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Research Article

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Mutation of the Glc-2 Gene May Confer Dominant Ivermectin Resistance

Keywords

Ivermectin: a broad-spectrum anti-parasitic drug frequently prescribed to treat worm infestations

Drug resistance: a phenomenon wherein selective pressure applied by a drug filters susceptible pathogens and leaves resistant pathogen unharmed; leads to reduced drug efficacy

glc-2: a key gene in parasitic worms that encodes an alternate subunit for a membrane channel; reduces channel recognition by ivermectin

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Abstract

Background: Ivermectin is a widely used anti-parasitic drug that binds to and activates glutamate-gated chloride channels (GluCls), giving it its nematocidal (nematode-killing) properties. Due to excessive use of ivermectin, frequent cases of resistance to this nematicide are being reported, suggesting that ivermectin is beginning to lose its efficacy. This project seeks to study whether a mutation of the glc-2 gene, which encodes for a β subunit of the GluCl channel, confers ivermectin resistance. We hypothesize that a glc-2 mutation achieves nematicide resistance by creating a defective GluCl channel that cannot bind to ivermectin.

Methods: We used classical genetics to obtain the desired mutants from stock worms. We then tested the worms for resistance profile using ivermectin sensitivity assays. Finally, we examined in vivo interactions by expressing relevant RNA in a heterologous system and performed electrophysiological recordings.

Results: We were able to demonstrate that presence of the defective glc-2 leads to increased resistance profiles when given the chance to associate with select GluClα subunits (e.g. AVR-15). We also demonstrated that co-injection of glc-2 and glc-3 compromises GluCl response to L-glutamate, a critical indicator of channel functionality.

Conclusion: Our results lend strong support to our hypothesis that glc-2 is able to interact with certain α subunits of GluCl to confer ivermectin resistance. This finding provides a framework for future dominant ivermectin resistance studies.

Introduction

Onchocerciasis is a disease found almost entirely in Sub-Saharan Africa, where it poses significant health concerns, with an estimated incidence of at least 25 million infections and 123 million individuals potentially at risk. (1) Ivermectin was developed as a broad-spectrum antiparasitic for veterinary use that has proven to be extremely effective in the treatment of this disease in humans, and was subsequently marketed for human use under the trade name Mectizan. Since its establishment in 1987 by Merck & Co., the Mectizan Donation Programme has achieved impressive gains in onchocerciasis control. (2) Ivermectin has particularly attractive pharmacology as it targets glutamate-gated chloride channels (GluCls), which are invertebrate-specific and pose minimal repercussion to the mammalian host. (3) Ivermectin is undoubtedly one of the largest public health success stories of the 20th century, and today has widespread applications in the control of other nematodiases (e.g., elephantiasis) and ectoparasites (e.g., scabies); it remains a mainstay for parasite control in veterinary medicine and is currently on the List of Essential Medicines put forth by the World Health Organization (WHO). (4-6)

Unsurprisingly, due to ivermectin's appealing pharmacological properties and extensive applicability, the drug has been very intensively used. This has led to the emergence of resistance in many animal hosts, and most recently, in humans. (7-10) The continued effectiveness of ivermectin relies critically upon our ability to keep resistance under control. Our lab has been interested in the emergence of such resistance mechanisms, and using C. elegans as a predictive model, we were able to identify critical GluCl alpha (GluClα) subunit genes (avr-14, avr-15, glc-1, and glc-3) which, if mutated, confer recessive resistance to ivermectin in a synthetic manner. (11) Nonetheless, these results fail to account for cases in which the resistance appears dominant. (12) We recently isolated via mutagenesis vu16, a point mutation allele in the glc-2 gene that encodes for a β subunit of the GluCl channel. This mutation has proven to be particularly interesting, as it confers synthetic hyper-resistance to ivermectin in a semi-dominant fashion in a 50μg/mL ivermectin screen (unpublished data). Mutations in glc-2 may provide us with a better understanding of how dominant ivermectin resistance could emerge in an invertebrate model.

