# The Role of Glycoconjugates as Receptors for Insecticidal Proteins

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**Abstract**

Bacillus thuringiensis (Bt) proteins are an environmentally safe and effective alternative to chemical pesticides and have been used as biopesticides, with great commercial success, for over 50 years. Global agricultural production is predicted to require a 70% increase until 2050 to provide for an increasing population. In addition to agriculture, Bt proteins are utilised to control human vectors of disease – namely mosquitoes – which account for >700,000 deaths annually. The evolution of resistance to Bt pesticial toxins threatens the progression of sustainable agriculture. Whilst Bt protein toxins are heavily utilised, the exact mechanisms behind receptor binding and toxicity are unknown. It is critical to gain a better understanding of these mechanisms in order to engineer novel toxin variants and to predict, and prevent, future resistance evolution. This review focuses on the role of carbohydrate binding in the toxicity of the most utilised group of Bt pesticidal proteins – three domain Cry (3D-Cry) toxins.

## Introduction

*Bacillus thuringiensis* (Bt) is a gram-positive bacterium that produces a large variety of insecticidal δ-endotoxins during sporulation. These proteins may be lethal to insects and/or nematodes yet are innocuous to vertebrates and plants. Additionally, Bt proteins demonstrate species-specific activity, allowing for the eradication of harmful pests that destroy crops and spread disease without exterminating beneficial insect species. Bt proteins are an environmentally safe and effective alternative to chemical pesticides and have now been used as biopesticides for over 50 years. In addition, genes encoding Bt proteins have been incorporated in crops such as corn and cotton with huge commercial success Sandhu (2020). The exact mechanisms behind Bt protein(s) toxicity are unknown, and increasing understanding is critical for the development of new Bt proteins, and to counteract emerging field resistance.

Bt pesticidal proteins may be produced during sporulation (crystal and cytolytic proteins) or the vegetative growth phase and are generally organised into a number of categories based on structural families, according to a recently-revised nomenclature (Crickmore 2020). The 3D-Cry toxins form the largest known group and are also the most mechanistically well-characterised – especially those that are lepidopteran active. Following ingestion by invertebrates, 3D-Cry activity is proposed to occur by either of two models; the most-widely known sequential binding pore-forming (Bravo 2007; Rodriguez-Almazan 2009; Schnepf and Whiteley 1981) or the alternative G-protein mediated apoptotic signalling

pathway model (Castella 2019; Mendoza-Almanza 2020; X. Zhang 2006). In the sequential binding model, Cry crystals are solubilised in the specific pH and physiological conditions of the insect gut, producing monomeric protoxins. The monomers are subsequently activated by host proteinases, yielding activated Cry proteins, which bind target receptors on the brush border membranes of midgut epithelial cells. This is followed by cleavage within the α-helical domain I by host proteinases, triggering toxin oligomerisation to form a pre-pore structure necessary for insertion into the phospholipid bilayer to form a channel. This culminates in cell death via colloid-osmotic lysis. There is increasing evidence that other routes to pore formation via receptor binding may exist and that the sequential binding model may not be a universal pathway (Endo 2022; D. Sun 2022; Vachon 2012). The signalling model differs in that there is no pore insertion, with cell death induced, instead, via the activation of an apoptotic signalling cascade – although this is not a widely accepted hypothesis.

Although significantly different at the amino acid level, active 3D-Cry proteins have a characteristic conserved 3-domain architecture (D-I to D-III) indicative of a similar mechanism of action. Crystal structures are available for a number of activated 3D-Cry (Cry1Aa (Grochulski 1995), Cry1Ac (Derbyshire 2001), Cry2Aa (Morse 2001), Cry3Aa (Heater 2020), Cry3Bb1(Galitsky 2001), Cry4Aa (Boonserm 2006), Cry4Ba (Boonserm 2005), Cry5Ba (Hui 2012), Cry7Ca1 (Jing 2019), and Cry8Ea1 (S. Y. Guo 2009) along with a number of mutant and chimeric forms) and all show a conserved structural arrangement. Domain I is linked to pore formation and consists of a helical bundle with a central hydrophobic helix-α5, associated with initialising membrane insertion, encapsulated by six amphipathic helices. Domain II and III are associated with receptor binding and are β-sheet rich domains resembling lectins. Both domains present structural homology to carbohydrate binding proteins, such as lectin jacalin and sialidase, respectively. This structural similarity implies that carbohydrate residues may play a critical role in receptor binding for 3D-Cry proteins – although the exact mechanisms by which this occurs remain somewhat unknown. The 3D crystal structure of the Cry1Ac1 protoxin has recently been elucidated, presenting four cysteine-rich prodomains (D-IV to D-VII) (Evdokimov 2014). Domains IV and VI are alpha helical bundles that resemble spectrin or bacterial fibrinogen-binding complement inhibitor, whilst D-V and D-VII are beta-rolls that closely resemble the carbohydrate-binding moieties seen in sugar hydrolases of Family 6 carbohydrate binding module – and similar to that seen in D-II and D-III. Aside from a few recent investigations (Pena-Cardena 2018;

Zghal 2017), prodomain studies have largely indicated that it is dispensable for insecticidal activity, and instead has roles in optimising crystal formation, packing different toxin variants into the same crystal, stability, selective solubilisation, and ensuring synchronous delivery through oligomerisation (Evdokimov 2014; Hofte and Whiteley 1989; Luthy and Ebersold 1981).

Cry proteins are usually highly selective to their target insect orders, and it is unusual to find a Cry protein that effectively targets more than one order – although exceptions exist, such as Cry2Aa which has activity against Lepidoptera (Donovan 1988) and Diptera (Yamamoto and Mclaughlin 1981b), and Cry1Ba which has been shown to target Hemiptera (Fernandez-Luna 2019),Lepidoptera (Simpson 1997), Diptera and Coleoptera (Zhong 2000). As well as the unique domain structure in individual Cry proteins, target selectivity is determined by the presence of the receptor proteins and lipids in the target insect midgut. A relatively strong understanding of this process has been derived in Lepidoptera, where several protein types have been identified to function as Cry receptors, including; cadherin-like proteins (CAD; (Gahan 2001; Nagamatsu 1998; Vadlamudi 1993; Vadlamudi 1995)), GPI-anchored aminopeptidases (APN; (S. S. Gill 1995; Knight 2004; Rajagopal 2002; Sangadala 1994)), GPI-anchored alkaline phosphatases (ALP;(Jurat-Fuentes and Adang 2004; Sangadala 1994)), and ABC transporters (Sato 2019). Similar receptors have been identified in other orders, for example mosquitoes (Diptera) utilise cadherins (Cry4Ba, Cry11Ba and Cry11Aa), APNs (Cry11Ba), and ALPs (Cry11Aa). A series of more recent work has identified that glycosphingolipids (GSLs) can also function as Cry5B and Cry14A receptors and mediate toxicity in the nematode *Caenorhabditis elegans* (Griffitts 2003; Griffitts 2005).

Resistance development against insecticidal toxins is a common phenomenon, and a wide array of resistance mechanisms has been identified from both laboratory and field studies (Peterson 2017). The most common mechanism appears to be altered Cry binding to receptors (Ferre and Van Rie 2002). Cadherins have received substantial attention due to their commonality as lepidopteran receptors and major mutations causing significant resistance to Cry1Ac have been identified in multiple strains of *Heliothis virescens* (Gahan 2001), *Pectinophora gossypiella* (Fabrick and Tabashnik 2012; Fabrick 2014; Morin 2003; Tabashnik 2004; Tabashnik 2005) and *Helicoverpa armigera* (Xu 2005; Y. Yang 2006; Y. Yang 2007; H. Zhang 2013; J. Zhao 2010), yet it is clear that cadherin binding and expression can be identical between resistant and susceptible strains (Bel 2009; Siqueira 2006). This, alongside other studies, has led to the common hypothesis that a combination of

other putative Cry binding moieties, such as APNs, ALPs, GSLs etc, may be required for full toxicity.

