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Nicotine-sensitive acetylcholine receptors are relevant pharmacological targets for the control of multidrug resistant parasitic nematodes

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A B S T R A C T

The control of parasitic nematodes impacting animal health relies on the use of broad spectrum anthelmintics. However, intensive use of these drugs has led to the selection of resistant parasites in livestock industry. In that respect, there is currently an urgent need for novel compounds able to control resistant parasites. Nicotine has also historically been used as a de-wormer but was removed from the market when modern anthelmintics became available. The pharmacological target of nicotine has been identified in nematodes as acetylcholine-gated ion channels. Nicotinic-sensitive acetylcholine receptors (N-AChRs) therefore represent validated pharmacological targets that remain largely under-exploited. In the present study, using an automated larval migration assay (ALMA), we report that nicotinic derivatives efficiently paralyzed a multiple (benzimidazoles/levamisole/pyrantel/ivermectin) resistant field isolate of *H. contortus*. Using *C. elegans* as a model we confirmed that N-AChRs are preferential targets for nornicotine and anabasine. Functional expression of the homomeric N-AChR from *C. elegans* and the distantly related horse parasite *Parascaris equorum* in *Xenopus* oocytes highlighted some striking differences in their respective pharmacological properties towards nicotine derivative sensitivity. This work validates the exploitation of the nicotine receptors of parasitic nematodes as targets for the development of resistance-breaking compounds.

1. Introduction

The control of gastro-intestinal nematodes of veterinary importance is mainly based on the use of broad spectrum anthelmintics such as levamisole, benzimidazoles and avermectins. Multiple resistant isolates could therefore represent a major threat for animal health as well as for production sustainability. For example, the haematophagous parasite *Haemonchus contortus* (barber pole worm), that is one of the most prevalent and pathogenic trichostrongylid species affecting small ruminants worldwide, has developed multiresistance against these three main classes of anthelmintics, thus stressing the need for the development of novel resistance-breaking drugs (Van Wyk et al., 1999; Mortensen et al., 2003; Kaplan, 2004; Peter and Chandrawathani, 2005). In this respect, nicotine-sensitive acetylcholine receptors of parasitic nematodes appear to be pharmacological targets of prime interest. Acetylcholine is a major excitatory neurotransmitter in both vertebrates and invertebrates. Acetylcholine receptors are members of the cys-loop ligand-gated ion channel superfamily and consist of five subunits arranged around a central pore (Unwin, 2005). Each subunit possesses an N-terminal extracellular domain containing a dicysteine loop followed by four transmembrane regions (TM1–TM4) of which TM2 lines the ion channel. In nematodes, the muscular acetylcholine receptors fall into two pharmacological classes that are preferentially activated by the cholinergic agonist levamisole (L-type) or nicotine (N-type) respectively. These cholinergic agonists induce a prolonged activation of muscular AChR causing spastic paralysis of the worms, which are either killed as with the free-living nematode *Caenorhabditis elegans* or expelled from the host organism in the case of *H. contortus*. (Aceves et al., 1970; Aubry et al., 1970; Harrow and Gration, 1985).

The molecular composition of L-AChR and N-AChR was first deciphered in the model nematode *Caenorhabditis elegans*. The main *C. elegans* L-AChR is a heteromeric receptor composed of five subunits encoded by the *unc-38*, *unc-63*, *lev-8*, *unc-29* and *lev-1* genes respectively (Lewis et al., 1980, 1987; Fleming et al., 1997; Culetto et al., 2004; Towers et al., 2005). Co-expression of these five distinct L-AChR subunits together with three additional *C. elegans* ancillary proteins led to the robust expression of a functional *C. elegans* L-AChR in *Xenopus laevis* oocytes (Boulin et al., 2008). The recombinant *C. elegans* L-AChR was found to be sensitive to levamisole (Lev) but insensitive to nicotine (Nic). The *C. elegans* N-AChR is a homomeric receptor composed of five identical subunits encoded by the *acr-16* gene and requires only the RIC-3 ancillary protein to enhance its functional expression in *Xenopus* oocytes (Ballivet et al., 1996; Halevi et al.,. 2003). In contrast with the L-AChR subtype, the recombinant *C. elegans* N-AChR was found to be very responsive to Nic whereas Lev did not induce any response. Similarly, the recombinant N-AChR made of the ACR-16 subunits from the pig parasitic nematode *Ascaris suum* presented the same differential response between Lev and Nic as observed for its *C. elegans* counterpart

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(Abongwa et al., 2016).

Whereas the L-AChRs are targets for several anti-parasitic drugs (for review Wolstenholme and Neveu, 2017) there is currently no anthelmintic on the market targeting the N-AChRs. However, several decades ago, nicotine has been used as an anthelminthic in livestock (Waller et al., 2001; McKellar and Jackson, 2004), therefore validating nicotine-sensitive AChR from nematode as potent anthelmintic targets.

In the present study, using an automated larval migration assay (ALMA), we provide evidence that nicotine and nicotine derivatives targeting AChR are able to paralyze a multiple drug-resistant isolate of *H. contortus*. In addition, using recombinant N-AChRs expressed in *Xenopus* oocytes, we deciphered their mode of action. Our results suggest that compounds targeting the N-AChR are potentially able to control levamisole, pyrantel and ivermectin-resistant parasites and need to be further explored.

2. Material and methods

2.1. Ethics statement

All animal care and experimental procedures were conducted in strict accordance with the European guidelines for the care and use of laboratory animals and were approved by the ethical committee from Indre et Loire under experimental agreement 6623 provided by the French Veterinary Services.

2.2. Nematodes

Haemonchus contortus L3 larvae from the Weybridge and Kokstad isolates were obtained as previously described (Delannoy-Normand et al., 2010). In the present study, the anthelmintic susceptible Weybridge isolate (UK) was used as a reference (Roos et al., 1990). Kokstad is a field isolate from South Africa, which is resistant to benzimidazoles, ivermectin and levamisole (Neveu et al., 2007; de Lourdes Mottier, M. & Prichard, R. K. 2008; Ménez et al., 2016). However, for Kokstad isolate, pyrantel resistance status remained to be determined. In that respect, for the present study, sheep were infected with 10 000 Kokstad L3 larvae and were subsequently treated with a full dose of Levamisole (7.5 mg/kg of bodyweight) at 14 days post infection (dpi) and a full dose of pyrantel (20 mg/kg of bodyweight) at 19 dpi and finally with a full dose of ivermectin (0,2 mg/kg of bodyweight) at 24 dpi. Host faeces were collected 35 days post infection and positive fecal egg counting after treatments confirmed the Lev/Pyr/Ivm multi-resistant status of the Kokstad isolate. L3 larvae corresponding to the multiple resistant adult's progeny were harvested from coprocultures and used for automated larval migration assays. Benzimidazole susceptibility or resistance status of the Weybridge and Kokstad isolate was investigated by performing egg hatch assays as described by Coles et al. (1992) using thiabendazole (TBZ). The test performed in triplicate confirmed the Weybridge isolate TBZ-susceptibility (ED_{50}: 0.024 ± 0.001 µg/ml) and the Kokstad isolate TBZ-resistance (ED_{50}: 0.437 ± 0.205 µg/ml).

