown that the co-expression of LCCH3 could alter the pharmacological properties of the RDL receptor. For example, Dm-RDL was picrotoxin-sensitive and bicuculline-insensitive, whereas Dm-RDL/Dm-LCCH3 was picrotoxin-insensitive and bicuculline-sensitive (Zhang et al., 1995). Interestingly, lower IC50 for fluralaner and fipronil, but higher IC50 for dieldrin were observed in blocking GABA-activated current when two variants of C. suppressalis RDL were co-expressed in Xenopus oocytes than when each variant was expressed individually (Sheng et al., 2018). This points out the fact that reconstitution of receptors with different variants of the same subunit further complicates the expression profile of GABA receptors in arthropods.

Concerning in situ expression, Dm-rdl and Dm-lcch3 were not found in the same tissues of the nervous system of drosophila (Aronstein et al., 1996). These two subunits were assembled and functional in Xenopus oocytes, indicating that the functionality of GABA receptors in this
heterologous expression system does not systematically imply their existence \textit{in vivo}. In the case of \textit{Phh-grd}, \textit{Phh-rdl} and \textit{Phh-lcch3}, results of relative expression showed that the three subunits were present in all developmental stages and in the different parts of the adults (head, thorax and abdomen). Similar results were observed in \textit{L. striatellus}, \textit{C. suppressalis} and \textit{A. mellifera}, where \textit{grd}, \textit{lcch3} or \textit{rdl} subunits were detected during the whole life cycle and in different parts of the insects (Wei et al., 2017; Jia et al., 2019; Henry et al., 2020), but mainly in larvae of \textit{D. melanogaster} (Knipple and Soderlund, 2010), whereas \textit{rdl} was essentially found in the head of \textit{Musca domestica} (Kita et al., 2019). The relatively similar expression pattern of \textit{Phh-grd} and \textit{Phh-lcch3} during developmental stages and in different parts could explain their tendency to form heteromeric cation-selective receptors that could play an important physiological role in the nervous system of human louse. One of them being their role as excitatory mediators during developmental stages of neuronal wiring or under pathological conditions (Henry et al., 2020). Fipronil and ivermectin antagonized all receptors tested here and killed human lice (Lamassiaude et al., 2021). Relationship between receptor targeting and pediculicide activity could be confirmed using RNAi strategy as shown for \textit{L. striatellus}, in which injection of \textit{rdl} dsRNA reduced fipronil-induced mortality (Wei et al., 2015).

In summary, this work allowed us to clone and characterize \textit{Phh-grd} and \textit{Phh-lcch3} subunits of GABA receptors from human body louse. These results revealed that \textit{Phh-grd}, \textit{Phh-lcch3} and \textit{Phh-rdl} were expressed throughout the developmental stages and in different tissues. At the functional level, heteromeric receptors constituted by \textit{Phh-GRD1/Phh-LCCH3}, \textit{Phh-GRD1/Phh-RDL} and \textit{Phh-LCCH3/Phh-RDL} were able to respond to GABA in a concentration-dependent manner with variable sensitivities to picrotoxin, fipronil and ivermectin. Altogether, these findings provide major basis for future investigations on LGIC to understand the mechanisms of action of pediculicides as well as their roles in resistance to insecticides.
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Authorship Contributions

**Participated in research design:** Hashim, Charvet, Neveu, Dimier-Poisson, Debierre-Grockiego, Dupuy.

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**Wrote or contributed to the writing of the manuscript:** Hashim, Charvet, Ahmed, Neveu, Dimier-Poisson, Debierre-Grockiego, Dupuy.
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Footnotes

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This article has supplemental material.
Legends for Figures

Fig. 1. Genomic and transcript organization of *Phh-grd* (A) and *Phh-lcch3* (B). Exons are represented by black boxes in expanded views of the contigs DS235886 with the annotated *Phh-grd* CDS (A, top drawing) and DS235842 with the annotated *Phh-lcch3* CDS (B, top drawing). The numbers indicated on DS235886 and DS235842 correspond to the final annotation of the genes. Positions of the primers are indicated by white arrows; 5’ and 3’ extremities are indicated by black arrows. On the bottom drawings are shown the final organizations of the *Phh-grd* (A) and *Phh-lcch3* (B) transcripts.

Fig. 2. Multiple sequence alignments conducted in clustal omega then viewed and annotated by Jalview software. (A) Alignment of deduced amino acid sequences of Phh-GRD1, Phh-GRD2, Phh-GRD3 with the sequences of Vd-GRD (KY748054), Am-GRD (NP_001292813.1), Tc-GRD (XP_015834304.1), Dm-GRD (NP_524131.1) and Phh-RDL (QRX38896.1). (B) Alignment of deduced amino acid sequences of Phh-LCCH3 with the sequences of Vd-LCCH3 (KY748055), Tc-LCCH3 (NP_001103251.1), Dm-LCCH3 (NP_996469.1), Am-LCCH3 (XP_026298403.1) and Phh-RDL (QRX38896.1). Transmembrane domains (black frames), signal peptides of Phh-GRDs and Phh-LCCH3 (grey frames), C-C loops (arrows), GABA binding loops (A to F, black lines), conserved motifs for ion selectivity (AD at positions -1’, -2’ and F at position 13’ for Phh-GRDs, SA at position -1’, -2’ and T at position 13’ for Phh-LCCH3, asterisks), mutations F148Y and E206G in GABA binding site of Phh-GRD (highlighted in red), mutations R100D, S165G and F193Y in GABA binding site of Phh-LCCH3 (highlighted in green), mutation S117P of Phh-GRD2 and mutations W295C, K365R, Y366C and G374R of Phh-GRD3 (highlighted in black), are indicated.