This review will focus on appraisal of the literature surrounding the relevance of carbohydrate moieties in eliciting the insecticidal action of 3D Cry proteins. In addition to the aforementioned Cry5B and Cry14A, there is ample precedent for the role of glycoconjugates as receptors for protein toxins – as is the case for cholera toxin (Holmgren 1975; Kabbani 2020), aerolysin (Abrami 2002), shiga toxin (Smith 2006) and ricin (Sandvig 1976). To understand how Cry toxins exploit carbohydrate moieties for toxicity in more detail, we will also provide a beginner's overview to the current understanding of the structural diversity, biosynthesis and function of insect glycoconjugates, as well as comparing insect glycopatterning to the better characterised pathways and glycoconjugate species present in mammals.

## Glycoprotein glycans in insects and nematodes

The addition of an oligosaccharide chain to a protein backbone (glycosylation) is an extremely common posttranslational modification in eukaryotes. A substantial array of studies have concluded that glycoprotein moieties play critical roles in cell signalling, cell migration, cell-cell interactions, blood group determination and immune cell trafficking – with changes in N-glycosylation associated with diverse disorders including cancers (Kodar 2012), Crohn's disease (Verhelst 2020) and diabetic kidney disease (Bermingham 2018). The distinct and divergent glycosylation patterns observed are driven by an orchestra of glycosidases and glycosyltransferases, which differ in terms of substrate specificity, and both temporal and spatial expression. The exact size and structure of the oligosaccharide can dramatically alter the biophysical properties of the protein - effectively significantly diversifying the functions of a single gene product.

As with vertebrates, insects and nematodes demonstrate both major forms of glycosylation; N-linked (attached to Asn in an Asn-X-Ser motif, where X is not Pro) and O-linked (attached via Ser/Thr). As in mammals, insect and nematode N-linked glycosylation begins in the endoplasmic reticulum (ER) with the cotranslational transfer of a dolichol-linked precursor oligosaccharide to the asparagine side chain of the consensus sequence within a nascent protein. This precursor is subsequently processed in multiple stages to form mature variants in the ER and Golgi. O-glycosylation also occurs in the ER, Golgi and,

occasionally, the cytoplasm but unlike N-linked does not begin with a common oligosaccharide precursor.

The vast majority of knowledge on insect glycoconjugates comes from the model organism *Drosophila melanogaster* (order Diptera), although there are now, collectively,a generous number of studies on the glycomes of species within the orders Lepidoptera (Cabrera 2016; Fuzita 2020; Stanton 2017), Hemiptera (Scheys 2019), Hymenoptera (Hykollari 2019), andNematoda (Cipollo 2005; Paschinger 2008; Vanbeselaere 2018; C. Wang 2021). Genome completion of *Drosophila* and random mutagenesis studies have enabled the elucidation of putative genes for glycoconjugate biosynthesis and the functional impact of altering glycan patterning (Seppo and Tiemeyer 2000; ten Hagen 2009).

**N-linked protein glycosylation**

All N-glycans share the same pentasaccharide core, termed paucimannose ($Man_3GlcNAc_2$), - a core conserved from protozoan to metazoan. After the dolichol-linked precursor oligosaccharide ($Glc_3Man_9GlcNAc_2$) has been transferred to the protein, resident ER glucosidases and mannosidase remove three glucose residues and a mannose residue, respectively. For most glycoproteins, mannose residues are further trimmed in the Golgi generating a high mannose structure ($Man_5GlcNAc_2$), followed by GlcNAc transferase (GlcNAcT-1) mediated conversion into a hybrid glycan ($GlcNAcMan_5GlcNAc_2$), and mannosidase II mediated conversion into $GlcNAcMan_3GlcNAc_2$. In invertebrates, this glycan can be trimmed further to generate paucimannose ($Man_3GlcNAc_2$, **Figure 1**) – an N-glycan that has only rarely, and relatively recently, been detected in vertebrates (Balog 2012; Lattova 2010; Zipser 2012). These initial trimming stages can be followed by additional enzymatic steps to add diverse sugar residues and generate more complex N-glycans.

Initial studies on N-linked glycans in *Drosophila* larvae and cultured *Drosophila* S2 cells showed a predominance of high ($Man_5GlcNAc_2$) and paucimannose ($Man_3GlcNAc_2$) moieties, suggesting an absence of more complex glycans (G. F. Parker 1991; Williams 1991). These simple N-glycans can be fucosylated via α1-6 and α1-3 linkages to the reducing terminal N-GlcNac. This is divergent from vertebrates where, although N-glycans have paucimannose as a core, the simplest N-glycan is chiefly $GlcNAcMan_3GlcNAc_2$. Furthermore, vertebrates only fucosylate N-glycans at the α1-6 linkage. Later work, after completion of the *Drosophila* genome, elucidated candidate glycosyltransferases required for the generation of more complex glycans. This, combined with improved analytical

techniques, led to several mass spectrometry-based studies which established the presence of hybrid, biantennary, and triantennary *Drosophila* glycoproteins – including sulphated, glucuronylated, and sialylated structures (Aoki 2007; Koles 2004; North 2006) - although the degree of sialyation is hotly debated (Ghosh 2018; Marchal 2001), with the only published studies reporting N-linked sialylated structures at a 0.01% or unquantifiable level (Aoki 2007; Koles 2007).

Although simple N-glycans ($Man_5GlcNAc_2$, $Man_3GlcNAc_2Fuc$) have been predominantly observed throughout *Drosophila* embryogenesis, the exact profile of N-linked glycans is shown to be both spatially and temporally controlled (Aoki 2007; Aoki 2008). This is indicative of stage and tissue-specific glycoprotein requirements and an associated regulation of glycosylation machinery, which can shift the balance between paucimannose and complex structures. More than 40 distinct glycoprotein species, all containing a paucimannose core, have now been identified in *Drosophila,* yet as observed in the earlier studies, these complex glycans are only present as minor components, with the vast majority remaining as unmodified high mannose or paucimannose structures. This is again distinct from mammals, where complex N-glycans with abundant sialylation are predominant. This invertebrate-specific abundance of paucimannose has been partially explained by the elucidation of a *Drosophila* hexosaminidase - β-N-acetylglucosaminidase, encoded by the gene *fused lobes* (*fdl*) (Aumiller 2006; Geisler 2008; Leonard 2006). This enzyme removes GlcNAc residues that are added by N-acetylglucosaminyltransferase I (GlcNAcT-I), resulting in formation of paucimannose (and its fucosylated derivatives), whilst blocking progression to more complex glycans. Human isoenzymes (*HEXA* and *HEXB*) have been shown to drive paucimannosidic protein production in neutrophils (Ugonotti 2022), through a noncanonical cascade that is only proposed to occur in limited tissues and (patho)physiological conditions (Chatterjee 2019; R. Parker 2021) – unlike the constitutive and ubiquitous utilisation of this pathway in invertebrates.

Several groups have utilised mass spectrometry to analyse glycoproteins in another well-characterised model organism, the nematode *C. elegans*; a body of work that has been reviewed in great detail by Paschinger *et al.* (Paschinger 2008). As with *Drosophila*, its well characterised genetics helped identify candidate enzymes associated with the synthesis of hybrid and complex glycans; homologues of N-acetylglucosaminyltransferase I (S. Chen 2002; Zhu 2004), II (S. Chen 2002), and V (Warren 2002). Mass spectrometric analysis of *C. elegans* N-glycans has shown, as in *Drosophila*, an abundance of high-mannosidic class

glycoproteins ($Man_{5-9}GlcNac_2$). Paucimannosidic structures ($Man_3GlcNAc_2\,Fuc_{0-3}$) are also copious in *C. elegans*, in which, as in *Drosophila*, the core can be fucosylated via α1-6 and α1-3 core linkages (Cipollo 2005; Hanneman 2006; Haslam 2002; Natsuka 2005; Paschinger 2004). Despite the similarities, these studies also highlight several distinctive and unique features of *C. elegans* N-glycan species. For example, *C. elegans* glycan species can by fucosylated at, up to, 3 residues on the $Man_{2-3}GlcNAc_2$ core and 5 fucose residues on the mature glycan (**Figure 1iii)** (Paschinger 2019). More complex *C. elegans* glycans can link phosphorylcholine (PC) groups to a core or terminal GlcNAc. This modification is thought to be relatively frequent in the glycoproteins of *C. elegans* and other nematodes compared to other invertebrates (Martini 2019; Stanton 2017), and associated with immunomodulatory properties (Harnett 1998; Pineda 2014) and/or be related to nematode growth and development (Lochnit 2005). Longitudinal studies in *C. elegans* have noted the N-glycan profile was distinct at each developmental stage studied, and an increased degree of N-glycan complexity and PC-presence in the L1 and Dauer stages – *C. elegans* stages associated with significant lifestyle changes (Cipollo 2005). Roughly 150 different N-glycan species have been identified in *C. elegans* and, as with *Drosophila*, the relative proportion of higher order glycans is low, suggestive of a gene acting in a homologous way to the *Drosophila fdl*. Recent studies have shown that mutant *C. elegans* with a partial deletion of a β-N-acetylhexosaminidase (*hex-2*), produce proportionally less paucimannose (Gutternigg 2007), although significant amounts are still detectable indicating the existence of supplementary *C. elegans* β-N-acetylhexosaminidase genes (*hex-3, -4, -5*).