As GluClα subunits seem to play a role of great importance in ivermectin binding (all relevant GluCl mutations identified thus far encode for α subunits, as mentioned previously), we hypothesize that the vu16 point mutation achieves resistance by creating a defective GLC-2 that associates with select α subunits constituting the GluCl, thereby "poisoning" channel function. This poisoning mechanism has been similarly described to account for resistance to levamisole, thus providing a strong precedent for our hypothesis. (13) We validated our hypothesis using a combination of genetics, drug assays and electrophysiology.

Methods

To avoid confusion, italicized terms denote the gene(s) mutated in a given strain (if wild-type forms were used, they will be followed with 'wt'); capitalized words, with the exception of GLC-2 (which is the defective subunit encoded by glc-2), denote a functional subunit in its wild-type form.
Unless otherwise indicated, the specific mutant alleles of the genes of interest were:

Found on Chromosome I	Found on Chromosome V
glc-2 (vu16), avr-14 (vu47), dpy-5 (e61), unc-57	*avr-15 (vu227), glc-1 (pk54::Tc1), glc-3 (ok212), unc-76 (e911)*

Table 1. Mutant alleles used in the course of this research project, either directly affiliated with the experiments or are used as genotyping markers.

Creating vu16 quadruple mutants using classical genetics

To test for potential associations of GLC-2, we used genetic crosses to introduce glc-2 into a triple mutant background, with three of the known GluClα-encoding genes mutated and one retained in its wild-type form. All of the triple mutants were available from previous experimental crosses, therefore a total of four new strains were made.

MUTANTS TO CONSTRUCT	STRAINS USED AS CONTROL
glc-2(vu16) avr-14(vu47) I; avr-15(vu227) glc-1 (pk54) V	avr-14(vu47) I; avr-15(vu227) glc-1 (pk54) V
glc-2(vu16) avr-14(vu47) I; glc-3(ok212) avr-15(vu227) V	avr-14(vu47) I; glc-3(ok212) avr-15(vu227) V
glc-2(vu16) avr-14(vu47) I; glc-2(ok212) glc-1 (pk54) V	avr-14(vu47) I; glc-2(ok212) glc-1 (pk54) V
glc-2(vu16); glc-3(ok212) avr-15(vu227) glc-1 (pk54) V	glc-3(ok212) avr-15(vu227) glc-1 (pk54) V

Table 2. glc-2 is brought into backgrounds with classical glutamate-gated chloride channel subunit mutations A total of 8 strains were used in the ivermectin sensitivity assays, including the 4 vu16-containing quadruple mutants and their corresponding wild-type glc-2 controls, in order to test for potential GLC-2 associations with each individual GluClα subunit implicated in IVM resistance.

Of available strains, we chose the D621 mutant strain (glc-2 avr-14 I; him-8(e1489) IV; avr-15 glc-1 V), which was heterozygous for him-8. Progeny from 15 worms were used to eliminate him-8.

To obtain glc-2 avr-14 I; glc-3 avr-15 V, we first crossed N2 males to JD3 (dpy-5 I, unc-76 V), heterozygous male F1s to JD624 (avr-14 I; avr15 glc-3 V), then F2 male progeny to glc-2 avr-14 I; unc-76 V. We constructed this strain in parallel by crossing JD621 to JD3, singling wild-type looking progeny, and eliminating him-8. Next, we singled out unc F2s to ensure that no F3 dpy progeny could be found. From this cross, We singled wild-type looking worms that threw F4 dpy. From these dpy-containing plates, we singled 15 wild-type looking F4s, and eliminated unc, dpy and selected candidates on 20 ng/mL ivermectin. We confirmed mutations via either gel electrophoresis (glc-3) or Sanger sequencing (glc-2, avr-14, avr-15).

To obtain glc-2 avr-14 I; glc-3 glc-1 V, we heat shocked our stock strain JD366 (avr-14 I; glc-3 glc-1 V) as described to generate males. (20) We crossed these to JD3, and selected male F1 progeny and crossed them to glc-2 avr-14 I; unc-76 V. We then singled wild-type looking F2 worms that threw dpy, then wild-type looking F3s from these plates. We then eliminated unc, dpy worms. We confirmed mutant alleles by PCR followed by either gel electrophoresis or sequencing.