Fig. 3. Evolutionary relationships of *Apis mellifera* Am-LCCH3 (XP_026298403.1), *Drosophila melanogaster* Dm-LCCH3 (NP_996469.1), *Laodelphax striatellus* Ls-LCCH3 (KX355312), *Nasonia vitripennis* Nv-LCCH3 (NP_001234895.1), *Pediculus humanus humanus* Phh-LCCH3 (OM128126), *Tribolium castaneum* Tc-LCCH3 (NP_001103251.1), *Varroa destructor* Vd-LCCH3 (KY748055), Am-GRD (NP_001292813.1), Dm-GRD (NP_524131.1), Ls-GRD (KX355313), Nv-GRD (NP_001234887.1), Phh-GRD (OM128123), Tc-GRD (XP_015834304.1), Vd-GRD (KY748054),
Am-RDL (AJE68941), Dm-RDL (NP_523991), Ls-RDL (BAF31884.1), Phh-RDL (MT321072), Tc-RDL (NP_001107809), and Vd-RDL (KY748050). Phh-β1 (unpublished sequence) was used to root the tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1050 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 21 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 365 positions in the final dataset. Evolutionary analyses were conducted with MEGA7 tool.

**Fig. 4.** Relative expression of *Phh-grd*, *Phh-rdl* and *Phh-lcch3* throughout the development stages: nits (A), L1, L2 and L3 larvae (B) and in head, thorax and abdomen of adults (C) of human body louse. Expression of the transcripts was quantified by RT-qPCR, calculated by the comparative CT method $2^{-\Delta CT}$ and normalized to the expression of actin as endogenous control. The differences between expression of the receptor subunits are not significant (ANOVA test followed by Tukey’s multiple comparison test).

**Fig. 5.** Functional expression of Phh-GRD1/Phh-LCCH3, but not of Phh-GRD2-3/Phh-LCCH3 receptors in *X. laevis* oocytes. (A) Current trace obtained on oocytes injected with cRNA of Phh-GRD1/Phh-LCCH3 in response to GABA in the range from 0.3 to 300 µM. Oocytes were clamped at -60 mV. Application time of 10 s is indicated by the bars. (B) GABA concentration response curve for Phh-GRD1/Phh-LCCH3 (n = 17 oocytes). Data were normalized to the maximal effect obtained by 100 µM GABA. (C) Means +/- SD of representative current traces obtained on oocytes co-injected with Phh-GRD2/Phh-LCCH3 (n = 13) and Phh-GRD3/Phh-LCCH3 (n = 15) in response to 30 to 300 µM GABA. Oocytes were clamped at -60 mV. Application time of 5 s is indicated by the bars.

**Fig. 6.** Functional expression of Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL receptors in *X. laevis* oocytes. Current trace obtained on oocytes injected with cRNA of Phh-GRD1/Phh-RDL (A) and
Phh-LCCH3/Phh-RDL (B) in response to GABA in the range from 0.3 to 300 µM. Oocytes were clamped at -60 mV. Application time of 10 s is indicated by the black bar. (C) Means +/- SD of GABA concentration-response curves for Phh-RDL, Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL (n = 20, n = 9, n = 7 oocytes, respectively). Data were normalized to the maximal effect obtained with 100 µM GABA. (D) Reverse potential ion-concentration relationship (means +/- SD) for sodium ions of Phh-GRD/Phh-LCCH3-expressing oocytes in the presence of 100 µM GABA and decreasing concentrations of Na⁺ (100 to 0 mM).

Fig. 7. Antagonistic effects of fipronil, picrotoxin and ivermectin on GABA receptors expressed in *X. laevis* oocytes. Representative current traces from single oocyte expressing Phh-GRD1/Phh-LCCH3 receptor alone or with co-application of 10 µM picrotoxin, 1 µM fipronil and 1 µM ivermectin (A). Relative current evoked by 100 µM GABA alone (= 100%) or with co-application of 10 µM picrotoxin (PIC, B), 1 µM fipronil (FIP, C) and 1 µM ivermectin (IVE, D) on oocytes expressing Phh-RDL, Phh-GRD1/Phh-LCCH3, Phh-GRD1/Phh-RDL and Phh-LCCH3/Phh-RDL (n = 4 to 7 as indicated on each figure). No significant differences (P > 0.05) were calculated between the results of the different receptors with picrotoxin (PIC, B) or fipronil (FIP, C), while P value = 0.0048 for Phh-RDL vs. Phh-LCCH3/Phh-RDL and for Phh-GRD1/Phh-LCCH3 vs. Phh-GRD1/Phh-RDL and P value = 0.0002 for Phh-GRD1/Phh-LCCH3 vs. Phh-LCCH3/Phh-RDL with 1 µM invermectin (IVE, D) (one-way ANOVA followed by Tukey’s multiple comparisons test).