Considering the number of N-glycan structures identified, alongside the potential modifications, the structural N-glycan diversity in insects and nematodes is vast, as is the repertoire of associated roles and locations (cell surface, ion channels, adhesion, extracellular matrix among others). In fact, apart from the lack of sialylation, structural diversity is reported as comparable to that of mammals (Walski 2017). Furthermore, inter-species diversity is also clear. This is highlighted by a recent comparative study showing minimal overlap in the N-glycoprotein profiles from four phylogenetically diverse insecta; the flour beetle (*Tribolium castaneum,* Coleoptera), the silkworm (*Bombyx mori*, Lepidoptera), the honeybee (*Apis mellife*ra, Hymenoptera) and the fruit fly *D. melanogaster* (Diptera) (Vandenborre 2011). The relevance of this diversity is yet to be fully understood with many questions remaining on establishing synthetic pathways, determining the functional relevance of N-glycans, and understanding the spatio temporal control throughout a life cycle. Indeed,

shifts in glycoconjugate expression could play an important role in determining species susceptibility to a range of glycoconjugate binding toxins.

**O-linked protein glycosylation**

O-linked glycan diversity appears to be one of the most varied sets of posttranslational modifications across organisms and begins with the initial monosaccharide moiety linked to the (glyco)protein via the oxygen atom of serine or threonine (O-S/T). These initial monosaccharides can be O-Xyl, O-Glc, O-GalNAc (mucin-type), O-Man, O-GlcNAc or O-Fuc (**Figure 2 A-F**). Mucin-type O-linked glycosylation appears to be the predominant form in *Drosophila* (the best characterised insect species), for which the core structures and associated biosynthetic stages are conserved in vertebrates (as shown in **Figure 2C**). Mucin-type glycans can be categorised by different core structures. In *Drosophila*, unmodified core-1 structures (Galβ1-3GalNAcα1-O-S/T or the 'T-antigen') are predominant (North 2006). Core-1 structures modified with glucuronic acid (GlcA), core-2 structures (GlcNAcβ1-6(Galβ1-3)GalNAcα1-O-S/T), and a less-well characterised HexNAc-GalNAc core structure are also present in a comparatively reduced abundance (where Hex = any 6 carbon monosaccharide) (Aoki 2008; Breloy 2008). Lectin binding and mass spectrometry based characterisation of the O-glycan profiles in lepidopteran (Sf9 from *Spodoptera frugiperda*, Mb from *Mamestra brassicae* & Tn from *Trichoplusia ni*) and dipteran (S2 from *D. melanogaster*) cell lines (Lopez 1999; Thomsen 1990), as well as larvae from two mosquito species (*Aedes aegypti* and *Anopheles gambiae*) (Kurz 2015) have also all demonstrated a prevalence of mucin-type core 1 & 2 structures.

As with N-glycans, an extension of the core O-glycan structure to generate more complex patterning appears to be proportionally reduced in arthropods – in comparison to their mammalian counterparts (Fristrom and Fristrom 1982; Kramerov 1996; North 2006; Theopold 2001). Further structural complexity and species-specific diversity is achieved through post synthetic modifications. For example, glucuronylated and sulphated O-glycans are observed in *Drosophila* (Breloy 2008), *Ae. aegypti*, *An. gambiae,* and various lepidopteran cell lines (**Figure 2 Gi**) (Garenaux 2011; Gaunitz 2013), and phosphoethanolamine is linked to HexNAc residues in wasps and mosquitoes (**Figure 2 Giii**) (Garenaux 2011; Kurz 2015). Insect and nematode glycan diversity could also be heavily influenced by the environment. Indeed, cell media composition has been indiciated to influence the O-glycosylation potential of a range of insect cell lines significantly (Lopez

1999), and an upregulation of mucins (a glycoprotein class where >50% have O-glycosylation), has been reported in the nematode *Laxus oneitus* under conditions of anoxia (Paredes 2022). The exact role of the environment and substrate scavenging in the role of insect glycan synthesis remains to be determined.

Our understanding of the most common O-glycans (O-GalNAc, mucin-type) has been significantly aided through the elucidation of 14 putative *Drosophila* UDP-GalNAc:Polypeptide N-acetylglucosaminyltransferases (pgants)–homologs of the mammalian enzymes required for the initial transfer of GalNAc from the UDP-GalNAc to the Ser/Thr hydroxyl group (Gerken 2008; Ten Hagen 2003a; Ten Hagen 2003b). Biochemical analysis has shown functional conservation between mammalian and *Drosophila* orthologues with some pgants acting as glycopeptide transferases (GalNAc modified substrate) and others as peptide transferases (unmodified peptide substrate). Additionally, *pgant* genes are shown to be spatially and temporally regulated throughout *Drosophila* development, suggesting a distinct regulation of O-glycan patterning (Tian and Ten Hagen 2006). Demonstrating the functional importance of appropriate O-glycosylation, *pgant35A Drosophila* mutants show embryonic, larval, and pupal lethality – the first demonstration of O-linked mucin-type glycosylation being essential for viability (Schwientek 2002b; Ten Hagen and Tran 2002). Further studies with *pgant35A* maternal mutants showed reduced localisation of mucin-type glycans on the apical and luminal surfaces of the developing respiratory system and a loss of tracheal integrity (Tian and Ten Hagen 2007). Lethality is also observed in *Drosophila* that cannot generate the core-1 T antigen -(C1GalTa enzyme mutants) – potentially due to abnormalities in CNS morphogenesis (Lin 2008; Xia 2004).

Alternative O-linked structures (O-Man, O-Glc, O-GlcNAc, O-Fuc, O-Xyl; **Figure 2**) have been detected in *Drosophila* (Kurz 2015), mosquitoes (Kurz 2015), nematodes (Vanbeselaere 2018), lepidopteran cell lines (Lopez 1999) and hymenopteran tissues (Garenaux 2011), demonstrating divergent structures with distinct tissue distributions. Genetic studies investigating the effects of reduced transferase activity have repeatedly demonstrated the importance of this, more minor, glycan patterning (Ju and Cummings 2002; Kelly and Hart 1989; Okajima 2003; Ten Hagen 2003a; Ten Hagen 2003b) and the conservation of functional pathways between eukaryotes. For example, *Drosophila* have two orthologues of the vertebrate O-mannosyltransferases (dPOMT1 and dPOMT2), encoded by *rotated abdomen (rt)* and *twisted, (tw),* which are both required for the mannosylation of protein substrates (Ichimiya 2004; Lyalin 2006). Mutations in either *Drosophila rt* or *tw,* causes defective muscular development and, as the name suggests, a rotated abdomen

phenotype. In humans, mutations in *Pomt* genes are associated with muscular dystrophies (Muntoni 2004b; Muntoni 2004a), highlighting the functional similarities of vertebrate and insect O-glycans. As another important example, O-linked fucose (and elongated b3-linked GalNAc generated via *Fringe*) residues are shown to play critical roles in embryonic development in insects and mammals through the glycosylation of Notch receptors and subsequent modification of Notch receptor ligand preferences (Okajima and Irvine 2002; Okajima 2003; Pandey 2019; Sasamura 2003). O-Xyl modification of serine residues represents the first stage in the synthesis of glycosaminoglycan-like O-glycans – linear polysaccharides consisting of a repeating two sugar-unit consisting of a 6-carbon acidic sugar (HexA) and an amino sugar $(HexNAcHexA)_n$. Nematodes, *C. elegans* and *O. dentatum*, have shown conservation of the common mammalian tetrasaccharide core (GlcAβ1-3Galβ1-3Galβ1-4Xylβ-O-Ser) (Guerardel 2001; Yamada 1999), and also shown the addition of galactose and phosphorylcholine (Vanbeselaere 2018). These nematode glycosaminoglycans (GAGs) are demonstrated to be important for development, with the mutation of *C. elegans* xylosyltransferases (*sqv-2* & *sqv-6*) inhibiting GAG biosynthesis, altering vulval morphogenesis and zygotic cytokinesis, and maternal-effect lethality (Hwang 2003). GAG-like glycans have also been identified in *Drosophila* (Yamada 2002), and have been associated with development and facilitating pathogen invasion (Baron 2009; Park 2003). As with N-glycans, the elucidation of currently unknown insect biosynthetic enzymes will help us to dissect the molecular function of *O*-glycans and the relevance of various structural features.