The first step in obtaining glc-2 I; glc-3 avr15 glc-1 V was to generate a strain with only glc-2 on the first chromosome. To do so, we crossed N2 males to unc-57, crossed F1 heterozygous males to JD429 (glc-2 dpy-5 avr-14 (ad1302) I; avr-15(ad1051) glc-1 V), and singled wild-type looking F2s that threw both unc and dpy progeny. The F2 singled worms were allowed to self-fertilize and we isolated 200 F3 progeny to locate an absence of either unc or dpy phenotypes in the F4 population, which is indicative of recombination (occurs at roughly 2% frequency).

The next step involved eliminating all the undesired mutations from chromosome V. First, we crossed N2 males to JD3. Then we crossed F1 males to the glc-2 mutant. We singled wild-type looking F2s that threw both unc and dpy, and eliminated unc, dpy via propagation. We brought the glc-2 background into JD300 (glc-3 avr-15 glc-1 V) to get the desired strain, and confirmed all mutations via PCR followed by sequencing or gel electrophoresis.

Ivermectin Sensitivity Assays

We performed ivermectin sensitivity assays as described by Dent et al. with slight modifications. (14) We prepared eggs using the standard alkaline method and resuspended in M9 buffer (42mM Na2HPO4, 22mM KH2PO4, 86mM NaCl to 1L dH2O), and 50-100 eggs plated to each NGM plate. Each triple mutant was compared to its quadruple mutant counterpart with the extra vu16 mutation introduced. We determined the percentage of gravid adults after 4 days of growth at ambient temperature (~25oC) with the individual agar plates divided into 9 sectors to facilitate counting. Concentrations of ivermectin were 0.01ng/mL to 50µg/mL. We constructed concentration-response curves using Igor Pro software (Wavemetrics, Lake Oswego, OR, USA) and fitted to the Hill Equation provided in their function set:

$$\text{base} + (\text{max} - \text{base}) \div [1 + (\text{xhalf} \div \text{x})^{\text{rate}}]$$

To ensure consistency, all agar plates were uniformly seeded with ~0.5mL of HB101 bacteria. Experimental and control strains for each set of mutants were L1 synchronized and counted simultaneously.

RNA Expression Followed by Two-Electrode Voltage Clamp (TEVC)

We selected Xenopus laevis females without past surgical history from stock and extracted oocytes under anaesthesia (0.15% MS-222, Sigma, Oakville, ON). The oocytes were subsequently immersed in 2mg/mL collagenase (Sigma) in OR2 buffer (82mM NaCl, 2mM KCl, 1mM MgCl2, 5mM Hepes to pH 7.5) for 2hr at ~25oC with shaking to remove the follicular layers and facilitate injection. We transferred partially de-folliculated oocytes to a 10cm agar plate containing ND96 (96mM NaCl, 2mM KCl, 1mM MgCl2, 1.8mM CaCl2, 5mM Hepes, pH 7.5) and replaced the buffer 3 times to ensure removal of any residual follicular layer.

We extracted RNA using the mMessage mMachine T7 kit (Life Technologies, Carlsbad, CA, USA). Next, we injected each oocyte with 500ng of cRNA (250ng if two cRNA types were simultaneously injected) using a Drummond Nanoject microinjector (Broomhall, PA, USA) equipped with 90mm flared microinjection needles (Harvard Apparatus Canada, Saint-Laurent, QC). We injected fifteen to twenty eggs per experiment to ensure there were enough expressing oocytes for data acquisition. Table 3 depicts the GluCl cRNA that were injected, along with a short description for the rationale.