Adult *Parascaris equorum* were obtained as described in Courtot et al., (2015). *Caenorhabditis elegans* experiments were carried out on the Bristol N2; *acr-16 (ok789)* and *lev-8(ok1519)* strains obtained from the *Caenorhabditis* Genetics Center (CGC).

2.3. Automated larval migration assay

The automated larval migration assay (ALMA) used for the present study was adapted from the technology previously designed for *H. contortus* L2 motility monitoring (Blanchard et al., 2018) with minor modifications. Using a Quanta Master spectrofluorometer (Horiba PTI, NJ, USA), larval motility was estimated by measuring *H. contortus* L3 auto-fluorescence resulting from ultraviolet excitation. . Motility assays were performed using 7500 *H. contortus* L3 larvae. Worms were transferred into a 5 mL glass tube and left for 15 min to concentrate by gravity. The supernatant was removed and replaced by 2 ml of tap water or anthelmintic solution. After 20 min, the tube was inverted on a 20 µm sieve. After a stabilization time of 60 s, the fluorescence accumulation (correlated to the number of larvae migrating through the sieve) was measured during 5 min . Each set of experiment was performed in triplicate.

2.4. cDNA synthesis

Total RNA was prepared from the distinct nematode species using 50 µL of pelleted L3 larvae of *H. contortus* or 50 adults *C. elegans* or cross-section (5 mm thick) from the mid body region of an individual adult worm of *P. equorum*. Frozen samples were ground in liquid nitrogen and homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was isolated according to the manufacturer's recommendations. RNA pellets were dissolved in 25 µL of RNA secure resuspension solution (Ambion, Austin, TX, USA) and DNase-treated using the TURBO DNA-free kit (Ambion). RNA concentrations were measured using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand cDNA synthesis was performed on 1 µg of total RNA using the superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations.

2.5. Cloning of complete coding cDNA sequences of acr-16 from H. contortus and P. equorum

To identify the cDNA sequences from *acr-16* homologs in *H. contortus* and *P. equorum*, nested polymerase chain reactions (PCR) were performed on respective first-strand cDNA templates with the Phusion High fidelity Polymerase (New England Biolabs) and PCR products were cloned into the transcription vector pTB207 (Boulin et al., 2008) using the In-Fusion®HD cloning kit (Clontech). For *H. contortus*, the following primers were designed based on the *Hco-acr*-16 mRNA sequence available in Genbank (accession number EU051823): Hc-ACR16-F-Xho1 (ATGTGGAGCTTGCTGATCGC) and Hc-ACR16-R-Apa1 (CTAGGCGACCAGATATGGAG). For *P. equorum*, a blast search (Altschul et al., 1997) with Asu-ACR-16 as a query against the partial *P. equorum* genomic sequence database (available at https://www.sanger.ac.uk/resources/downloads/helminths/) retrieved contig NODE_2631440_length_27442_2.804278 as the best hit containing an incomplete sequence for the *acr-16* gene. Then, specific primers were designed to amplify the coding sequence of *Peq-acr-16* (FuPeq16ptbamF TCGTGT AATTGACGCTGCGTCT, FuPeq16ptbapaR CTATGCTATCGTGTAAGGC GCA, Peq-acr-16-F0 TTCAGAGTGATAACGCATAACGG, Peq-acr-16-Z1R GCAAATACGTTAGTGTAAGTATGG). The novel complete coding sequences of *acr-16* were named *Hco-acr-16* for *H. contortus* and *Peq-acr-16* for *P. equorum* according to Beech et al. recommendations (Beech et al., 2010) and were deposited to GenBank under the accession numbers MH806893 and MH806894, respectively.

2.6. Sequence analysis

Deduced amino-acid sequences were aligned using MUSCLE (Edgar, 2004). Signal peptide predictions were carried out using the SignalP 3.0 server (Bendtsen et al., 2004) and membrane-spanning regions were predicted using the SMART server (Schultz et al., 1998). Phylogenetic analysis was performed on deduced amino-acid sequence. Sequence from the signal peptide, the intracellular loop (between TM3 and TM4) and C-terminal tail were removed as they could not be aligned unambiguously. Maximal likelihood phylogeny reconstruction was performed using PhyML V20120412 (https://github.com/stephaneguindon/phyml-downloads/releases) and significance of internal tree branches was estimated using bootstrap resampling of the dataset 100 times. The accession numbers sequences used for the analysis are:

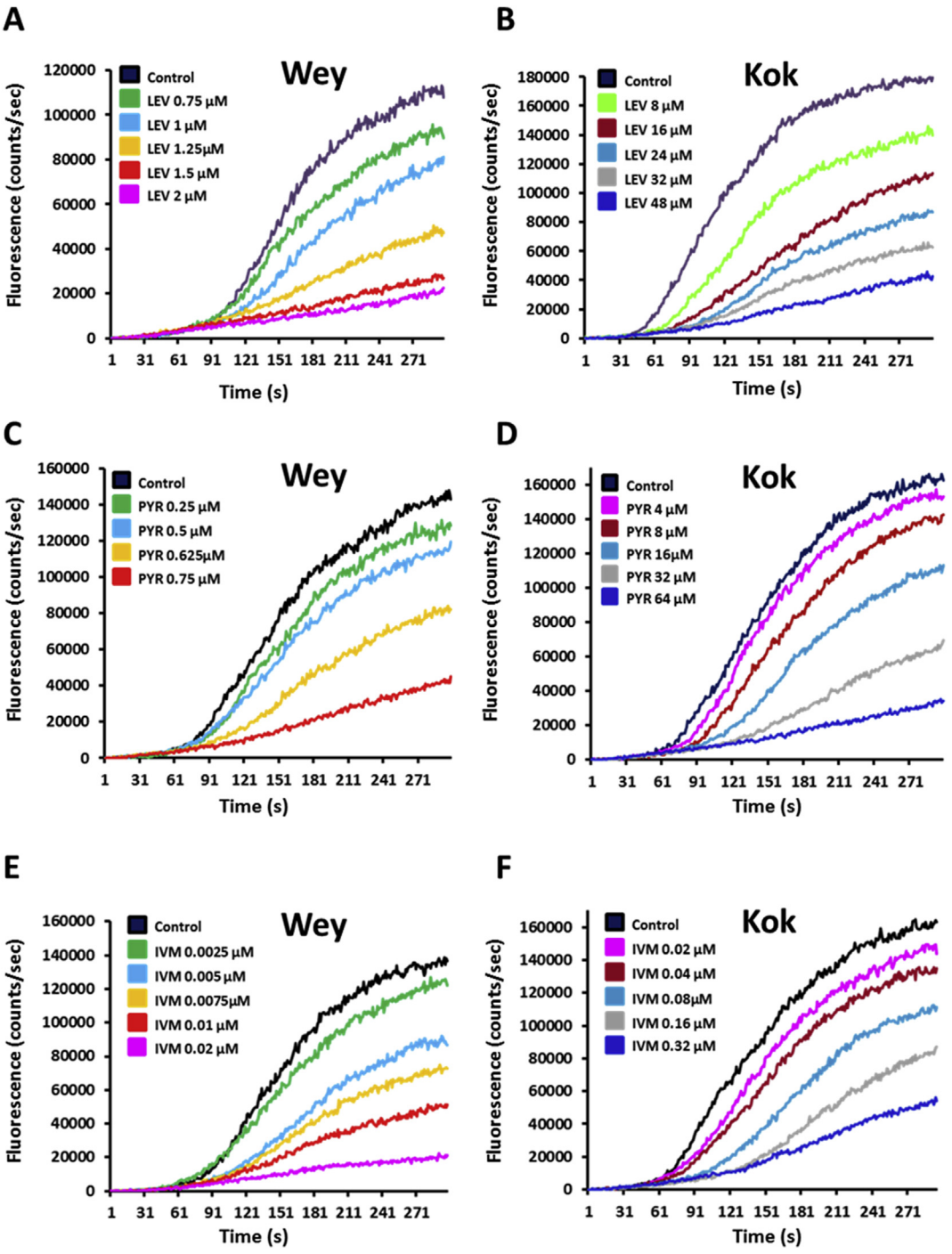

Fig. 1. Motility modulation of H. contortus L3 larvae exposed to levamisole, pyrantel or ivermectin. The automated larval migration assay (ALMA) was used to determine dose-dependent paralysis effect of Lev, Pyr or Ivm on the *H. contortus* L3 from Weybridge (A; C and E) or Kokstad isolate (B; D and F). Representative recording traces of the real-time fluorescence counting relative to the L3 migration during 5 min exposed to Lev (A and B); Pyr (C and D) or Ivm (E and F). Each trace corresponds to the mean data from 3 runs performed with 7500 L3 larvae. The controls correspond to untreated L3 larvae.

***Caenorhabditis elegans*:** ACR-5 NP_498437; ACR-6 NP_491354; ACR-7 NP_495647; ACR-8 NP_509745; ACR-9 NP_510285; ACR-10 NP_508692; ACR-11 NP_491906; ACR-12 NP_510262; ACR-13 (=LEV-8) NP_509932; ACR-14 NP_495716; ACR-15 NP_505206; ACR-16 NP_505207; ACR-17 NP_001023961; ACR-18 NP_506868; ACR-19 NP_001129756; ACR-20 NP_001122627; ACR-23 NP_504024; ACR-24 NP_001255866; DEG-3 NP_505897; DES-2 NP_001256320; EAT-2 NP_496959; LEV-1 NP_001255705;; UNC-29 NP_492399; UNC-38 NP_491472; UNC-63 NP_491533. ***Haemonchus contortus*:** Hco-ACR-16 MH806893; ***Parascaris equorum*:** Peq-ACR-16 MH806894.

2.6.1. Caenorhabditis elegans experiments

Worms were maintained at 20 °C on nematode growth medium (NGM) plates and fed on a bacterial lawn (*Escherichia coli* OP50). Paralysis assays were performed on gravid adults as previously described (Gottschalk et al., 2005).

2.7. Electrophysiology experiments

The pTB207 containing either the *C. elegans, H. contortus and P. equorum acr-16* cDNAs were linearized with the *Nhe*I restriction enzyme

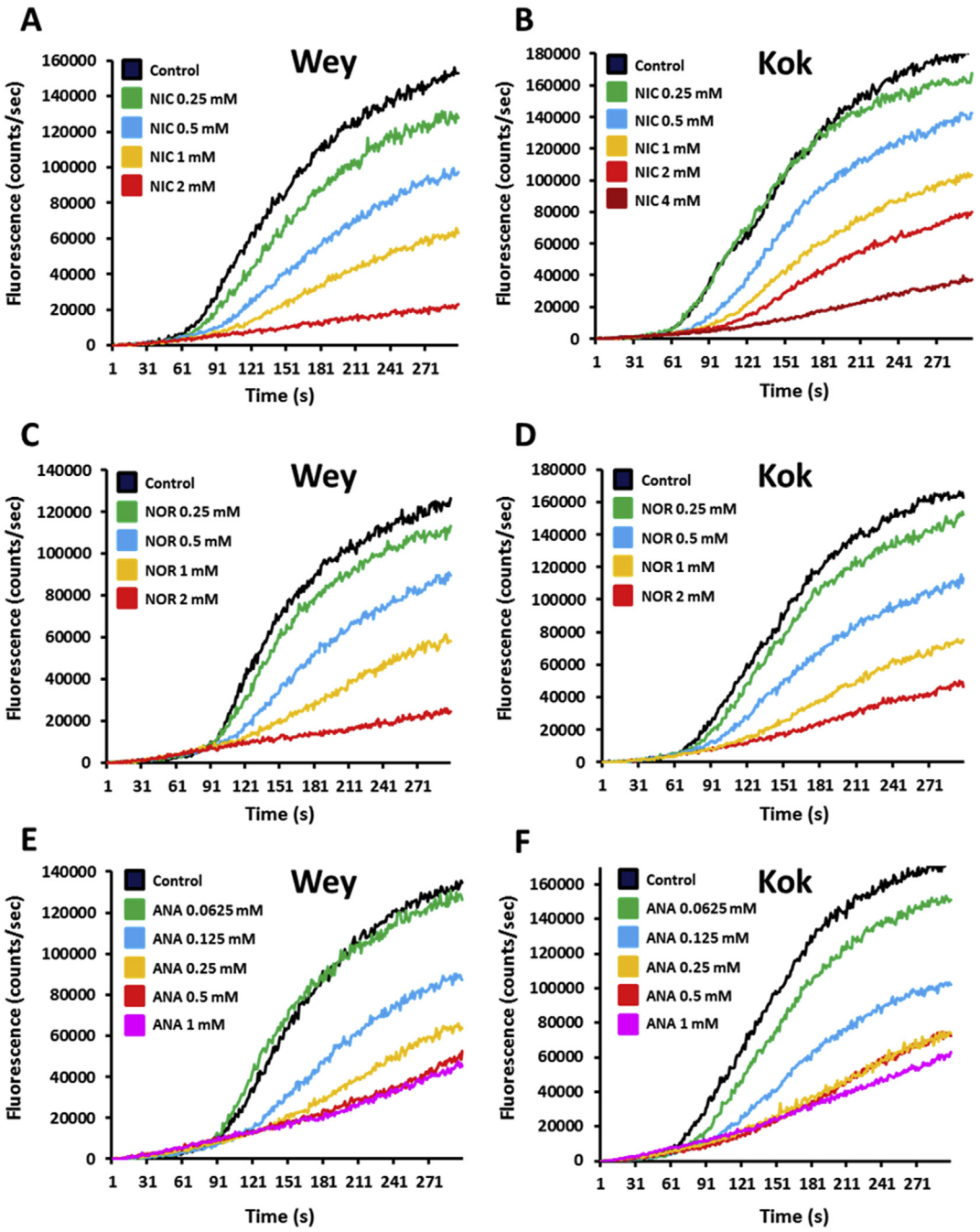

Fig. 2. Motility modulation of H. contortus L3 larvae exposed to nicotine, nornicotine or anabasine. The automated larval migration assay (ALMA) was used to determine dose-dependent paralysis effect of Nic, Nor or Ana on the *H. contortus* L3 from Weybridge (A; C and E) or Kokstad isolate (B; D and F). Representative recording traces of the real-time fluorescence counting relative to the L3 migration during 5 min exposed to Nic (A and B); Nor (C and D) or Ana (E and F). Each trace corresponds to the mean data from 3 runs performed with 7500 L3 larvae. The controls correspond to untreated L3 larvae.