## Glycolipids in insects and nematodes

Glycolipids are lipids with a carbohydrate attached via a glycosidic bond, with known roles in maintaining cellular membrane integrity, facilitating cell-to-cell and intracellular signalling, initiating host immune responses, and determining blood groups. Glycosphingolipids (GSLs) are a subclass of glycolipid where the carbohydrate group is covalently attached to a ceramide backbone moiety (a sphinganine that is amide linked to a fatty acid; **Figure 3**). GSLs are of particular interest when considering potential receptor functions, as they are known toxin receptors (Geny and Popoff 2006), and found enriched in cellular membrane microdomains (lipid rafts) that act as specialised platforms for signal transduction and protein/lipid transport (D. A. Brown and London 1998; Simons and Ikonen 1997).

Initial investigations into insect GSLs in 1973 by Luukkonen *et al.*, showed an absence of complex GSLs in cells cultured from *Aedes albopictus* (Luukkonen 1973). However, later reports identified the first GSLs in arthropods, by utilising 2D high-performance thin-layer chromatography (HPTLC) to indicate the presence of glucosylceramide (GlcCer) and mannosyl-glucosylceramide (Man-GlcCer) in two closely related dipteran species; the larvae of the green-bottle fly, *Lucilia caesar*, and the pupae of the blowfly, *Calliphora vicina* (Dennis 1985b; Sugita 1982a). This was followed by several ground-breaking studies from Sugita, Hori, Dennis, Wiegandt and others, predominantly in the same dipteran species, showing arthropods form an 'arthro-series' of GSLs derived from a single, neutral, Manβ1,4Glcβ-ceramide core - termed mactosylceramide (MacCer) (Dabrowski 1990; Dennis 1985a; Dennis 1985b; Helling 1991; Sugita 1982b; Sugita 1982a; Sugita 1989; Sugita 1990; Weske 1990). This invertebrate-specific glycolipid signature is conserved in nematodes and insects but is divergent from vertebrates, where the majority of GSLs are derived from a lactosylceramide core (LacCer; Galβ1,4Glcβ-ceramide). Using a combination of HPTLC, sequential exoglycosidic digestion, methylation analysis, and direct-inlet mass spectrometry (MS), these aforementioned studies in dipteran insects went on to find neutral, acidic, and zwitterionic GSLs with increasing complexity and oligosaccharide length – all as extensions of the MacCer core. Dipteran GSLs were also identified to be frequently modified with phosphoethanolamine (*P*Etn) linked to C6 of GlcNAc, resulting in a zwitterionic core structure.

*D. melanogaster* has become the predominant choice for studying arthropod GSLs, with the biosynthesis pathways and structural variants now relatively well understood (**Figure 4**) – as summarised in greater detail by Aoki & Tiemeyer (Aoki and Tiemeyer 2010). Analysis of *Drosophila* GSLs indicated the presence of a similar family of variants to that observed previously in *L. caesar* and *C. vicina* (Callaerts 1995; D'Amico and Jacobs 1995; Fredieu and Mahowald 1994; Seppo 2000). However, there are noted *Drosophila* distinctions such as an increased proportion of longer GSLs that are substituted with two *P*Etn residues (Aoki and Tiemeyer 2010; Itonori 2005), and a 4-linked GalNAc (as opposed to a 3-linked GalNAc) in the longest characterised *Drosophila* GSL (Seppo 2000). Studies in other insects and nematodes have also indicated that a distinct species-specific GSL diversity is present (**Figure 4i-iv**). For example, although the MacCer core is most commonly extended with GlcNAc via a β1-3 linkage followed by GalNAc via a β1-4 linkage, *Drosophila* can extend with Gal, rather than GalNAc, followed by Glucuronic acid (GlcA) (**Figure 4i**) (Aoki and

373 Tiemeyer 2010). Additionally, the later steps of biosynthesis appear to diverge between
374 dipterans (*Drosophila* and *Calliphora*) and nematodes. In both these dipteran genera , the
375 common core tetrasaccharide (GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ-Cer) is extended by a
376 GalNAc, whereas *C. elegans* extends with an α1,3 linked Gal. Furthermore, the core GlcNAc
377 can be substituted with phosphorylcholine (PC) (**Figure 4iii**)– a modification that appears to
378 be conserved in parasitic nematodes (Gerdt 1999; Wuhrer 2000). Whether these distinctions
379 always reflect true species-specific GSLs or developmentally regulated expression in the
380 material studied (embryonic, larvae or pupae) is not completely clear. Indeed, GSL synthesis
381 is highly regulated in mammals - both spatially and temporally - with dysregulation
382 prevalent in disease such as storage disorders (Breiden and Sandhoff 2019) and cancers
383 (Furukawa 2019). The ability to diversify functional lipids significantly, early in the
384 biosynthesis pathway, may tailor GSLs for specific spatial or temporal functions – such as
385 development or toxin binding in localised regions of the insect gut. Temporal artificial
386 manipulation of GSL biosynthesis may be a useful approach for investigating toxin binding at
387 different stages in an insect's life cycle. For example, many mammlian studies have utilised
388 small molecule inhibitors of glycolipid biosynthesis pathways, and different cell culture
389 media additives are known to drastically alter cellular glycosylation profiles.
390 The presence of insect gangliosides (GSLs that contain one or more sialic acid
391 residue) remains controversial, as reviewed previously (Ghosh 2018; Marchal 2001). Whilst
392 little is known about insect sialylation, eukaryotic sialylation is well-studied and has diverse
393 roles in development of the central nervous system, immune response, cell death, cell
394 signalling pathways, host-virus interaction, as well as pathogenic implications in Alzheimer's
395 disease and cancer progression (Ghosh 2015; Schauer 2009; Teppa 2016; Varki 2008;
396 Yanagisawa 2015). Sialic acids, sialylated macromolecules and sialyltransferase (ST)
397 enzymes have been reported in a range of insects including *B. mori* (Kajiura 2015), *D.*
398 *melanogaster* (Koles 2004), *A. aegypti* (Cime-Castillo 2015; Di 2017), and *Galleria*
399 *mellonella* (Karacali 1997) but, despite this, insect investigations indicate that gangliosides
400 do not appear to be intrinsically present at a detectable level (Aoki 2007; Koles 2007).
401 Additionally, little is known about the synthesis or function of sialic acid moieties, and there
402 is no structural information surrounding STs. Arthro-series GSLs capped with GlcA on a
403 non-reducing terminal are common and have been identified in flies (*C. vicina & D.*
404 *melanogaster*) (Wiegandt 1992). GlcA carries a negative charge under physiological
405 conditions, prompting comparisons to the sialic acid-containing gangliosides of vertebrates

406 and the term ‘arthrosides’. Currently, there are very limited data to support a functional
407 comparison. Furthermore, sialic acids can be α2-8 linked to additional sialic acids whereas
408 GlcA dimers, to the best of our knowledge, have not been reported.