Injected RNA	Rationale
glc-2 (wt)	Control, it was already demonstrated that the wild-type glc-2 can form homomeric channels in oocytes*
glc-3 (wt)	Control, it was already demonstrated that glc-3 can form homomeric channels
glc-2 vu16	Control, it was already demonstrated that glc-2 vu16 is unable to form its own channel (unpublished)
glc-2 (wt) + glc-3 (wt)	Control, want to see whether co-injection of both cRNA leads to expression of heteromeric channels
glc-2vu16 + glc-3	Experiment, if hypothesis true, then the channels formed (if at all) would depict compromised functionality

Table 3. Oocyte injections and rationales A total of 5 oocyte group was each injected with a different set of cRNA (s) and subsequently recorded using electrophysiological techniques.
*Source: Cully et al. (1994)

We incubated injected oocytes at 15°C for 2 days before proceeding to recordings, where eggs were re-introduced to fresh ND96 saline and held at a membrane potential of -80mV. Voltages were recorded using a Axoclamp 2B voltage clamp (Axon Instruments, Foster City, CA, USA). Glutamate was dissolved in ND96 and diluted to different concentrations before being sequentially administered to oocytes. Strong responses, defined by a significant current dip (>500nA) upon application of 1mM glutamate, were indicative of functional GluCl channel formation and results were acquired using Clampex 10 Data Acquisition Module (Axon).

Results

Differential ivermectin resistance across the four different quadruple mutants

The control strains avr-14 (I); glc-3 avr-15 (V), avr-14 (I); glc-3 glc-1 (V), avr-14 (I); avr-15 glc-1 (V) and glc-3 avr-15 glc-1 (V) exhibited ivermectin EC50s of 123.23±9.23, 2.43±1.07, 500.31±26, and 5.87±0.171ng/mL respectively, results that are comparable to and consistent with past ivermectin sensitivity trials (unpublished findings). We observed a modest increase in resistance when we introduced glc-2 into avr-14 (I); glc-3 avr-15 (V), as denoted by a slight rightward shift of the concentration-response curve. In contrast, we observed hyper-resistance to ivermectin when glc-2 was introduced into either the avr-14 (I); avr-15 glc-1 (V) or avr-14 (I), glc-3 glc-1 background. Finally, glc-2 (I), glc-3 avr-15 glc-1 (V) and glc-3 avr-15 glc-1 (V) showed the same level of low synthetic resistance to ivermectin until a slight difference at maximal IVM concentration, a phenomenon that we failed to explain. The EC50s of glc-2 avr-14 (I); glc-3 avr-15 (V), glc-2 glc-2 avr-14 (I); avr-15 glc-1 (V) and glc-2 avr-14 (I); glc-3 glc-1(V) were indeterminable within our assessment limits, as they showed robust resistance and never dropped to baseline level, even at 50µg/mL ivermectin. However as a proof-of-principle, we believe that these initial data are sufficient. These results are summarized in Fig. 1.

Co-injection of vu16 with glc-3 compromises response to glutamate

To validate our findings further, we elucidated subunit interactions by co-injecting GluCl cRNAs into Xenopus oocytes. Since GluCl activation is mediated by glutamate, a robust response after its application is a reliable indicator of channel functionality. As a control, we administered glutamate to three un-injected oocytes, none of which responded (data not shown). GLC-2 GluClβ homomers responded to concentrations of glutamate as low as 50µM. GLC-3 homomers were slightly less sensitive, with responses recorded at 100µM glutamate. The heteromeric channel formed by GLC-3 and GLC-2 was significantly more sensitive to glutamate, with responses evident at 10µM. Glutamate concentrations ranged from 2µM-2mM and their effects in oocytes were concentration-dependent, consistent with past observations made in our lab with glutamate application to oocytes expressing Haemonchus contortus (Hc) GluClαs (15). Oocyte responses with the least noise interference for each group are shown in Fig. 2. Each recording was repeated three times in three separate oocytes.

None of the 10 oocytes injected with GLC-2vu16 cRNA responded to glutamate (data not shown). More interestingly, however, glc-2 co-injection with glc-3 wt dramatically compromised the glutamate response, to the point of nearly abolishing it. The highest glutamate concentration available (5mM) was unable to induce a current >200nA, a marginal response compared to the much larger currents (>1000nA despite much lower glutamate administration) recorded in the other experimental oocyte groups.

