The avermectin/milbemycin receptors of parasitic nematodes

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Abstract

Glutamate-gated chloride channels are the most important target of ivermectin and related compounds in parasitic nematodes. A small family of genes encode subunits of these channels, allowing the assembly of multiple channel subtypes; the subunit composition of most of the native receptors is unknown. The members of the gene family vary between species, making extrapolation from *C. elegans* to parasites difficult. Expression of recombinant receptors in *Xenopus* oocytes can identify subunits that have the ability to co-assemble into novel channels, but localisation data, ideally at the single-cell level, is required to confirm that these subunits are expressed in the same cells and tissues. Fortunately, recent advances in this area are starting to make this information available; this information is adding to our understanding of how the drugs act and of the possible subunit combinations that create their targets in vivo.

Keywords: Nematode; ivermectin; glutamate; anthelmintic; macrocyclic lactones

1. Introduction

Parasitic nematodes remain a serious threat to human and animal welfare, both as the causative agents of chronic and debilitating diseases and also by causing dramatic losses to livestock and crop productivity (Coyne et al. 2018; Gilleard et al. 2021; Veesenmeyer, 2022)Control of these organisms is based largely on the use of chemical anthelmintics and these have greatly reduced the overall impact of parasitic nematode infection on human and animal health, though this is far from complete and for parasites of livestock and companion animals control is threatened by the emergence of drug-resistant nematodes (von Samson-Himmelstjerna et al. 2021; Noack et al. 2021; Raza et al. 2019; Kenyon et al. 2017). One of the most important families of anthelmintics is the macrocyclic lactones, also known as the avermectin/milbemycins (A/Ms), (Campbell, 2012) and the discovery and development of ivermectin led to the award of half of the 2015 Nobel Prize in Physiology or Medicine to William Campbell and Satoshi Ōmura (Molyneux and Ward, 2015). These drugs are very widely used in veterinary medicine and ivermectin is a key part of control and elimination efforts for lymphatic filariasis, onchocerciasis and Strongyloides (Lakwo et al. 2020; Ashour 2019). Though ivermectin is the sole member of the family currently licensed for use in humans – moxidectin is in phase 3 clinical trials (Milton et al. 2020) – a number of other drugs have been developed and licensed for use in animal health, including moxidectin, doramectin, abamectin, milbemycin oxime and selamectin.

2. Early studies on *C. elegans*

It quickly became apparent that ivermectin acts on the nematode nervous system, and researchers at Merck identified glutamate-gated chloride channels (GluCls) as the ivermectin target in the free-living nematode, *Caenorhabditis elegans* (Arena et al., 1992), having first demonstrated the existence of a specific high-affinity binding site (Cully and Paress, 1991). Shortly afterwards, Cully et al. (1994) identified two cDNA clones that, when expressed together in *Xenopus* oocytes, produced GluCls which were potentiated by low concentrations of ivermectin. The 'α' subunit, now called GLC-1, produced ivermectin-sensitive channels when expressed alone, whereas the 'β' subunit, now referred to as GLC-2, produced ivermectin-insensitive GluCls; when expressed together the two subunits formed channels that were sensitive to both ligands. These early studies revealed two recurring themes of research into the interaction between avermectins and their targets; the subunit composition of the native channels and whether the drug exerts its effects by direct activation of the channels or via potentiating the effects of glutamate, perhaps by acting as an allosteric modulator. Sequence analysis of GLC-1 and GLC-2 showed that they were members of the pentameric ligand-gated ion channel (pLGIC) family, closely related to mammalian glycine and $GABA_A$ receptor subunits (Cully et al., 1994). Structural studies on GLC-1 complexed with ivermectin showed that the drug binds to the channel domain of the receptor, holding it in an open configuration, a site consistent with the

hydrophobic nature of the molecule (Hibbs and Gouaux, 2011). Further genetic and molecular studies identified another four GluCl genes, *glc-3*, *glc-4*, *avr-14* and *avr-15* (Table 1) (Horoszok et al. 2001; Cully et al. 1996; Laughton et al. 1997; Dent et al. 1997; 2000; Vassilatis et al. 1997). All except *glc-4* encode subunits that can be expressed to form homomeric glutamate- and ivermectin-sensitive chloride channels. Two, *avr-14* and *avr*-15, are alternatively spliced and in each case only one of the splice variants has been successfully expressed (Dent et al. 1997; 2000; Laughton et al. 1997; Vassilatis et al. 1997); the functions of AVR-14A and AVR-15A remain obscure. In general, glutamate application results in a rapid and reversible opening of the channel, whereas ivermectin opens the channels slowly and either irreversibly or with very slow channel closing (Wolstenholme and Rogers, 2005).