409 In addition to the sugar component of GSLs, it must also be noted that the ceramide (a
410 sphingoid base backbone linked to a fatty acid) backbone composition also differs between
411 invertebrates and mammals. Mammalian sphingoid bases tend to be longer (generally C18)
412 (Sullards 2003), whereas insect sphingoid bases are generally reported as C14 and C16 and
413 are amide linked to shorter fatty acid chains (Oswald 2015) (**Figure 3A&B**). In many
414 arthropods, ceramide phosphoethanolamine (CPE) is the bulk sphingolipid (**Figure 3D**)
415 (Panevska 2019), whereas only trace amounts of CPE have been detected in mammalian cells
416 (Bickert 2015) and Nematoda (Satouchi 1993) which, instead, favour sphingomyelin
417 synthesis (a ceramide with a phosphocholine group; **Figure 3C**). Distinct biophysical
418 properties have been observed between sphingomyelin and CPE in terms of membrane-order
419 parameters (Bjorkbom 2010; Terova 2005) and the ability to interact with cholesterol and
420 form lipid-rafts (Bjorkbom 2010; Ramstedt and Slotte 2006), suggesting they have differing
421 biological roles (Dawaliby 2016). It may be that these GSL backbone differences play a part
422 in determining binding specificity of insecticidal proteins, yet, to the best of our knowledge,
423 this has not been investigated.

424 As with vertebrates, the complexity of insect and nematode GSLs occurs along
425 common biochemical pathways via specific, glycosyltransferase-catalysed, sequential
426 addition of monosaccharides. Elucidation, and manipulation, of these glycosyltransferases
427 has provided an insight into GSL function and utility. The first committed step in GSL
428 synthesis is through the addition of glucose to ceramide via glucosylceramide synthase
429 (GlcCer). Knockdown of an embryonic *Drosophila* GlcCer homolog caused increased
430 apoptosis, indicating a requirement for GSLs – at least during development (Kohyama-
431 Koganeya 2004). Catalysing the second and third steps in *Drosophila* GSL synthesis are two
432 genes *brainiac (brn)* and *egghead (egh)* - initially proposed to act in the same functional
433 pathway based on similar developmental phenotypes exhibited by their respective mutants –
434 namely an over proliferation of neural cells and enlarged peripheral nerves. The *brn* gene was
435 determined to encode a β1,3GlcNAc transferase directed to transfer GlcNAc preferentially to
436 the Manβ1,4Glc core structure (Muller 2002; Schwientek 2002a), and *egh* to encode a β1,4-
437 mannosyltransferase to form MacCer (**Figure 4**) (Wandall 2003). Both *Brn* and *egh* mutants
438 are lethal, implying a requirement for second and third step sugar addition. Interestingly,

inhibiting the fourth step in GSL synthesis – via null mutation of β1,4N-acetlygalactosaminyltransferases (β4GalNAcTB/β4GalNAcTA) is not lethal, although still causes defects including the ventralisation of ovarian follicle cells (Y. W. Chen 2007). *Drosophila* α1,4-N-acetylgalactosaminyl transferase (α4GTI) synthesises the ceramide-pentahexoside (Mucha 2004), although as fourth step (β4GalNAcTB/β4GalNAcTA) mutants are still viable, this is also presumably non-essential for viability. Toxicity studies in the nematode *C. elegans* (discussed in greater detail below) have found genes homologous to *brainiac* and *egghead*, *bre-5* and *bre-3* respectively.

As with N and O glycans, it is clear that an increasing range of glycolipid structural variants is being identified in insects and nematodes, even if these more complex structures do not make up the majority of the total pool. Key to deciphering the molecular function of these glycoconjugates is the elucidation of glycosyltransferases. Altering glycolipid biosynthesis pathways - through manipulation of glycosylatransferase activity via gene silencing or inhibitory compounds - will help to inform approaches towards current, and novel, methods of pest control.

## Glycoconjugates as membrane receptors for insecticidal and nematocidal toxins

The role of host cell membrane glycoconjugates as toxin receptors has ample precedent (Zuverink and Barbieri 2018). Toxins that rely on glycoprotein binding include pertussis toxin (Stein 1994) and aerolysin (Diep 1998). Examples of protein toxins shown to use lipid-moieties to facilitate entry include the pore-forming toxins lysenin (via sphingomyelin (Yamaji 1998) and cholesterol-dependent cytolysins (Tweten 2005), Shiga toxin (via glycosphingolipid Gb3 (Okuda 2006; Shin 2009), and cholera toxin (via GM1a ganglioside (Wernick 2010). Lipid microdomains are also implicated in toxin binding due to the high concentration of GSLs present. For example, cholera toxin-induced membrane curvature is shown to be dependent on both the multiplicity and specific geometry of GM1a binding sites (Kabbani 2020), and Shiga toxin is localised to Gb3 in lipid rafts (Smith 2006). Some toxins, such as members of the Botulinum toxin family, utilise both a ganglioside and a protein receptor, whereas others, such as ricin, bind a specific carbohydrate moiety that can be present on either a glycolipid or a glycoprotein (Fu 1996; Zuverink and Barbieri 2018). Below we will discuss the existing research surrounding the role of glycoconjugates on insecticidal and nematocidal 3D-Cry protein toxin activity. Lectins are carbohydrate binding proteins which are, individually, highly specific to a distinct sugar group (Cummings and

Etzler 2009). Lectins have been incredibly useful, and widely used, in elucidating the sugar binding properties of various insecticidal toxins; those discussed in this review are summarised in **Table 1**.

Several of the studies, discussed below, utilise cellular models to investigate 3D-Cry binding affinity and toxicity. In these studies, it is worthwhile to consider the impact of pH, as 3D-Cry proteins are solubilised and activated in the midgut lumen due to selective pH conditions (Knowles 1994). In the literature, the insect midgut is often referred to as alkaline – a characteristic that is often cited to assist in conferring insect species selectivity. Indeed, the majority of Dipteran and Lepidopteran species assessed have an alkaline midgut (approx. pH 8.0 – 10.0), although there are exceptions such as *Marasmia trapezialis* (pH 7.0 – 7.2), *Pieris rapae* (pH 7.3 – 7.6), and *Corcyra cephalonica* (pH 7.0 – 7.6) (Berebaum 1980). Furthermore, there are often differences between the posterior and anterior midgut regions, such as *A. aegypti* and *Aedes canadensis* mosquito larvae (approx. pH 8 in the gastric caecum, > pH10 in the anterior midgut, pH 7.5 in the posterior midgut) (Boudko 2001; Dadd 1975). In contrast, other insects can have a mildly acidic midgut such as Coleoptera, *Leptinotarsa decemlineata* (pH 6.5 – 5.36) (Krishnan 2007) and *Diabrotica virgifera virgifera* (pH 5.75) (Kaiser-Alexnat 2009). In terms of cell culture experiments the pH will be determined by buffer or culture media (which are frequently more acidic than mammalian media, approx. pH 6.2 – 6.5). In many experiments the toxin in question is solubilised and activated before addition to cells, via extracted 'midgut-juice' or artificially with buffer and proteinases - which in theory should negate the need for 'mid-gut' conditions for solubilisation and activation but may alter the binding affinities via protonation states of key residues.

## Cry1A (Cry1Aa, Cry1Ab & Cry1Ac)

### *Binding to BBMVs show Cry1Ac binds in a GalNAc-dependent manner*

The Cry1A subclass of lepidopteran-specific toxins are of great commercial importance and the most well studied 3D Cry toxins. The earliest glycoconjugate binding studies were performed using endotoxin isolated from Bt serovar. *kurstaki* HD-1 (Btk HD-1), which was later confirmed to contain three distinct Cry1A proteins that share >76% aa identity as protoxins; Cry1Aa, Cry1Ab and Cry1Ac (Hofte and Whiteley 1989). These early studies proposed the occurrence of a common Cry insecticidal pore-forming action (Hofmann 1988a; Hofmann 1988b), yet identified mechanistic heterogeneity dependent on individual Cry