Discussion

GLC-2 potentially interacts with GLC-1, AVR-15 and GLC-3, but not AVR-14

We separately mutated three GluClα subunits while maintaining one functional subunit. glc-2 avr-14 (I); glc-3 avr-15 (V), glc-2 avr-14 (I); glc-3 glc-1 (V) and glc-2 avr-14 (I); avr-15 glc-1 (V) worms all exhibited greater resistance to ivermectin upon substitution of glc-2 wild-type background with glc-2. This result suggests that GLC-2vu16 likely interacts with wild-type GLC-1, AVR-15, and GLC-3, respectively, and that increase in ivermectin resistance resulted from disruption of GluCl channel integrity upon heteromeric association with the defective GLC-2vu16 protein. Since the presence of GLC-2vu16 does not seem to affect ivermectin resistance of the strain glc-3 avr-15 glc-1 (V), then by the same reasoning GLC-2vu16 and wild-type AVR-14 subunits are unable to associate with each other. Thus, from our initial concentration-response results, we propose that GLC-2vu16 can form heteromeric channels with GluClα subunits, and that a mechanism exists that determines which subunits it can interact with.

Another interesting observation is that the level of synthetic ivermectin resistance conferred is variable, depending on which subunits are available for interaction (e.g., moderate increase for GLC-1 and substantial increase for AVR-15/GLC-3). While the exact mechanism is poorly understood, we believe that it may be attributable in part to the localization of the different α subunits. Through past mapping and laser ablation studies, we were able to construct a hypothetical model that proposes different pathways to attain ivermectin sensitivity. (12) A slightly modified depiction of the model is shown in Fig. 3. Since AVR-15 subunit-containing channels are localized in the pharynx, it plays a much greater role mediating essential physiological functions, especially feeding. Meanwhile, AVR-14 and GLC-1 are localized in the extrapharyngeal neurons, which play somewhat of a supplementary role, making it more difficult to develop hyper-resistance upon interaction with GLC-2vu16. It is also noteworthy that glc-1 is C. elegans specific, and as such may have evolved new functionality in other tissues, thereby reducing its ability to interact with GLC-2. The exact expression profile of GLC-3 is debated and not shown in the model, but evidence suggests that it is localized in the olfactory AIY interneurons, which may play an important role in olfaction-regulated turning behaviour (16).

GLC-2 interacts with and poisons GLC-3 in a heterologous system

Our concentration-response data can only provide a preliminary assessment of ivermectin resistance upon glc-2 incorporation into a mutant background strain. Ion channel expression is necessary to provide further insights into possible in vivo subunit interactions. Activity of glc-2 wt/glc-3 wt co-injection is different from their individual expressions, thus demonstrating that GLC-2, in its wild-type form, does form a heteromeric channel with GLC-3. As such, the lack of significant channel activity when glc-2 was co-injected with glc-3 wt was particularly noteworthy. These co-injection results have three possible interpretations: [1] GLC-2vu16 cannot assemble with GLC-3. While this is a plausible scenario, it does not explain our data as GLC-3 should still be expressed normally, and we would expect recordings consistent with a GLC-3 homomeric channel; [2] GLC-2vu16 is able to form functional heteromers with GLC-3. This is very unlikely, since that would again lead to robust current recordings with glutamate administration; [3] GLC-2 is able to associate with GLC-3, but creates a heteromeric channel with compromised functionality. Our data strongly support the last possibility, a result that is consistent with our poisoning hypothesis. Glutamate activation was possible with GLC-3 subunits alone, but this ability was severely compromised when a defective GLC-2 was introduced.

Limitations

Time was the most significant limitation for our project. Obtaining the quadruple mutants was particularly challenging and time-consuming. Additionally, the glc-2 (without avr-14) background on chromosome I was extremely difficult to acquire due to low recombination rates, and there were technical issues screening for the glc-1 mutant allele using PCR. The ivermectin sensitivity assays and electrophysiological recordings had internal replicates, but were performed once. Validation of our hypothesis would require additional technical replicates. The oocyte recordings investigated channel expression with only GLC-3 and GLC-2; co-injection with avr-15 wt, avr-14 wt and glc-1 wt has not been adequately examined.