Fig. S1: Multiple sequence alignments of Phh-GRD1 (OM128123), Phh-GRD2 (OM128124), Phh-GRD3 (OM128125), Phh-RDL (MT321072) and Phh-LCCH3 (OM128126), conducted in clustal omega then viewed and annotated by Jalview software. Transmembrane domains (black frames), C-C loop (arrows), GABA binding loops (A to F, black lines), motifs for ion selectivity (AD, SA and PA at positions -1’, -2’ and F, T, T at position 13’ for Phh-GRD, Phh-LCCH3 and Phh-RDL, respectively (asterisks), mutations F148Y and E206G in GABA binding site of Phh-GRD (highlighted in red), mutations R100D, S163G and F193Y of Phh-LCCH3 (highlighted in green), amino acids conserved in
GABA binding site of all subunits Y 113 and Y 263 (numbering of Phh-GRD) were highlighted in dark violet.

**Fig. S2:** Functional expression of Phh-GRD1/Phh-LCCH3 receptor in *X. laevis* oocytes. Representative current traces from an oocyte injected with cRNA of Phh-GRD1/Phh-LCCH3 challenged with 30 µM GABA, acetylcholine, glutamate, glycine, aspartate, histamine and serotonin.
| Primers | Sequences (5'-3') |
|---------|------------------|
| *Phh-grd* 5' RACE first PCR | CGAAATCGGGCCCATGCTTC |
| *Phh-grd* 5' RACE nested PCR | CGGGCCATGCTTCGTACC |
| *Phh-grd* 3' RACE first PCR | GACTTTATCAAGATGGACGAGTTC |
| *Phh-grd* 3' RACE nested PCR | GGACGAGTTTTATTCCAGC |
| *Phh-grd* full forward | ATTTTCCGTGTTGGAATTTTTTTTC |
| *Phh-grd* full Xho1 forward<sup>a</sup> | CTGGCGGCCCGCTCGAGATGGCGTCGATGTTCGGA |
| *Phh-grd* full reverse | GAATACATAGTAAAAATAAATAAAT |
| *Phh-grd* full Apa1 reverse<sup>a</sup> | GAATACATAGTAAAAATAAATAAATGGGCCCGAGCTTGATCTGGT |
| *Phh-lcch3* 5' RACE first PCR | CGTTGTCGTGAAACGCCATACC |
| *Phh-lcch3* 5' RACE nested PCR | TTTCAAGACAAAATCACCGCC |
| *Phh-lcch3* full Xho1 forward<sup>a</sup> | CTGGCGGCCCGCTCGAGGGAATGATGATGCAATGCAGC |
| *Phh-lcch3* full Apa1 reverse<sup>a</sup> | GCAATGAGTAGTAAATATTTATACGGGCCCGAGCTTGATCTGGT |

<sup>a</sup> Primers Xho1 and Apa1 were designed with extensions (in bold) for recombination with the expression vector PTB207 linearized by Xho1 and Apa1.
TABLE 2

Primers designed for qPCR for *Phh-grd, Phh-lcch3, Phh-rdl* and *β-actin*

| Primers   | Sequences (5’-3’)       | Amplicon sizes (bp) |
|-----------|-------------------------|---------------------|
| *Phh-grd* forward | GGTGGGAAGCAAGAAGGAC    | 155                 |
| *Phh-grd* reverse  | CCGAAATAACATTCAACCGAACC |                     |
| *Phh-lcch3* forward | GGGTATAACCACGGTACAAAC | 171                 |
| *Phh-lcch3* reverse | CTTGCTCCCCAATGTATAG   |                     |
| *Phh-rdl* forward  | GCCAAAAAGTAGATTTATGGCG | 174                 |
| *Phh-rdl* reverse  | GTACCTCCTTTGGAATGAGC  |                     |
| *β-actin* forward  | TGCCACATGCTATTCTCCGT   | 60                  |
| *β-actin* reverse  | CGGCAGTGGTATGGAATGAA  |                     |
Figure 1
Figure 2
Figure 3
Figure 4

(A) Nits

Expression relative to actin

Actin  GRD  RDL  LCCH3

(B) Larvae

Expression relative to actin

L1  L2  L3

(C) Adults

Expression relative to actin

Head  Thorax  Abdomen

Actin  GRD  RDL  LCCH3
Figure 5
Figure 6

A
Phh-GRD1/Phh-RDL

B
Phh-LCCH3/Phh-RDL

C

D

Reverse Potential (mV)

Log [Na⁺] (mM)
Figure 7