505 proteins, target species, and putative binding 'receptors'. Of note, early studies using the Btk
506 HD-1 strain also likely contain other Cry proteins including Cry2Aa2, Cry2Ab2 and Cry1Ia3.
507 The relevance of glycoconjugates in eliciting toxin activity was recognised early on,
508 with Knowles *et al.* showing that GalNAc and GlcNAc binding-lectins (SBA and WGA,
509 respectively) neutralised activity of lepidopteran-active δ-endotoxin proteins from Btk strain
510 HD-1 in a lepidopteran cell line (CF1) isolated from the Cry1A-susceptable cabbage butterfly
511 (*Choristoneura fumiferana*) (Knowles 1984). Using the same model, they went on to identify
512 the first putative Cry 'receptor' - a 146 kDa cell-surface glycoprotein capable of binding both
513 SBA and δ-endotoxin (Knowles and Ellar 1986). Dennis *et al.*, first proposed that glycolipids
514 were responsible for modulating δ-endotoxin actions, through demonstrating Btk HD-1 toxin
515 binding to distinct *C. vicina* pupal GSLs – of which some species contained a relevant
516 terminal GalNAc residue (Dennis 1986). In these studies, they isolated both total neutral and
517 total acidic glycolipid fractions, and isolated neutral GSL components that they probed using
518 a thin layer chromotography (TLC) overlay technique to detect binding of both the protoxin
519 and activated forms of Btk HD-1 proteins. Although Btk HD-1 contains a number of toxins
520 (Yamamoto and McLaughlin 1981a), the authors only used the ~130 kDa proteins – most
521 likely representing a mix of Cry1 proteins. Multiple binding partners were observed in both
522 glycolipid fractions, with the main component – bound by both the protoxin and activated
523 forms – being Galα1-3GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1-4Cer (denoted as 5B by the
524 authors). Although both the protoxin and activated form were shown to bind strongly to the
525 Gal-terminal 5B glycolipid, the toxin showed a decrease in binding specificity after
526 activation, with an increased number of glycolipids bound and an increase towards
527 glycolipids with terminal GalNAc residues. Different binding patterns between the pro and
528 active forms would indicate the binding of protoxin would not block activity of the activated
529 protein through competition for binding. When reading these works, it is important to
530 consider that these binding experiments utilised models containing cells derived from non-
531 target tissues, which potentially present glycoconjugates found predominantly outside of the
532 midgut, and in an altered abundance. Brush border membrane vesicles (BBMVs) prepared
533 from larval midguts provided a more '*in vivo'* representation and became common in the
534 field for investigating toxin binding to apical microvilli. Using BBMVs or gut tissues,
535 isolated from a range of lepidopteran species, several investigations confirmed a range of
536 specific Cry1A binding sites with nM affinity constants (Denolf 1993; Ferre 1991;
537 Garczynski 1991; Jaquet 1987; Van Rie 1989, 1990; Wolfersberger 1990). In many cases the

level of Cry1A toxicity was shown to correlate with binding affinity (Denolf 1993; Garczynski 1991; Hofmann 1988b; Van Rie 1989, 1990). For example, Cry1Ab and Cry1Ac recognise the same receptor on *Ostrinia nubilalis* BBMV, yet the former has an 11-fold higher affinity which correlates with a 10-fold higher toxicity (Denolf 1993). The importance of these binding sites was further illustrated by work in a field population of *Plutella xylostella*, where resistance to Cry1Ab was associated with loss of BBMV binding sites (Ferre 1991). Furthermore, these studies illustrated frequent receptor heterogeneity and the existence of multiple binding sites, with increased binding site concentration also associated with increased toxicity (Garczynski 1991; Van Rie 1989, 1990). For example, *H. virescens* larvae show three different populations of binding site, one which binds Cry1Aa, Cry1Ab and Cry1Ac, a second which binds Cry1Ab and Cry1Ac, and a third restricted to Cry1Ac binding. This correlates with the pronounced larvicidal difference between Cry1A variants (Ac > Ab > Aa) (Van Rie 1989, 1990). Receptor proteins originally identified from ligand binding studies in BBMV have since been purified and characterised. Two major forms of putative Cry receptor have been identified, namely cadherin-like receptors (CAD) (Vadlamudi 1993; Vadlamudi 1995), and aminopeptidase-N (APN) family receptors (Knight 1994; Sangadala 1994) – both shown to be glycosylated. Other receptor families for insecticidal toxins include alkaline phosphatase (ALP) (Arenas 2010; Jurat-Fuentes and Adang 2004; Krishnamoorthy 2007; McNall and Adang 2003; Ning 2010) and ATP-binding cassette (ABC) transporter protein (L. Chen 2018b; Z. Guo 2015; Y. Wang 2019; C. Wu 2019; Xiao 2014). Roles for putative glycosylation sites in the latter two receptor families are less well explored – with no specific role for glycosylation reported for Cry1 ABC receptors.

However, toxicity does not always correlate with BBMV protein binding (Ferre 1991; Garczynski 1991; Van Rie 1990; Wolfersberger 1990). This is exemplified by Garczynski *et al.*, showing similar high affinity Cry1A binding to BBMVs isolated from both highly susceptible (*Manduca sexta* & *H. virescens*), moderately susceptible (*Helicoverpa zea*), and tolerant (*S. frugiperda*) lepidopteran larvae (Garczynski 1991). Kumaraswami *et al.,* and others, demonstrated BBMV proteins isolated from either susceptible or resistant populations of *P. xylostella* have the same Cry1A binding capacity, yet resistant insect-derived BBMV and gut tissue had a significant reduction in neutral GSLs, indicating these glycolipids can mediate toxin susceptibility (Higuchi 2007; Kumaraswami 2001). In resistant *P. xylostella* populations, this was accompanied by decreased oligosaccharide length, with synthesis arrest at the pentasaccharide stage and a slightly reduced activity of Gal and GalNAc transferase, suggesting that more elaborate glycolipid moieties facilitate Cry1A toxicity (Kumaraswami

572 2001). More recent work by Ma *et al.,* supports the role of glycolipids in Cry1Ac binding and
573 tolerance (Ma 2012a). *H. armigera* larvae demonstrate enhanced tolerance to Cry1Ac if they
574 are pre-fed with LEC-8 – a galectin-like protein isolated from nematodes. Both LEC-8 and
575 Cry1Ac were shown to bind to gut glycolipids in a similar manner, implying that LEC-8
576 inhibits Cry1Ac glycolipid binding sites, thus mediating tolerance. The LEC-8 natural ligand
577 is unknown, but an inhibitory ELISA showed lactose can inhibit LEC-8 binding to *H.*
578 *armigera* gut glycolipids by 20%, and a mild inhibitory effect was observed with GalNAc,
579 galactose, mannopyranose, inositol and trehalose. LEC-8 has also been shown to interact with
580 Asialofetuin – a glycoprotein with terminal GalNAc residues (Nemoto-Sasaki 2008).
581 Differences in neutral sugar content between susceptible and resistant *M. sexta*
582 populations has been reported to correlate with Cry1A binding by a number of groups (Jurat-
583 Fuentes 2002; Sangadala 2001). Knowles *et al.*, solidified a role for a glycoconjugate in
584 Cry1A binding in insect gut epithelia (Knowles 1991). GalNAc addition completely
585 abolished Cry1Ac binding in *M. sexta*, partially in *H. virescens*, but had no effect on *Pieris*
586 *brassicae*. This correlated with SBA and Cry1Ac binding the same (glyco)protein in *M. sexta*
587 and *H. virescens*, but not *P. brassicae*, collectively indicating GalNAc is a component of the
588 Cry1Ac receptor(s) in some lepidopteran species, but glycoprotein interaction is not required
589 in others e.g., *P. brassicae.* The authors did not investigate the possibility of binding to
590 GalNAc present in glycolipids. Although much of the literature to date is focused on the role
591 of GalNAc in eliciting Cry1Ac toxicity, Haider *et al.,* have proposed the relevance of D-Glc
592 in eliciting Cry1 activity (Haider and Ellar 1987). Here, the authors showed the activity of a
593 trypsinised lepidopteran-specific preparation from *Bt* serovar. *aizawai* IC1 (containing a 55
594 kDa and a 58 kDa polypeptide) is completely inhibited in *M. brassicae* cells by D-Glc and
595 the D-Glc binding lectin – ConA. It is not clear exactly what protein toxins were expressed in
596 this preparation, although Cry1Ab7 is reported in this strain (Haider and Ellar 1988).
597 Conversely, glycolipid and sugar binding is also implicated in enhancing tolerance to
598 Cry toxins through the sequestration of toxin oligomers in the gut and subsequent prevention
599 of receptor binding in the midgut brush border (Hayakawa 2004; Ma 2012b; Ma 2012a). The
600 peritrophic membrane (PM) is the semipermeable lining of the insect midgut which, among
601 its functions, acts as protection from mechanical and pathogenic damage. Several studies
602 have indicated that compromising the integrity of the PM can enhance Bt toxin activity in
603 insect larvae, presumably through allowing more insecticidal protein to reach receptors at the
604 midgut epithelium brush border (Granados 2001). Hayakawa *et al.*, demonstrated that the
605 interaction of Cry1Ac with the PM can be inhibited with the addition of GalNAc in the