The A/M anthelmintics have a variety of different effects on *C. elegans*, some of which become apparent at different concentrations. These different concentrations could reflect the sensitivity of different receptors to the drug, or reflect their accessibility to externally applied compounds. The effects include body wall paralysis, inhibition of pharyngeal pumping and inhibition of egg laying (Dent et al. 2000; Pemberton et al. 2001; Hahnel et al. 2021). The variety of these effects could, in hindsight, be seen as indication of the variety of different GluCls that are expressed in the nematode, and for the number of different cell types in which they are expressed.

3. Identification of GluCl in parasitic nematodes

The existence of ivermectin-sensitive chloride channels in parasitic nematodes was first confirmed using electrophysiological approaches, though the high concentration used in some of these studies led to the conclusion that the drugs acted through GABA receptors, at least in part. Kass et al. (1980, 1984) showed that, in the ventral nerve cord of *Ascaris suum*, avermectin b1A (5µg/ml ≈ 5.7µM) blocks transmission between interneurons and excitatory motor neurons, and between inhibitory motor neurons and muscle, but not between excitatory motor neurons and muscle. They suggested that the interneuron transmission to excitatory motor neurons was GABA-ergic, but a different transmitter was involved in inhibitory neuromuscular transmission. They also reported indirect evidence for an effect on the central nervous system of this parasite (Kass et al., 1982). It was rapidly appreciated that the pharynx is an important target of the A/M anthelmintics in parasites (Geary et al. 1993), with glutamate and ivermectin activating chloride channels and inhibiting pumping activity in *Ascaris suum* (Adelsberger et al. 1997; Brownlee et al. 1997) and *Ascaridia galli* (Holden-Dye and Walker, 2006).

Given the importance of ivermectin and the other A/M in treating infections with parasitic nematodes, it is no surprise that efforts were made to identify orthologues of the *C. elegans* genes that are responsible for the currents identified physiologically. Degenerate RT-PCR approaches led to the identification of *avr-14* and *glc-2* orthologues from *Haemonchus contortus*, as well as an additional

subunit gene, now called *Hco-glc-5* (the nomenclature used is that of Beech et al. 2010), that does not have a clear *C. elegans* orthologue (Delany et al 1998; Jagannathan et al. 1999; Forrester et al. 2003); *avr-14* orthologues were also identified in many other species, including *Dirofilaria immitis*, *Cooperia oncophora*, *Cylicocyclus nassatus* and *Ostertagia ostertagi* (Yates and Wolstenholme, 2004; Njue et al. 2004; El-Abdellati et al. 2011; Tandon et al. 2006) and this gene seems to be present in all nematodes with a conserved pattern of alternative splicing. The Hco-AVR-14B and Hco-GLC-5 subunits bound radiolabelled ivermectin with high-affinity when expressed in mammalian cells, but the Hco-AVR-14A and Hco-GLC-2 subunits did not (Cheeseman et al 2001; Forrester et al. 2002); data that are consistent with the results obtained from expression in *Xenopus* oocytes (Forrester et al 2003; McCavera et al. 2009; Atif et al, 2017). As genome sequence data for parasitic nematodes have become available the full picture of the composition of the nematode GluCl family has become clearer, with an additional subunit gene, *glc-6*, identified in *H. contortus* and other trichostrongyles (Glendinning et al. 2011). Four subunit genes, *avr-14, glc-2*, *glc-3* and *glc-4* seem to be conserved in the species examined to date, *avr-15* and *glc-5* are present in species from multiple phylogenetic clades with *glc-1* and *glc-6* having a more limited distribution (Lamassaiude et al., 2021). The conservation of *glc-4* is perhaps noteworthy as there is little evidence regarding the function of the GLC-4 subunit; it has never been reported to form channels when expressed in vitro from any species. In *C. elegans*, *glc-4* expression was downregulated by space flight and RNAi of the gene produced extended life span and defective dauer formation (Honda et al. 2012).