We have shown a "poisoning" effect of GLC-2vu16 on heteromeric channels with GLC-3 in Xenopus oocyte electrophysiology. If this occurs in vivo, it can explain the partially dominant hyper-resistant phenotype observed. We have yet to demonstrate that this is in fact the case, but can state that glc-2 is a potential key player in ivermectin pharmacology.
nels with GLC-3 in Xenopus oocyte electrophysiology. If this occurs in vivo, it can explain the partially dominant hyper-resistant phenotype observed. We have yet to demonstrate that this is in fact the case, but can state that glc-2 is a potential key player in ivermectin pharmacology.

Future Directions

Our goals will seek to address the current limitations with our dataset. One priority is to expand our recording experiments to include cRNA co-injections of glc-2 with avr-15 wt, avr-14 wt and glc-1 wt. Our hypothesis would be supported if we can show that AVR-14 expression is unaffected by the presence of GLC-2, while all other co-injection groups exhibit a much reduced or non-existent glutamate response.

Our second goal is to generate four additional quadruple mutants of glc-2 using the mutation ok1047, which is a null whole gene deletion rather than a single point mutation. (18) This means we can study mutants that do not produce GLC-2, and we will screen these strains in parallel with the glc-2 vu16 quadruple mutants and the glc-2 wt GluClα triple mutants. For our poisoning hypothesis to be supported, we would expect to see that the glc-2 deletion mutants are as sensitive to ivermectin as the triple mutants, and do not exhibit the hyper-resistance associated with glc-2 vu16.

Conclusion

Ivermectin remains a critically important drug for the treatment of parasitic diseases. With the current focus shift of African Programme for Onchocerciasis Control (APOC) from containment to elimination, and plans to increase ivermectin mass administration to the public, understanding the mechanisms of ivermectin resistance is paramount. (19) Not only will it help provide insight on current drug administration practices, but it will also aid in the development of new compounds that can bypass the structural GluCl mutations (e.g., using medicinal chemistry to optimize access to truncated target sites). Our preliminary results with glc-2 have helped shed new light on this area, and set the foundation for future research in dominant ivermectin resistance, as well as mechanisms of parasite resistance in general. With ongoing research in this direction, it is our hope that ivermectin can continue being a therapeutic success as it has been forty years past, forty years into the future.

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Figures

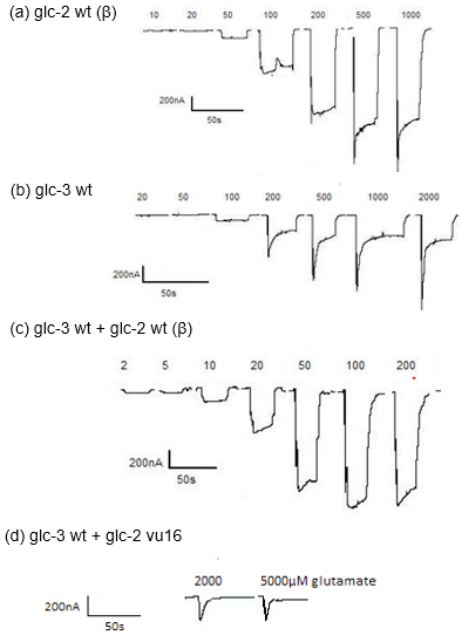

Figure 2. Glc-2 association compromises response to glutamate when co-injected with glc-3. Electrophysiological recordings (TEVC) from RNA-injected oocytes and tested for L-glutamate response at progressively increasing concentrations. Oocytes injected with glc-2 wt (a), glc-3 wt (b) or co-injected with glc-3 wt and glc-2 wt (c) exhibited much greater L-glutamate sensitivity than those co-injected with glc-3 wt and glc-2 vu16 (d).