Cry1Ac tolerant lepidopteran species, *B. mori*. Upon addition of GalNAc, Cry1Ac passes through the PM significantly quicker, and at a similar rate to the *B. mori* active toxin, Cry1Aa - although the authors did not demonstrate if this renders *B. mori* Cry1Ac susceptible (Hayakawa 2004). Ma *et al.*, have suggested that binding of Cry toxin to glycolipids in lipophorin – lipoprotein particles that transport lipids in insect haemolymph – increases Cry toxin tolerance (Ma 2012b). They demonstrated that D-II of Cry1Ac monomers binds glycolipids from lipophorin particles, and forms Cry1Ac oligomers in the presence of glycolipids isolated from both *H. armigera* and *G. mellonella* cell-free plasma and midgut tissue. Cry1Ac addition to *G. mellonella* lipid particles induced aggregation - an interaction through which, the authors suggest, Cry1Ac is sequestered to the gut lumen. This study also used TLC to show the main Cry1Ac glycolipid binding species present in *H. armigera* gut tissue migrated to a similar position as globoside Gb4 ($GalNAc_3\beta1$-2Gal$\alpha$1-4Gal$\beta$1-4Glc$\beta$1-1-Cer) – which has a terminal GalNAc.

The exact mechanistic basis for Cry1A toxicity remains unclear. A large body of data shows insecticidal activity is dependent on much more than a single receptor interaction, but with the exact insect system, toxin oligomerisation state, multicomponent complexes, and tissue localisation all having profound effects on toxicity. The most established mechanism for Cry1A appears to be that of sequential binding during which a toxin monomer is recognised by a cadherin-like receptor causing a conformational change which facilitates pre-pore oligomer formation (and distinct types of pre-pore may be possible even for the same toxin (Gomez 2014)), and the subsequent binding to APN enabling membrane insertion. Multiple and complex receptor binding is not uncommon in the toxin field outside of 3D-Cry proteins, for example diphtheria (Hasuwa 2001) and protective antigen (Scobie 2003) are determined to utilise more than one receptor. Furthermore, as discussed in the introduction, the role of the prodomains in toxicity is yet to be fully elucidated. Aside from the commonly hypothesised roles in toxin stability, formation, and stabilisation (Derbyshire 2001), the structure of Cry1Ac1 protoxin D-V and D-VII have four predicted ligand binding sites for galactose, N-acetylglucosamine, mannose, and xylose (Zghal 2017), presenting the possibility that D-V and D-VII could interact with glycans in the gut, and may be involved in protoxin recognition of a receptor. In support of this idea, a recent study by Peña-Cardeña *et al*, has demonstrated the C-terminal protoxin domain of Cry1Ab provides additional binding sites for ALP and APN receptors, resulting in a higher binding affinity of the protoxin, which correlates with increased toxicity - compared to the activated form (Pena-Cardena 2018).

***APN and APN glycosylation in mediating Cry1A binding & activity***

Utilising protoxin affinity chromatography and anion-exchange chromatography, Knight *et al.*, purified a glycoprotein (APN1) present in the midgut target tissue of *M. sexta* that was bound by Cry1Ac and SBA, but not Cry1B (Knight 1994). Sequencing of the bound glycoprotein revealed sequence similarity to the APN family - a heavily glycosylated zinc aminopeptidase that is a common feature of the insect midgut and, therefore, often used to assess BBMV purity. APNs have since been extensively studied as Cry receptors and many different lepidopteran variants have been characterised - although not all bind Cry proteins. APNs are divided into 8 phylogenetic classes (Crava 2010; Fonseca 2015; Hughes 2014), with single insect species able to express multiple receptors from different classes. APN isoforms that bind Cry1Aa (Masson 1995), Cry1Ab (Denolf 1997; Masson 1995), and Cry1Ac (Angelucci 2008; S. S. Gill 1995; Luo 1997; Nakanishi 2002; Valaitis 1995; P. Wang 2005b) have been discovered in multiple lepidopteran species – although current evidence suggests only Cry1Ac binds via GalNAc, recognised by moieties present in a surface cavity in D-III, that is not conserved in Cry1Aa or Cry1Ab (Burton 1999; de Maagd 1999b; Jenkins 2000; Masson 1995). Putative Cry toxin receptors have been identified in APN classes 1 – 5, although recently APNs from class 6 and 8 have been implicated in mediating toxicity of Cry1Ab, Cry1Ac & Cry1Ca in *Chilo suppressalis* larvae (Y. Sun 2020).

The crystal structures of Cry1Ac and Cry1Ac in complex with GalNAc have been published (**Figure 5**) (Derbyshire 2001) and although this has provided evidence of D-III involvement in GalNAc binding, exactly where the GalNAc receptor ligand is located on APN is unknown. Sequence analysis of class 1 Cry1Ac-binding *M. sexta* APN isoforms showed the presence of 4 - 7 potential N-linked glycosylation consensus sites and 13 putative O-glycosylation sites (Knight 1995; Knight 2004; Stephens 2004). Ten of the putative O-linked sites are predicted in a Thr/Pro rich region of the C-terminus, thought to form a 'stalk' that raises the active site above the membrane. Lectin recognition of these *M. sexta* Apn1-linked glycans indicated the presence of fucosylated and high mannose N-glycans (ConA, AAA, GNA, UEA1 lectin binding), and O-linked glycans (SBA lectin binding) (Denolf 1997; Knight 2004). As presented in **Figure 6**, the presence of N and O-linked glycosylation sites can be predicted by sequence analysis. Comparing the sequences of Cry-binding lepidopteran midgut APNs we see the number of N-glycosylation sites does not vary dramatically between classes (0 – 6 sites per protein), and the positioning of these sites is somewhat similar - especially between members of the same class. The number of O-linked

673 sites does differ dramatically between sequences (1 – 46 sites), with class 1 & 3 sequences
674 containing substantially more consensus sites (13 – 46) than class 2, 4 & 5 (1 – 6). Previous
675 analysis of lepidopteran APN sequences using an earlier version of O-glycosylation site
676 prediction software (NetOGlyc v3.1, opposed to v4.0) predicted no consensus sites for class 2
677 receptors (Pigott and Ellar 2007).
678 Individual species of N-linked glycoconjugates on the 120 kDa *M. sexta* Apn1 have
679 been identified through MALDI-TOF/TOF tandem mass spectrophotometry coupled with
680 lectin binding and exoglycosidase digestion. These included the common insect
681 paucimannose structure ($Man_3GlcNAc_2$) linked to Asn609, and highly fucosylated structures
682 at the other three consensus sites (Asn295, Asn623 and Asn752). These glycans were shown
683 to display up to a trifucosylated core and fucosylated antennae structures ($Fuc_{1-3}GlcNAc$).
684 This predominance of Fucα1,3GalNAc-Asn is further indicated by the resistance of APN to
685 PNGase F – an enzyme that cleaves all asparagine-linked oligosaccharides unless the core
686 contains an α1,3 fucose (Stephens 2004). It is unlikely that these high-fucose glycans are
687 responsible for Cry1Ac binding as they lack terminal GalNAc residues, suggesting it is the C-
688 terminal O-site glycans that might determine Cry1Ac binding. Supporting this hypothesis,
689 Cry1Ac is not reported to bind to any class 2 lepidopteran APNs – a class which has
690 significantly fewer predicted O-linked glycosylation sites and no C-terminal stalk region
691 (**Figure 6**) (Pigott and Ellar 2007). Although O-glycosylation sites have been hypothesised to
692 be critical for Cry1Ac activity, there is evidence of Cry1Ac binding and activity in APN
693 classes with comparatively low numbers of O-glycosylation consensus sites. Cry1Ac can
694 bind to a class 4, 110 kDa APN present in *H. virescens* BBMV, that does not contain a C-
695 terminal stalk and is not recognised by SBA (Banks 2001). Furthermore, a class 5 APN
696 isolated from *Athetis lepigone* (AlAPN5) has recently been identified as a putative functional
697 receptor mediating Cry1Ac toxicity (L. Y. Wang 2017b). This may indicate that the increased
698 O-glycosylation sites seen in class 1 & 3 are not responsible for Cry1Ac toxicity, although, to
699 the best of our knowledge, it is unknown whether Cry1Ac binding to AlAPN5 is GalNAc-
700 dependent. Further investigations are required to determine if glycosylation is required for
701 Cry1Ac binding to class 2 & 5 APNs, or if these receptors work via a GalNAc-independent
702 route.
703 An array of studies has shown lepidopteran APNs, of all classes, are attached to the
704 membrane via glycosyl-phosphatidylinositol (GPI) anchors (Denolf 1997; S. S. Gill 1995;
705 Hua 1998; Knight 1995; Valaitis 1995). GPI-anchors contain carbohydrate-rich structures,