4 Progress towards the elucidation of the subunit composition of GluCl

As with many receptors, there is a gap between the properties of the channels formed in vitro by recombinant channel subunits and those of the receptors that are actually present in nematode tissues. One of the major reasons for this is our ignorance of the subunit composition of the native receptors; GluCl are members of the pentameric ligand-gated ion channel (pLGIC) family and, as with other members of this family, the functional channels formed in vivo could be homomeric or heteromeric. In addition, no auxiliary proteins, analogous to those that influence the trafficking and assembly of nematode nicotinic acetylcholine receptors (Boulin et al. 2008; 2011; 2012) have so far been reported for the GluCls and we do not know if any such proteins exist. Deciding whether two or more subunits associate to form a channel is not straightforward, especially if one of the components is capable of forming a homomeric receptor. It is necessary to demonstrate clear physiological or pharmacological differences between the homomeric and putative heteromeric channels, however even if this can be conclusively shown, this just demonstrates that the subunits can biochemically associate, not that they actually do so in vivo. For that, it is necessary to show that the subunits are expressed in the same cells and, until recently, this was difficult. Recent advances in single-cell transcriptomics (Taylor et al.,

2021) and related techniques make the prospect of aligning subunit expression and electrophysiology much more realistic.

4.1 Co-expression data

The very first identification of GluCl cDNAs from *C. elegans* showed that different subunits, GLC-1 and GLC-2, could combine to form a heteromeric channel distinct from those formed by either GLC-1 or GLC-2 homomers (Cully et al, 1994), an observation repeated for AVR-15 and GLC-2 (Vassilatis et al. 1997). In these studies, the distinction between homomeric and heteromeric channels was relatively straightforward, based on the different responses of the channels to both glutamate and ivermectin. In the latter case, the reported expression of both AVR-15 and GLC-2 in the pharynx (Vassilatis et al. 1997; Laughton et al. 1997) strengthened the case for the incorporation of both subunits into the same receptor. However, there have been few later studies attempting to express combinations of GluCl subunits. Wang et al. (2021) recently published genetic evidence that, in *C. elegans*, mutations in *glc-3* and *glc-4* both altered the response of worms to elevated temperatures. Both subunits were expressed at the presynaptic region of AIY interneurons. Since GLC-4 does not form channels when expressed alone, this combination of expression and genetic data may indicate that GLC-3 and GLC-4 combine to form the relevant GluCl in AIY. The single cell transcriptomic data for AIY (Taylor et al. 2021) confirms that *glc-3* is highly expressed in this cell type and that *glc-4* mRNA is also present, though at lower levels. A third GluCl gene, *avr-14,* is also expressed in AIY but mutations in this gene did not have the same effect as those in *glc-3* or *glc-4* (Wang et al. 2021), perhaps implying that AVR-14 might be part of a different GluCl in these cells.

For GluCl of parasitic nematodes, there have not been many reports of the successful expression of heteromeric GluCl. In both *C. oncophera* and *H. contortus* the AVR-14B and GLC-2 subunits form functional heteromeric channels (Njue et al. 2004; Atif et al. 2019), and the existence of this subunit combination is supported by immunolocalization data from *H. contortus* (Portillo et al. 2003) showing expression of these subunits in similar locations in inhibitory motor neurons. Very recently Lamassiaude et al. (2021) compared the expression of the GluCl of three nematodes in *Xenopus* oocytes: *C. elegans* and the parasites *Parascaris univalens* and *Brugia malayi*. They found that *avr-14*, *glc-2*, *glc-3* and *glc-4* were present in all three species. Based on expression of combinations of cRNAs expressed in the oocytes these authors presented strong evidence that AVR-14B and GLC-2 could co-assemble into a novel GluCl in all three species. GLC-2 and GLC-3 from *C. elegans* and *P. univalens,* but not *B. malayi*, also combined to form heteromeric channels. They also showed some data that might suggest that co-expression of Pun-GLC-4 with Pun-GLC-3 led to the formation of functional GluCl and that Cel-GLC-4 could interfere with the expression of Cel-GLC-2 channels. In the case of the GLC-2/GLC-3 channels, these were not directly activated by ivermectin or moxidectin

but the drugs did potentiate the response to L-glutamate, an observation that has been made for several other GluCls.