(Thermofisher) and used as templates for cRNA synthesis using the T7 mMessage mMachine kit (Ambion). In parallel, cRNAs for the ancillary proteins Hco-RIC-3, Hco-UNC-50 and Hco-UNC-74 were also synthesized and mixed with the respective *acr*-16 cRNAs. *Xenopus laevis* defolliculated oocytes were obtained from Ecocyte Bioscience (Germany). The oocytes were injected in the animal pole with a total volume of 36 nL of cRNA mix containing 50 ng/μL of each cRNA in RNase-free water using the Drummond nanoject II microinjector. Microinjected oocytes were incubated at 20 °C for 48H before recording. Two-electrode voltage-clamp recordings were carried out using an Oocyte Clamp OC-725C amplifier (Warner instrument) on oocytes being voltage-clamped at −60 mV. The electrophysiology experiments were performed on BAPTA-free oocytes as the previous study investigating the ACR-16 N-AChR from *Ascaris suum* showed a calcium permeability (P_{Ca}/P_{Na}) of 0.4, indicating that the calcium ion is not the major ion going through the ACR-16 channel (Abongwa et al., 2016). In accordance, we found no statistical differences in the EC_{50} values for ACh- and Nic-elicited currents resulting from BAPTA-AM-free and BAPTA-AM-treated oocytes, thus downplaying a putative confounding effect of calcium-activated chloride channels on whole-cell current responses. Acetylcholine and nicotine were dissolved in recording buffer (100 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$.2H2O, 5 mM HEPES, pH 7.3). Nornicotine and anabasine were prepared first in DMSO and diluted subsequently in recording buffer so that DMSO final concentration was less

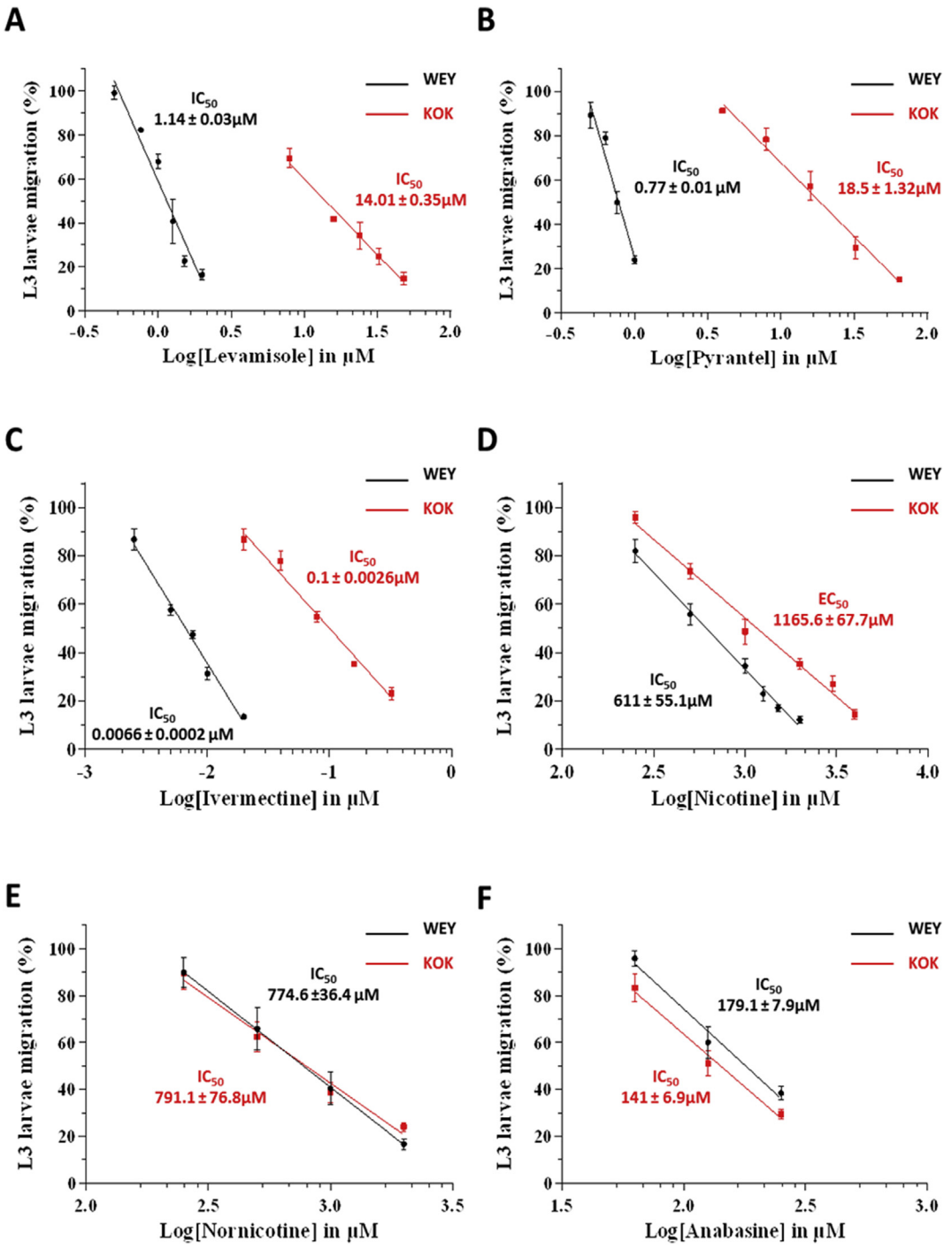

Fig. 3. Determination of the dose response relationships for levamisole (A); pyrantel (B); ivermectin (C); nicotine (D); nornicotine (E) and anabasine (F) on *H. contortus* L3 larvae using the automated larval migration assay (ALMA). Results are shown as the mean ± se from 3 distinct ALMA assays performed with 7500 *H. contortus* L3 larvae from the Weybridge isolate (in black) or Kokstad isolate (in red). IC_{50} values are indicated on the graphs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

than 0.1%. Currents were recorded and analyzed using the pCLAMP 10.4 package (Molecular Devices). EC_{50} values were determined using non-linear regression on normalized data (1 mM ACh as maximal response) using GraphPad Prism® software.

3. Materials

Acetylcholine chloride (ACh), ivermectin; (−)-tetramisole hydrochloride (levamisole), (−)-nicotine hydrogen tartrate, pyrantel citrate, (±)-nornicotine, anabasine were purchased from Sigma-Aldrich.