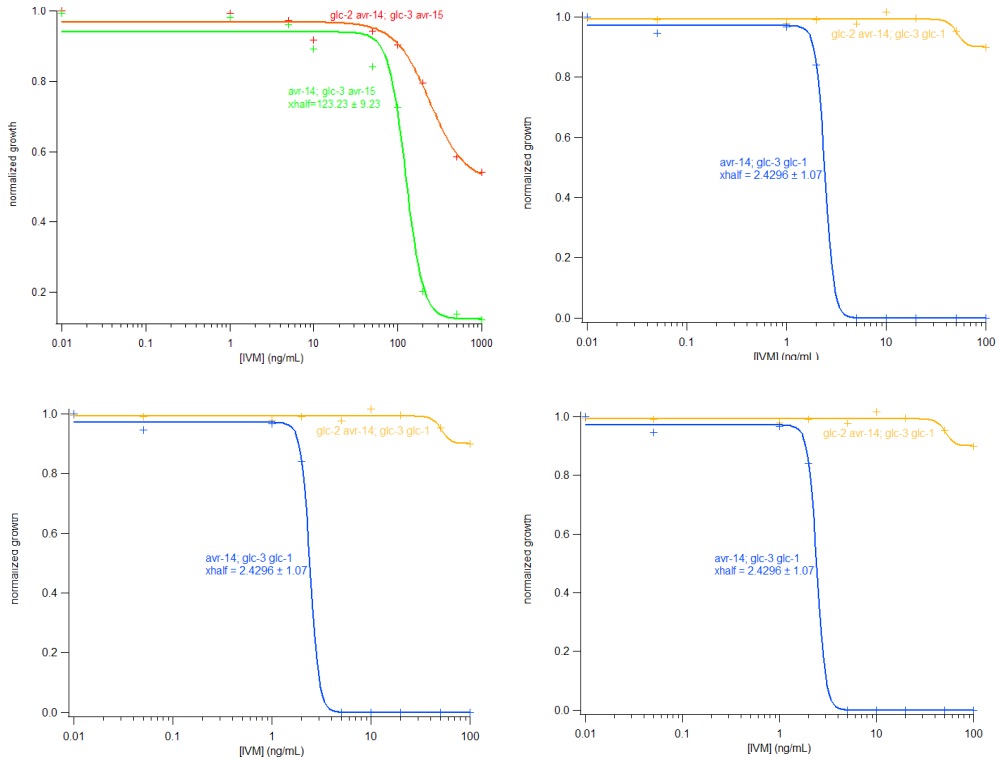

Figure 1. A defective GLC–2 interacts and poisons select subunits that constitute the GluCl channel. Our results reveal that a defective GLC–2 leads to a modest increase in resistance with a functional GLC–1 (a), a substantial increase with a functional AVR–15/ GLC–3 (b and c), and no change at all with a functional AVR–14 (d).

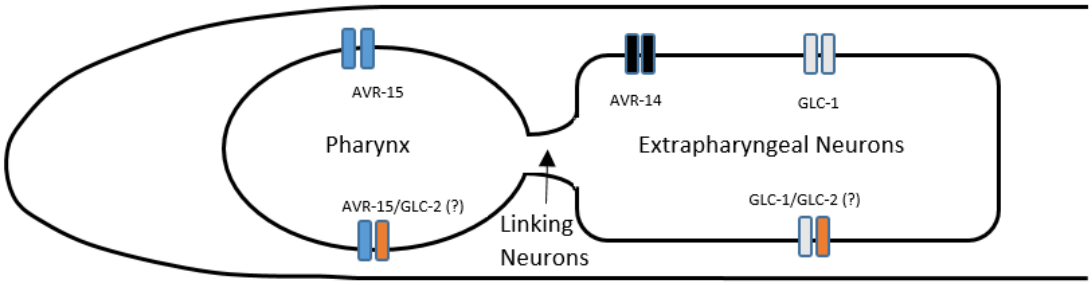

Figure 3. Postulated sites of interaction of GLC–2 in the ivermectin pathway AVR–15 subunits are expressed in pharyngeal GluCls while AVR–14 and GLC–1 subunits are expressed in extrapharyngeal neurons. The exact expression profile of GLC–3 is currently debated and thus not shown