4.2 Localisation data

The expression patterns of GluCl in specific cells and tissues have been determined by several means. Until recently, most of the data from *C. elegans* was obtained using reporter gene methods, where an easily identified protein, often green fluorescent protein (GFP) or a derivative, is expressed in transgenic worms under the control of the putative promoter. Using this approach, the expression of AVR-15 and GLC-2 in the pharynx was demonstrated (Laughton et al., 1997; Vassilatis et al. 1997), explaining the ivermectin sensitivity of pharyngeal pumping in *C. elegans* and parasites. AVR-14 is expressed widely in the nervous system, including in the motor and sensory circuits (Dent et al. 2000; Glendinning et al. 2011). GLC-3 is expressed in AIY, interneurons that integrate signals from the amphid sensilla (Chalasani et al. 2007; Wang et al. 2021). There is little information in the published literature concerning the expression patterns of GLC-1 and GLC-4. In a related approach, Liu et al. (2004) used a putative promoter sequence from *Hco-glc-5* to drive expression of GFP in *C. elegans* and detected its presence in the MC and M2 pharyngeal neurones, but not in pharyngeal muscle cells.

Expression of GluCl subunits in parasitic species has been mostly studied using specific antibodies or in situ hybridisation techniques, both of which can be technically challenging. In *H. contortus*, expression of AVR-14, GLC-2 and GLC-5 has been reported from parts of the motor nervous system and the nerve ring (Delany et al. 1998; Jagannathan et al. 1999; Portillo et al. 2003) including GABAergic motor neurons. AVR-14 was also reported to be expressed in the dorsal nerve cord of *Ascaris suum* (Jagannathan et al. 1999). Interestingly, AVR-14A immunoreactivity was detected in *H. contortus* amphid neurons (Portillo et al. 2003) which are deranged in some resistant isolates of this parasite (Freeman et al. 2003). Using in situ hybridisation, Li et al. (2014) showed that Bma-AVR-14 is expressed in the reproductive tissue of adult *Brugia malayi*, perhaps explaining the sterilising effects of ivermectin filarial nematodes. In contrast, Moreno et al. (2010) used specific antibodies to localise AVR-14 to structures in the vicinity of the excretory/secretory pore of *B. malayi* microfilariae; a possibly surprising result that could explain the inhibition of parasite protein and vesicle secretion following ivermectin treatment in vitro (Moreno et al. 2010; Harischandra et al. 2018; Loghry et al. 2020). It also stimulated renewed speculation that an important part of the anthelmintic action of the A/Ms was due to their interference with parasite mechanisms of immune evasion (Wolstenholme et al. 2016).