4. Results

4.1. Automated larval migration assay (ALMA) confirms the multidrug resistant status of the H. contortus Kokstad isolate

We recently reported the development of the ALMA technology (Automated Larval Migration Assay), a spectrofluorometric-based approach to quantify the migration rate of the *H. contortus* L2 larvae (Blanchard et al., 2018). Here, we adapted the ALMA to the L3 stage in order to use it for testing the *in vitro* anthelmintic activity of several

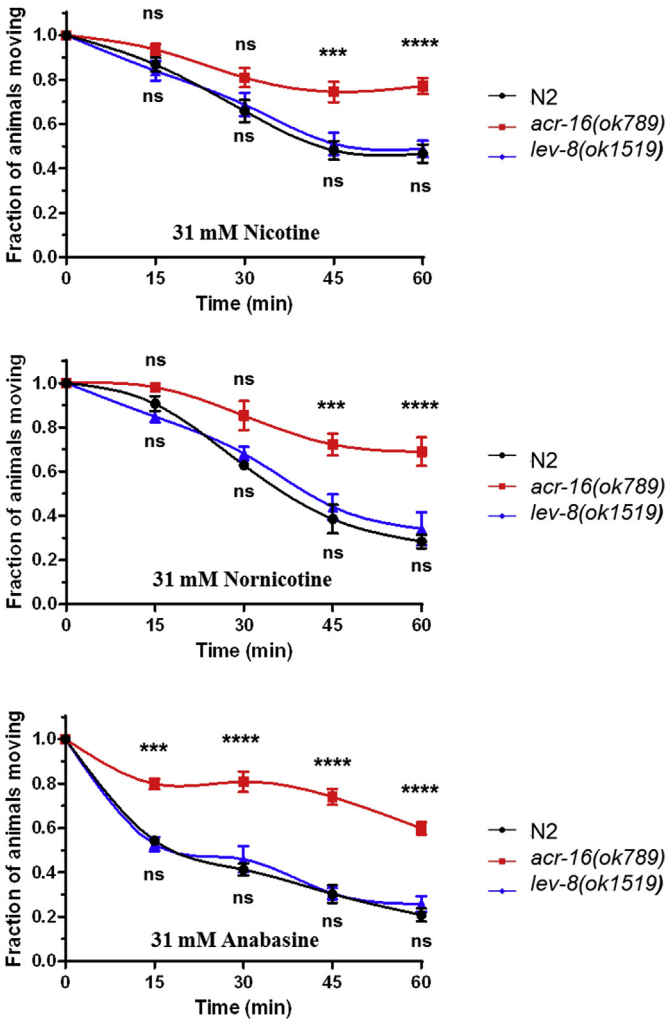

Fig. 4. Effects of nicotine, nornicotine or anabasine on the motility of C. *elegans*. Paralysis assays were performed on N2, acr-16 (ok789) and lev-8 (ok1519) *C. elegans* strains in agar plate containing 31 mM of nicotine (A), nornicotine (B) or anabasine (C) after 15, 30, 45 and 60 min drug exposure. Paralysis was scored based on the absence of worm's movement in response to prodding. Data are the mean ± SEM of n = 12, ****p < 0.0001, ***p < 0.001, **p < 0.01 and *p < 0.05, one way ANOVA with Bonferroni post-hoc test between N2 and the mutant strains.

drug standards. Interestingly, as previously reported for L2 larvae, we found a highly significant relationship between the fluorescence measured and the accumulation of L3 larvae that migrated into the recording chamber ($R^2 = 0.9977$) after 5 min (S1 Fig.). Therefore, we were able to measure the L3 migration and the *in vitro* effect of anthelmintics by quantifying the increase in fluorescence against time in the absence or presence of drug.

In order to determine their respective Lev, Pyr and Ivm susceptibility, ALMA assays were performed on *H. contortus* L3 from the anthelmintic-susceptible Weybridge (Wey) and the Lev/Pyr/Ivm multi-resistant Kokstad (Kok) isolates (Figs. 1–3; S2 Fig.). In the absence of drug application, a similar pattern of migration kinetic was observed between both isolates. In contrast, dose-response assays performed with Lev, Pyr and Ivm revealed a drastic reduction of drugs efficacy on the Kok L3 in comparison with Wey worms (Fig. 1; Fig. 3; S2 Fig.). The IC_{50} values of Lev, Pyr and Ivm were 1.14 ± 0.03 µM, 0.77 ± 0.01 µM and 6.6 ± 0.2 nM for Wey *versus* 14.01 ± 0.35 µM, 18.5 ± 1.32 µM and 100 ± 2.6 nM for Kok, respectively. Based on the respective IC_{50} values, the calculated resistance factors (Kelly and Hall, 1979) between Kok and Wey were 12.3, 23.9 and 15.1 confirming the Lev, Pyr and Ivm resistance status of the Kok isolate and the drug susceptibility of the Wey isolate as previously determined *in vivo* (see Material and methods section).

4.2. Nicotine and nicotinic derivatives paralyze both lev-susceptible and lev-resistant isolates

The ALMA assay was then used to compare the effect of a set of nicotinic compounds including, nicotine (Nic), nornicotine (Nor) and anabasine (Ana) on *H. contortus* L3 from the Wey and Kok isolates (Fig. 2; Fig. 3; S2 Fig.). The application of Nic, Nor and Ana led to migration reductions of both Wey and Kok L3. Surprisingly, whereas Kok and Wey L3 presented a similar response to Nor (IC_{50} Wey Nor (774.6 ± 36.4 µM), IC_{50} Kok Nor (791.1 ± 76.8 µM)); Kok worms were less susceptible to Nic (IC_{50} Wey Nic (611 ± 55.1 µM), IC_{50} Kok Nic (1165.6 ± 67.7 µM)) but more responsive to Ana than their Wey counterparts (IC_{50} Wey Ana (179.1 ± 7.9 µM), IC_{50} Kok Ana (141 ± 6.9 µM)). These results confirmed the anthelmintic activity of the three drugs and provided a first evidence that nicotinic compounds are efficient on the Lev/Pyr/Ivm resistant worms.

4.3. N-AChRs are relevant drug targets for the control of levamisole and pyrantel resistant worms

In order to get first insights about the mode of action of nicotine and nicotinic-derivatives on Lev/Pyr resistant worms, we used the free living nematode *Caenorhabditis elegans* as a model. In addition to the wild type strain Bristol N2, two *C. elegans* mutant strains lacking respectively the L-AChR or N-AChR subtype (i.e. *lev-8(oK1519)*, or *acr-16(oK789)* respectively) were used in the presents study. Note that *lev-8* null mutants were chosen among other L-AChR subunit invalidated mutant, as these worms are not impaired in their locomotion and are Lev and Pyr-resistant, thus mirroring the phenotype of *H. contortus* Kokstad L3 larvae (Hernando et al., 2012; Blanchard et al., 2018).