More recently much more information has become available through the use of transcriptomics data from single cells or small regions of tissue. For *C. elegans*, single cell transcriptomic data for all the individual neurons of L4 larvae is available at https://cengen.org (Taylor et al. 2021). In a similar approach, Airs et al. (2022) have used spatial transcriptomics to associate anthelmintic targets, including GluCls, with the behaviour of *B. malayi*. Given the larger size of adult parasites, single cell approaches are much more challenging, so Airs et al. (2022) examined the anterior 1mm of adult female *B. malayi* and dissected micro-regions from it. They used RNA tomography to obtain cryosections with about 10 nucleated cells/section. One use of these data could be to identify genes that are expressed in the same place and whose products could, in principle, interact to form heteromeric channels. A detailed analysis of the data in these publications pertaining to the GluCls is beyond the scope of this short review but even a superficial examination revealed some points of interest. In *C. elegans*, expression of *glc-1* was detected in very few cells and tissues, including neurons associated with amphids, epidermis and intestine. Potential partners for GLC-1 were GLC-2 in the epidermis, perhaps supporting the original results of Cully et al. (1994) and GLC-4 in the amphid neurons AWA and intestine. The limited expression pattern of *glc-1* is perhaps consistent with the absence of orthologues of this gene in parasitic species, but it is of interest that the amphids have long been considered a potential route of entry for A/Ms into nematodes (Page, 2018) and population genetic data showed that variations in *glc-1*correlate with natural variation in drug sensitivity between wild *C. elegans* strains (Ghosh et al.). *glc-2* is expressed in several neuron classes associated with the pharynx, some interneurons and cells associated with sensory sensilla, as well as the epidermis. *glc-3* is expressed at highest levels in cells associated with the sensory system, including the integrating interneurons AIA and AIY, and the labial sensilla. It is also expressed in motor neurons innervating head muscles and in the connection between CNS and pharynx. Many of these cells also express significant levels of the other GluCl mRNAs. Expression of *glc-4* was found in a large number of cell types, despite the lack of evidence for any function for this gene. This included many different types of interneurons and motor neurons innervating the head muscles, plus some that innervate body wall muscle and the vulva. In almost all these cell types, *avr-14* and sometimes *avr-15* expression was also detected. The cell with highest levels of expression of *avr-14* was the head mesodermal cell (hmc), which is interesting as this cell is in intimate contact with the excretory gland cell and the two may be electrically coupled (Hall and Altun, 2008; Mathies et al. 2019). This raises the possibility that GluCl expressed in hmc could regulate excretion or secretion from the worm and this would explain the effects of ivermectin on this process. It is also possible that the immunoreactivity observed by Moreno et al. (2010) in *B. malayi* mf could be in the equivalent cell in the parasite and this could explain how IVM inhibited secretion. *avr-15* is also expressed in hmc and in the IL1 ciliated neurons, the cell type with the second highest level of *avr-14* expression, raising the possibility that either these cells express two distinct GluCls or that AVR-14 and AVR-15 might co-assemble. In other cell types *avr-14* expression was detected alongside *glc-2*, *glc-3* or *glc-4* and sometimes all three, which argues for the

expression of multiple forms of the GluCl on these cells or, alternatively, for a complex subunit composition (Figure 1). *Avr-15* is also expressed widely in neurons, especially in interneurons and head motor neurons as well as pharyngeal muscle. Although there are some cells where it is only co-expressed with *avr-14*, in most expression of either *glc-2* or *glc-4* was also detected. It is therefore complicated to predict the subunit composition of native GluCl based on these data. AVR-15 and GLC-2 are the only subunits present in pharyngeal muscle, as suggested by reporter gene studies, and are therefore likely to co-assemble. AVR-14, AVR-15 and GLC-3 are all capable of forming functioning homomeric channels and perhaps they can also be incorporated into heteromeric receptors, together with either GLC-2, GLC-4, or both as structural subunits (Figure 1). There is ample precedent for this complexity of subunit composition of pLGIC from nematode nicotinic acetylcholine receptors (Wolstenholme and Neveu, 2017).

These data from *C. elegans* confirm that GluCl have major roles in sensory perception, pharyngeal function, the regulation of movement, especially of the head, and in reproduction. They also support a role in excretion/secretion. The effects of ivermectin on parasitic species confirm many of these functions. The data of Airs et al. (2022) for *B. malayi* are largely consistent with this, though given the reduced complement of GluCl genes and subunits in the parasite, some differences are inevitable. *Bma-avr-14* and *Bma-glc-2* were enriched in body versus head tissues, with *Bma-glc-3* and *Bma-glc-4* more concentrated in the head. The spatial distribution of *Bma-avr-14* expression in the head region correlated best with that of *Bma-glc-3*, and *Bma-glc-3* was the most highly correlated partner for *Bma-glc-2* and *Bma-glc-4* as well. Their data provide some support for the existence of a Bma-GLC-2/Bma-GLC-3 receptor, as reported by Lamassiaude et al. (2021) and suggest that in this species GLC-3 might be a central component of native GluCls.

5 Other possible targets

It is perhaps not surprising that the avermectin/milbemycin drugs can interact with many different targets. Their effects on nematode GABA receptors have already been mentioned, and alleles of *Hco-lgc-37* have been associated with ivermectin resistance and changes in the effects of ivermectin on a recombinant GABA receptor formed by co-expression with Cel-GAB-1 (Feng et al. 2002). Ivermectin has effects on some nicotinic receptors too, acting as a positive allosteric modulator (Krause et al. 1998). In addition, a wide range of other targets has been reported (reviewed by Laing et al. 2017; Martin et al. 2021), mostly at concentrations much greater than those which are effective against nematodes. Some of these effects may account for the in vitro activity of ivermectin against viruses, including SARS-2, at very high concentrations: for example, Caly et al. (2020) showed inhibition in vitro at 5µM IVM, compared to normal plasma concentration 1000-fold lower than this for anthelmintic effects. However, there is overwhelming evidence that the anthelmintic effects of

ivermectin, moxidectin etc. result from their actions at GluCls, and that the complexity of those effects is reflected in the complexity of the subunit composition, expression patterns and functions of those receptors.

6 Conclusions

There is overwhelming evidence that the anthelmintic effects of the avermectin/milbemycins are mediated via their effects on nematode GluCls. However, much remains to be understood about the subunit composition and exact localisation of these receptors, and how these might differ between parasitic nematode species. Studies on *C. elegans* have yielded, and continue to yield, valuable insights but are likely to be insufficient to completely understand how the drug interacts with parasites.

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483 **Table 1 Glutamate-gated chloride channel subunit genes**

Gene	Subunit properties	Expression pattern in *C. elegans*
avr-14	Alternatively-spliced. AVR-14 B forms glutamate and IVM-gated channels.	Widely expressed in nervous system
avr-15	Alternatively-spliced. AVR-15B forms glutamate and IVM-gated channels and co-assembles with GLC-2.	Expressed in pharynx and widely in nervous system.
glc-1	Forms IVM-gated channels. Co-assembles with GLC-2 to form glutamate-gated channels.	Expressed in amphid neurons.
glc-2	Forms glutamate-gated channels. Co-assembles with GLC-1 or AVR-15B to form IVM-gated channels.	Expressed in pharynx
glc-3	Forms glutamate and IVM-gated channels.	Expressed in interneurons downstream of sensory neurons
glc-4	Has not been shown to form channels	Widely expressed in nervous system
glc-5	Forms glutamate and IVM-gated channels.	Not present in *C. elegans*. Expressed on ventral nerve cord and motor neuron commissures of *H. contortus*.
glc-6	Rescues mutations in IVM-sensitive GluCl.	Not present in *C. elegans*