Paralysis assays were performed as described by Gottschalk et al., (2005) on agar plate containing 31 mM Nic, Nor or Ana (Fig. 4). Whereas N2 worms and *lev-8* mutant motilities were affected by Nic, Nor and Ana, the *acr-16* mutant lacking the N-AChR subtype was significantly less sensitive to the drugs. Taken together, these results support the hypothesis that the nematode N-AChR subtype including the ACR-16 subunit contributes to the anthelmintic effect of Nic, Nor and Ana.

4.4. Nicotinic derivatives activate recombinant homomeric N-AChRs expressed in Xenopus oocytes

It has been previously reported that the ACR-16 AChR subunit from *C. elegans* and the distantly related pig parasite *Ascaris suum* are able to form homomeric functional N-AChRs when expressed in *Xenopus* oocytes with the RIC-3 ancillary protein (Boulin et al., 2008; Abongwa et al., 2016).

In order to further investigate the mode of action of nicotinic derivatives on nematode N-AChR, full-length cDNA sequences corresponding to *acr-16* were obtained from *C. elegans*, *H. contortus* and the horse parasite *Parascaris equorum*. An alignment of the ACR-16 subunit sequences from the three nematode species is presented in Fig. 5. All sequences shared features of an AChR subunit including a predicted signal peptide, a “cys-loop”, four transmembrane domains and the vicinal dicysteines characteristics of alpha subunits. Protein sequences were highly conserved between the Clade V and Clade III species with identities for the mature proteins, excluding the signal peptide sequence, ranging from 76% to 89%. The orthologous relationship between the *C. elegans* ACR-16 subunit with its counterparts from parasitic species was confirmed by a phylogenetic analysis (S3 Fig.). *Hco-acr-16* and *Peq-acr-16* sequences have been deposited in Genbank with accession numbers MH806893 and MH806894 respectively.

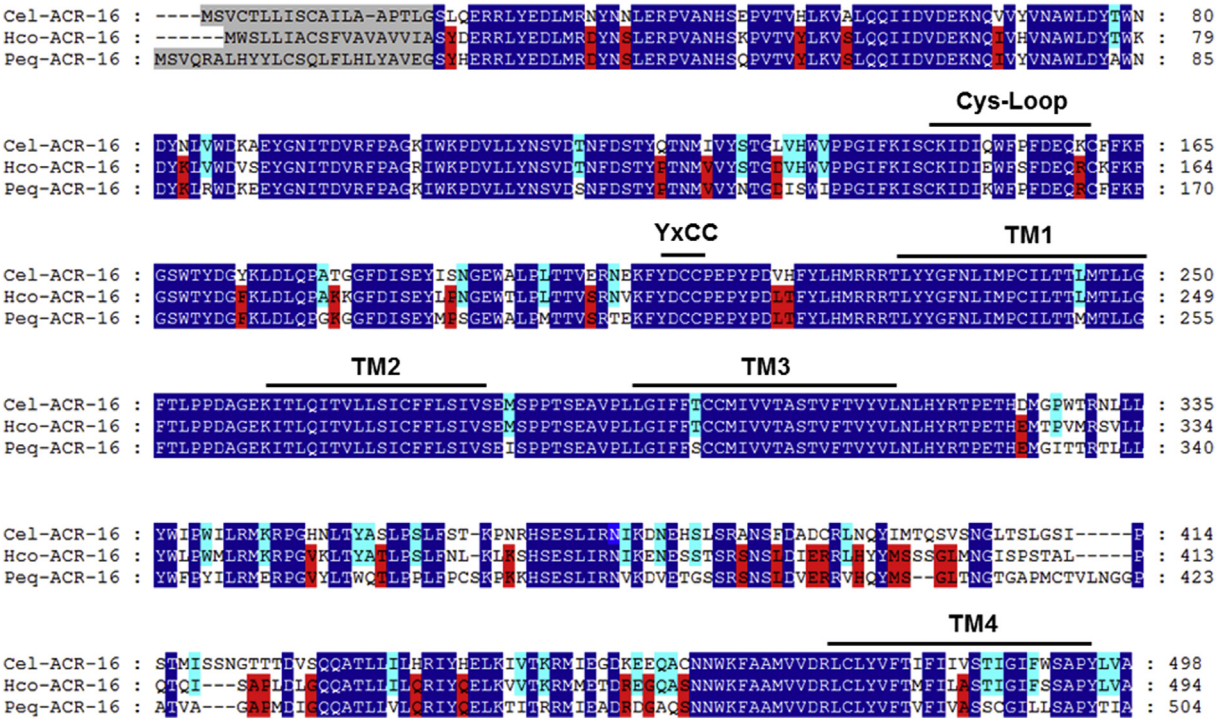

Fig. 5. Amino-acid alignments of ACR-16 subunit sequences from *Caenorhabditis elegans, Haemonchus contortus* and *Parascaris equorum*. acr-16 deduced amino-acid sequences were aligned using the MUSCLE algorithm (Edgar, 2004) and further processed using GeneDoc. Predicted signal peptide sequences are shaded in grey. Amino acids conserved between all the ACR-16 sequences are highlighted in dark blue. Amino acids specifically shared by ACR-16 homologs from parasitic species are highlighted in red. Amino acids specifically shared by Clade V nematode species (*C. elegans* and *H. contortus*) are highlighted in light blue. The cys-loop, the four transmembrane regions (TM1–TM4) and the primary agonist binding (YxCC) are indicated above the sequences. Cel (*Caenorhabditis elegans*), Hco (*Haemonchus contortus*), Peq (Parascaris equorum). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The cRNAs encoding ACR-16 from *C. elegans, H. contortus* or *P. equorum* were micro-injected in combination with cRNAs encoding the *C. elegans* RIC-3 ancillary protein in *Xenopus* oocytes. Two days after injection, we recorded robust currents in the μA range following the perfusion of 100 μM acetylcholine (ACh) in oocytes expressing ACR-16 from *C. elegans* and *P. equorum* demonstrating that the subunits assembled into functional AChRs. As previously reported for *C. elegans* and *A. suum* (Boulin et al., 2008; Abongwa et al., 2016), the *P. equorum* receptor made of ACR-16 displayed rapid and large activating inward currents with fast-desensitization kinetics which is a hallmark of the N-AChRs (Fig. 6). Nevertheless, Hco-ACR-16 failed to produce a functional receptor (n = 30), (S4 Fig.). Note that neither extending the expression time nor replacing RIC-3 from *C. elegans* by the RIC-3.1 and RIC-3.2 from *H. contortus* in the cRNA mix led to a functional N-AChR made of Hco-ACR-16 subunit (n = 16).