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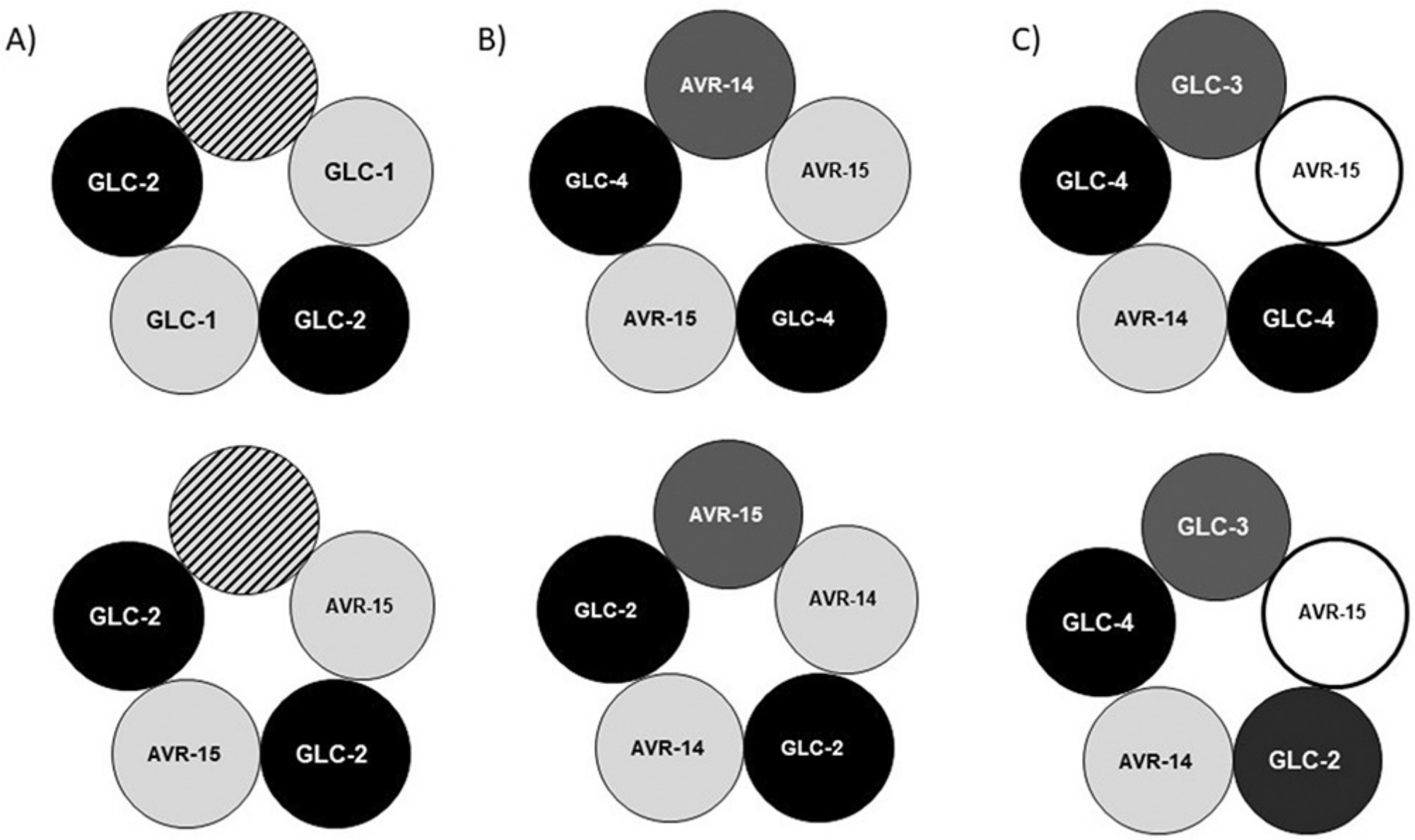
Figure Legend

Figure 1. Speculative subunit composition of native GluCl composed of two, three, four or five different subunits. For the sake of these illustrations GLC-2 and GLC-4 are treated as potential structural subunits, analogous to the 'β' subunits of the nicotinic acetylcholine receptors. A) Potential channels composed of two subunits: the GLC-1 + GLC-2 receptor was reported by Cully et al. (1994) and the AVR-15 + GLC-2 receptor by Vassilatis et al. (1997). In each case the stoichiometry is unknown, but the properties of the channel may vary depending on whether it 2:3 or 3:2, as shown for nicotinic receptors (Zwart and Vijverberg, 1998). B) Potential channels composed of three different subunits. Different possible combinations of AVR-14 and AVR-15 with GLC-2 or GLC-4 are shown, though others may of course exist. In *C. elegans*, *avr-14*, *avr-15* and *glc-2* transcripts were detected in RIB interneurons; *avr-14*, *avr-15* and *glc-4* transcripts were detected in head motor neurons and elsewhere. Data from https://cengen.org. C) Potential channels composed of four or five subunits. The expression of *avr-14*, *avr-15*, *glc-3* and *glc-4* transcripts was detected in AIA interneurons. The expression of the five subunits was detected in RIP interneurons and NSM pharyngeal neurosecretory-motor neurons (cengen.org).


A)
GLC-2
GLC-1
GLC-1
GLC-2
GLC-2
AVR-15
AVR-15
GLC-2
B)
AVR-14
GLC-4
AVR-15
AVR-15
GLC-4
AVR-15
GLC-2
AVR-14
AVR-14
GLC-2
C)
GLC-3
GLC-4
AVR-15
AVR-14
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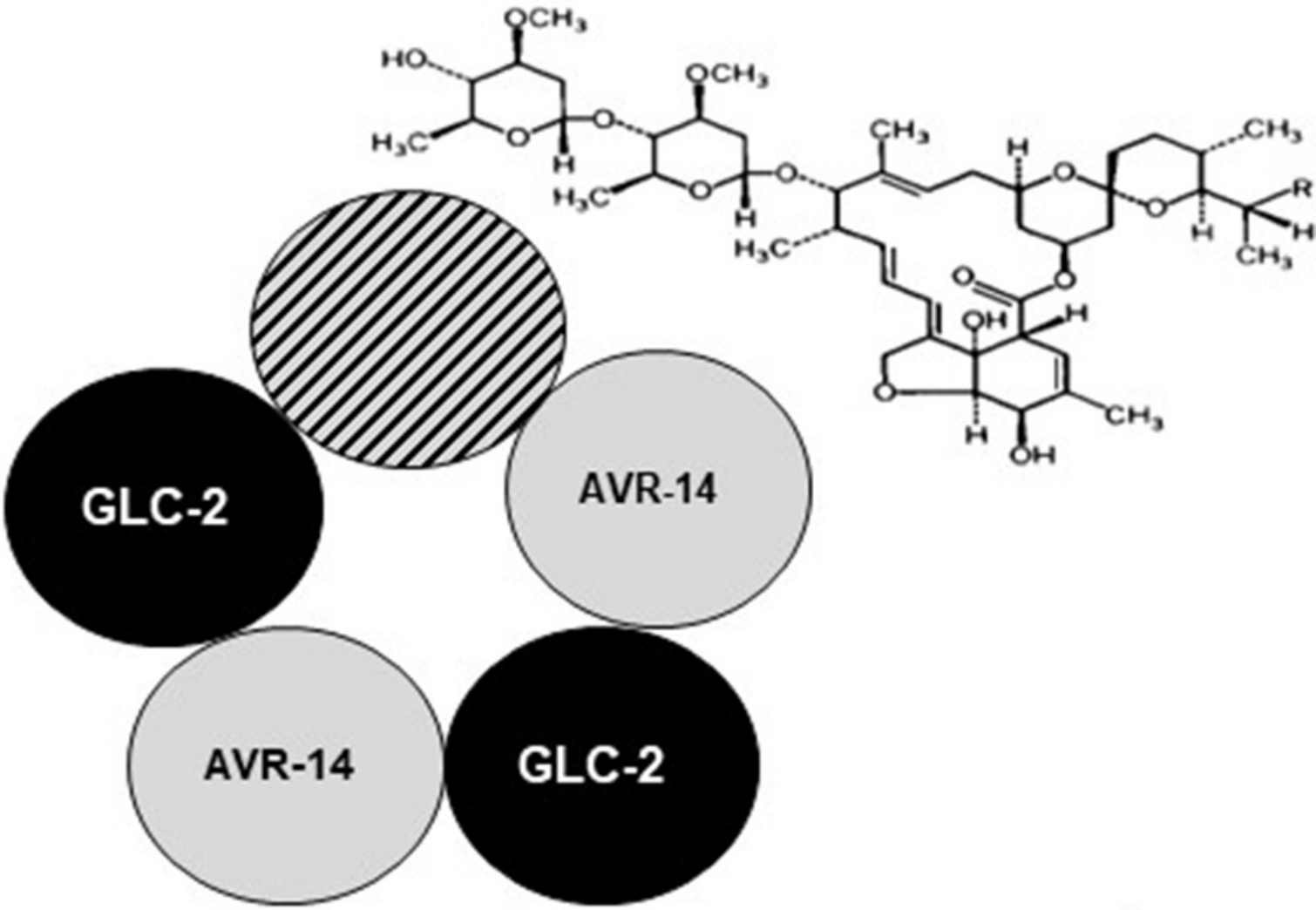
GLC-4
AVR-15
AVR-14
GLC-2


GLC-2
AVR-14
AVR-14
GLC-2