Next, we obtained the ACh concentration-response curve for Cel and Peq-N-AChR with maximal current amplitude elicited by 1 mM ACh (Fig. 7). The EC_{50} value of ACh was 6.4 ± 1.1 μM (n = 6) for Peq-N-AChR (Table 1) which was markedly more sensitive to ACh than the Cel-N-AChR (21.4 ± 1.1 μM) as well as the *P. equorum* morantel heteromeric receptor (34.9 ± 1.1 μM) made of the ACR-26 and ACR-27 subunits (Courtot et al., 2015). The pharmacological profiles of Cel-N-AChR and Peq-N-AChR were then established with Nic, Nor and Ana (Figs. 6 and 7). As expected, both receptors were highly responsive to 100 μM Nic with 79.5 ± 7.5% of ACh response (n = 16) for Cel-N-AChR and 96.6 ± 9.2% of ACh response (n = 8) for Peq-N-AChR. In addition, the perfusion of 100 μM Nor and Ana resulted in very similar currents as for Nic (Fig. 4). Interestingly, Nic and Ana were more potent than ACh in activating the *P. equorum* N-AChR as revealed by their respective EC_{50} values (2.9 ± 0.5 μM and 1.7 ± 0.1 μM, respectively), unlike Nor (34.9 ± 7.2 μM). The *C. elegans* N-AChR was more sensitive to Nic than ACh (15.1 ± 1.3 μM *versus* 22.0 ± 1.2 μM, respectively), showed a similar EC_{50} for Ana (27.5 ± 0.9 μM) and was less responsive to Nor (67.7 ± 6.6 μM). The Hill coefficient for the two N-AChRs ranged from 1.7 ± 0.2 (Nic, n = 8) to 2.7 ± 0.4 (Nor, n = 7) suggesting that more than one molecule must occupy the receptor to open the channel (Table 1). As a control, the nicotinic derivatives were also applied to the levamisole-sensitive receptors of *C. elegans* (Boulin et al., 2008) and *H. contortus* (Boulin et al., 2011). When perfused, Nic, Nor and Ana failed to induce significant response on oocytes expressing Cel-L-AChR and Hco-L-AChR-1 (S5 Fig.). Similarly, Nor and Ana did elicit very small currents on few oocytes while Hco-L-AChR-2 responded robustly to Nic (S5 Fig.). Altogether these results highlight some striking differences in the respective pharmacological properties of the *C. elegans* and *P. equorum* recombinant N-AChRs.

5. Discussion

In the present work, using the ALMA assay we first confirmed the multiresistance status of the *H. contortus* Kokstad isolate and demonstrate that nicotine and some nicotinic derivatives can efficiently paralyze the Lev/Pyr/Ivm-resistant worms. The ALMA technology has been originally designed to quantify subtle motility modification of *H. contortus* L2 larvae associated with gene silencing (Blanchard et al., 2018). Here we show that ALMA is also suitable to monitor *H. contortus* L3 motility allowing the determination of IC_{50} values for cholinergic agonists but also macrocyclic lactones. This result open the way for the systematic determination of resistance status in other *H. contortus* isolates and lays the basis for a novel drug screening approach for the identification of resistance breaking drugs.

Because different cholinergic agonists are selective for different nematode AChR subtypes, the cholinergic receptor diversity could be potentially exploited for the development of novel anthelmintic able to control resistant parasites (Martin et al., 2012; Beech and Neveu,

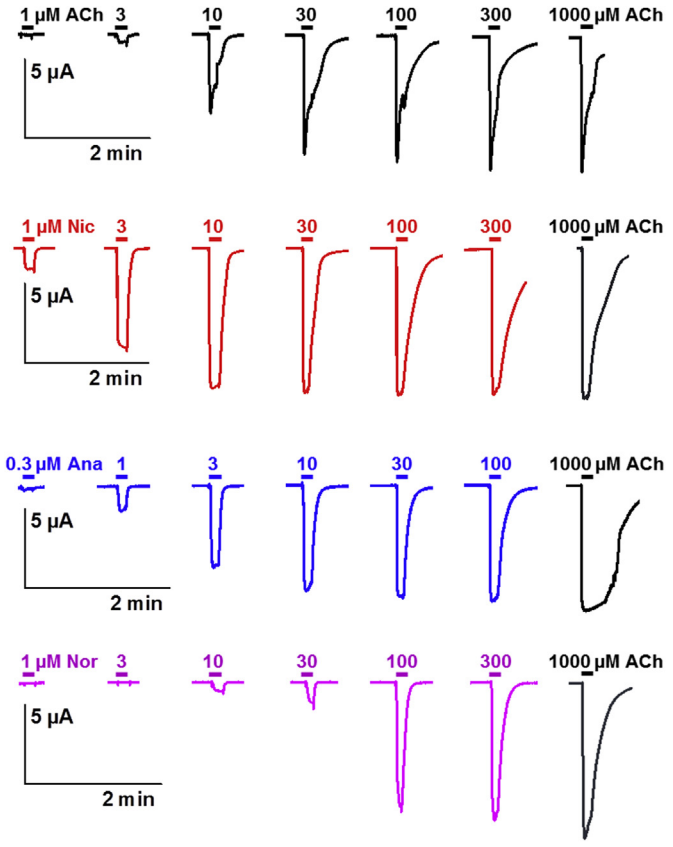

Fig. 6. Concentration-response relationships of acetylcholine and nicotine derivatives on the *P. equorum* N-AChR expressed in *Xenopus* oocytes. Representative current traces for single oocytes perfused with acetylcholine (ACh), nicotine (Nic), anabasine (Ana) and nornicotine (Nor). The concentration of agonist (µM) is indicated above each trace.

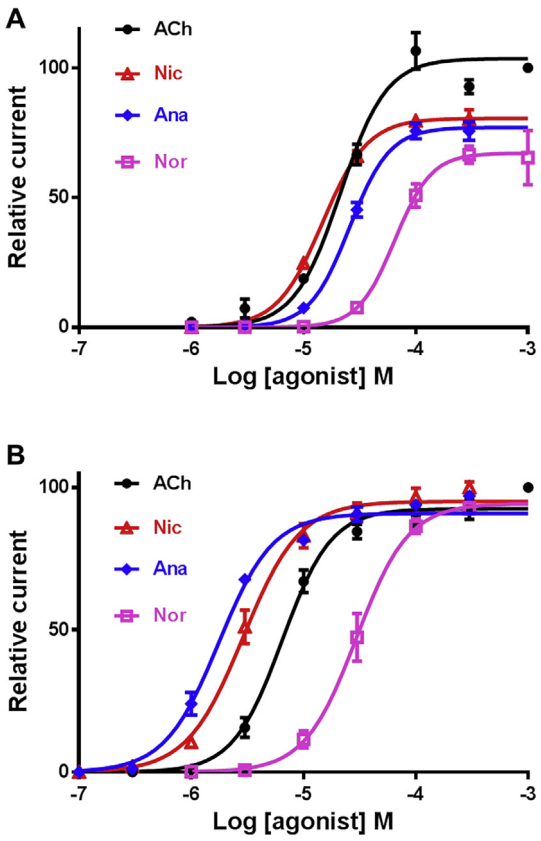

Fig. 7. Concentration-response curves of acetylcholine and nicotine derivatives on the *C. elegans* (A) and P. equorum (B) N-AChRs expressed in *Xenopus oocytes*. The N-AChRs were challenged with acetylcholine (ACh), nicotine (Nic), anabasine (Ana) and nornicotine (Nor). All responses are normalized to 1 mM Ach. Results are shown as the mean ± se.

2015; Wolstenholme and Neveu, 2017). In strongylid nematodes, Lev resistance has been shown to be associated with changes in binding characteristics or in the number of L-AChRs expressed in muscle cells (Sangster et al., 1988, 1998). In accordance with these observations, molecular investigations performed on Lev-resistant isolates from *H. contortus* identified truncated isoforms of two L-AChR subunits (i.e. ACR-8 and or UNC-63) associated with resistance (Neveu et al., 2010; Fauvin et al., 2010). In the pig parasitic nematode *Oesophogostomum dentatum* resistance to Lev has been characterized as the loss of a Lev receptor while nicotine-sensitive receptors were unaffected (Robertson et al., 1999). In accordance with this result, electrophysiological studies performed in *C. elegans* showed that genetic ablation of L-AChR resulting in Lev/Pyr resistance did not impact the functionality of ACR-16-containing receptors. In addition, in the present work, we showed that *C. elegans lev-8* null mutants are sensitive to nicotine, nornicotine and anabasine, whereas these worms are resistant to both Lev and Pyr (Blanchard et al., 2018). Taken together these results support the hypothesis that drugs targeting the nicotinic receptors including the ACR-16 subunit might be efficient at controlling Lev/Pyr-resistant parasites.

In *C. elegans*, the anthelminthic activity of nicotine at the neuromuscular junction is mainly mediated by the N-AChR which is, a homomeric receptor subtype made of the ACR-16 subunit (Richmond and Jorgensen, 1999; Touroutine et al., 2005). In accordance, in the present work we report that the nicotinic derivatives such as Nor or Ana induce a paralysis on wild-type and *Lev-8* null mutant worms whereas *acr-16* null mutants are resistant to these drugs highlighting the N-AChR as a major contributor of nicotinic derivative sensitivity in *C. elegans*. In addition, these results further support the use of drug targeting the N-AChR as a way to control Lev/Pyr-resistant nematodes.

Table 1
Summary of the EC_{50} and Hill coefficient values for acetylcholine and nicotine derivatives on the *C. elegans* and *P. equorum* N-AChRs expressed in *Xenopus* oocytes. Results are shown as the mean ± sd. The number of eggs recorded is indicated (n).

		Cel N-AChR	Peq N-AChR
Acetylcholine	EC50 (µM)	22.0 ± 1.2	6.4 ± 0.6
	Hill slope	2.1 ± 0.4	2.0 ± 0.2
	n	12	6
Nicotine	EC50 (µM)	15.1 ± 1.3	2.9 ± 0.5
	Hill slope	2.2 ± 0.2	1.7 ± 0.2
	n	11	8
Nornicotine	EC50 (µM)	67.7 ± 6.6	34.9 ± 7.2
	Hill slope	2.7 ± 0.4	1.9 ± 0.3
	n	7	9
Anabasine	EC50 (µM)	27.5 ± 0.9	1.7 ± 0.1
	Hill slope	2.4 ± 0.4	1.8 ± 0.2
	n	14	6

Interestingly, in parasitic species such as *H. contortus* and *O. dentatum*, a recombinant L-AChR subtype made of UNC-63, UNC-38 and UNC-29 (i.e. Hco-L-AChR-2 and Ode 29-38-63 respectively) subunits was found to be responsive to Nic (Boulin et al., 2011; Buxton et al., 2014). Here we reported that in contrast with Nic, Hco-L-AChR-2 is readily insensitive to both Nor and Ana. Even though the contribution of Hco-L-AChR-2 to Nic sensitivity *in vivo* remains to be elucidated, it is tempting to speculate that the reduced sensitivity to Nic observed in Kok (in comparison with Wey) could be associated with a putative impairment of this Hco-L-AChR-2 subtype. Nonetheless, such a reduced sensitivity to nicotine had no impact on Nor and Ana response of Kok L3

supporting the hypothesis that a putative N-AChR subtype including the ACR-16 subunit might be a preferential target for nicotine and nicotinic derivative in *H. contortus*.

In *C. elegans*, electrophysiological studies and expression in *Xenopus* oocytes strongly suggested that the ACR-16 subunit can associate to form a homopentameric channel both *in vivo* and *in vitro* (Ballivet et al., 1996; Touroutine et al., 2005). In the distantly related pig parasite *A. suum*, the ACR-16 subunit was also able to form a functional homopentameric channel when co-expressed in *Xenopus* oocytes with the RIC-3 ancillary protein. These results suggested that homomeric recombinant N-AChR made of ACR-16 should be obtained for other parasitic species such as *H. contortus* and the horse parasite *P. equorum* for which Pyr resistance is an increasing concern (Kaplan, 2002; Matthews, 2014; Lassen and Peltola, 2015). In accordance with this assumption, in the present study we report that the co-expression of the ACR-16 subunit from *P. equorum* with the RIC-3 from *C. elegans* led to the robust expression of a functional AChR. In comparison with the prototypical *C. elegans* N-AChR, the *P. equorum* N-AChR was found to be more responsive to Nic, Nor and Ana. Such differences could lay the basis for directed mutagenesis experiments in both *C. elegans* and *P. equorum* respective ACR-16 subunit that will provide critical information about the binding site of their respective homomeric N-AChR.

Interestingly, if Ana has been identified as the most potent agonist on the recombinant Peq-N-AChR, this nicotine alkaloid was also the most efficient on *H. contortus* Wey and Kok L3 as revealed during ALMA assays. However, because of its potential toxicity for the host (Lee et al., 2006), Ana is unlikely to be used as resistance breaking drugs for livestock. In that respect, the pharmacomodulation of Ana could represent an attractive approach to improve its efficacy as a potential anthelminthic. Recently, Zheng et al. (Zheng et al., 2017) showed that (S)-5-ethynyl-anabasine has higher agonist potency than other nicotine alkaloids on the recombinant *A. suum* N-AChR. If such studies open the way for the discovery of novel compounds targeting the N-AChR, there is now an urgent need to evaluate their efficacy on the parasites and also evaluate their potential toxicity for the host.

In contrast with *P. equorum*, into our hands the ACR-16 subunit from *H. contortus* failed to form a functional channel when co-expressed with RIC-3 from *C. elegans* but also using the RIC-3.1 and/or RIC-3.2 from *H. contortus* (data not shown). Here, we hypothesize that additional subunits or ancillary proteins are required to obtain a functional recombinant *H. contortus* N-AChR in *Xenopus* oocytes. Clearly, additional investigations are now required to further investigate ACR-16 containing receptors in *H. contortus*. In that respect recent progress concerning the efficient silencing of AChR subunit genes in *H. contortus* using RNAi will provide a valuable approach to decipher its role in nicotinic compound sensitivity *in vivo* (Blanchard et al., 2018).

In conclusion, we provide a proof of concept that drug targeting the nematode N-AChR can efficiently control Lev/Pyr-resistant parasites.

Research effort should now focus on the identification of a wider range of N-AChR from parasitic species laying the basis for the identification of novel compounds targeting these attractive targets.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2018.11.003.

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