often including core-linked GalNAc present at the membrane surface, leading to speculation that this may be a Cry1A binding epitope. However, removal of the GPI-anchor glycan moiety using phospholipase C (PLC) does not appear to alter binding activity (Masson 1995), although it does drastically reduce Cry1Ac pore-forming activity - as expected by loss of membrane association (Lorence 1997). GPI-anchored proteins, including APN, are preferentially clustered in glycolipid-enriched microdomains – specialised detergent-resistant membrane microdomains present in both mammals and insects that are enriched in cholesterol and GSLs. Chemical analysis of the 115 kDa *M. sexta* APN-associated lipid aggregate showed a predominance of neutral lipids, mainly diacylglycerol and free fatty acids (Sangadala 2001). The presence of neutral lipids is interesting given the aforementioned studies indicating a reduction in neutral GSLs in resistant populations of *P. xylostella* and *M. sexta* (Higuchi 2007; Kumaraswami 2001). Reconstitution of the 115 kDa *M. sexta* APN into liposomes showed increased Cry1Ac binding when the lipid aggregate was present, as well as preferential binding of Cry1Ac to lipid microdomains (Sangadala 2001). This concentration of APNs to lipid microdomains is hypothesised to facilitate toxin oligomerisation through the high density of binding epitopes. Oligomerisation of Cry1Ac and Cry1Ab is shown to facilitate membrane insertion and pore formation via significantly increasing the binding affinity to APN (approx. 100-fold over the monomeric form) (Pardo-Lopez 2006). Nevertheless, these lipid domains could also be required for protection from gut proteases or APN structural stabilisation. Furthermore, lipid rafts appear to be required for the pore-forming actions of GalNAc-insensitive Cry1Ab (Zhuang 2002), indicating they are not simply just enhancing toxicity via increasing GalNAc receptor concentration.

The exact role of APN and glycoconjugates in facilitating Cry1A toxicity is yet to be fully understood, with several studies indicating APN binding alone is not always enough to induce toxicity. For example, Banks *et al.* showed *Drosophila* S2 cells transfected with a novel 110 kDa APN from *H. virescens* conferred binding but did not induce pore formation (Banks 2003). Furthermore, removing APN binding does not necessarily eliminate all binding, with Lee *et al.*, showing APN competes for Cry1Ac binding with *Lymantria dispar* BBMV – but does not eliminate it (Lee 1996). However, a significant number of reports indicate APN is critical for pore-formation (M. Gill and Ellar 2002; Sangadala 1994; Schwartz 1997). For example, expression of the 120 kDa *M. sexta* APN in the mesodermal and midgut tissue of *Drosophila* is capable of rendering normally insensitive larvae susceptible to Cry1Ac (M. Gill and Ellar 2002). Furthermore, several studies show that artificial APN suppression confers Cry1A resistance in several Lepidoptera (Qiu 2017b; Y.

Sun 2020). Divergent outcomes between these experiments are partially explained by the use of different experimental systems conferring differing posttranslational modifications – especially when we know the gut tissue is the *in vivo* target. Carroll *et al.*, first proposed a GalNAc sensitive and a GalNAc insensitive Cry1Ac binding mechanism within the same gut, by exploring the difference in Cry1Ac binding to BBMV isolated from either the anterior (A-BBMV) or posterior (P-BBMV) midgut of a target insect, *M. sexta* (Carroll 1997). Cry1Ac binding to P-BBMV induced a faster rate of toxicity, compared to A-BBMV, but was substantially reduced by the presence of GalNAc, whilst A-BBMV binding was not. Furthermore, Cry1Ac binding to APN was concentrated in P-BBMV suggesting the GalNAc-sensitive mechanism involves APN, whilst the GalNAc-insensitive binding does not. Indeed, later studies by Banks *et al.* supported this idea showing that Cry1Ac recognised a distinct 110 kDa APN in *H. virescens*, where binding was not inhibited by GalNAc and the receptor itself did not bind SBA. Furthermore, a mutant Cry1Ac with an altered GalNAc binding pocket demonstrated enhanced binding to the 110 kDa APN variant, even though binding was abolished to the GalNAc-mediated 120 and 170 kDa *H. virescens* APN variant (Banks 2001).

As briefly discussed above, a model of how APN confers Cry1Ac toxicity is through a bivalent sequential binding mechanism, with an initial low-affinity, rapidly-reversed interaction (GalNAc-sensitive) followed by a slower high-affinity irreversible interaction (GalNAc insensitive) (Cooper 1998; Jenkins 2000). Combined mutational, binding and toxicity studies have enabled the identification of residues important for Cry1Ac binding to APN and GalNAc (**Figure 5 & Table 2**). Broadly, D-I is associated with insertion of the pore into the membrane, and APN binding epitopes are primarily localised to Cry1A D-II and D-III (Liu and Dean 2006; Rajamohan 1996c; Rajamohan 1996b; Rajamohan 1996a; Vachon 2004). Domain II has been shown to influence membrane insertion, via a high affinity interaction with APN, whereas D-III is hypothesised to be involved in host specificity and the initial low-affinity receptor recognition (de Maagd 1999b; de Maagd 1999a; S. J. Wu and Dean 1996) - such as the GalNAc-dependent binding mechanism of Cry1Ac (Burton 1999; de Maagd 1999b; Jenkins 2000). Indeed, sequence analysis has shown D-III to be markedly divergent in Cry1Ac compared to other related – non GalNAc binding – 3D Cry proteins (Bravo 1997; Thompson 1995).

The first phase of APN recognition is hypothesised to be through fast, low affinity D-III binding. This is supported by Lee *et al.*, who generated a series of alanine substitution mutations in the region of D-III unique to Cry1Ac (503 – 525 aa) and demonstrated that

774 binding affinity was significantly reduced, and to a relatively greater degree than toxicity
775 (**Figure 5.B**) (Lee 1999). Whilst some of these mutant residues are in direct contact with
776 GalNAc (Q509, R511, Y513,), others are not (S503, S504, N506, N510, W545) – but with
777 the exception of W545 are in close proximity to the binding pocket. It was not investigated
778 whether any of these mutations affect GalNAc binding, making it difficult to interpret
779 whether reduced mutant binding to BBMVs was through a loss of GalNAc binding. The
780 authors conclude that if D-III is predominantly involved in initial low-affinity APN binding,
781 then this will only compromise second phase high-affinity binding when it is reduced by at
782 least 5-fold. Burton *et al.* also reported substitution mutations in the unique region of Cry1Ac
783 D-III (N506D, Q509E & Y513A -the latter two having direct contact with GalNAc in the
784 crystal structure of the complex) resulted in reduced binding and slower pore formation, with
785 the triple mutation no longer inhibitable by GalNAc – yet no significant differences in
786 toxicity were observed. Further supporting that D-III binding is required for sequential D-II
787 binding, the mutation of a tryptophan residue (W545A) in D-III (**Figure 5.B**) can completely
788 abolish sequential binding of D-II to the *L. dispar* APN and recognition of GalNAc – of
789 particular note given W545 is not part of the GalNAc binding pocket (Jenkins 2000).
790 Interestingly, all Cry1Ac tryptophan residues are conserved in the closely related Cry1Ab,
791 except the D-III W545 residue (Rausell 2004). The complete loss of APN binding in *L.*
792 *dispar*, via the Cry1Ac W545A mutation, only caused a 50-fold decrease in activity, whereas
793 the same W545A mutation in *M. sexta* larvae did not abolish binding to APN, with little to no
794 loss in toxicity (Pardo-Lopez 2006). The work in *M. sexta* also demonstrated that GalNAc
795 binding to the Cry1Ac oligomer increases the exposure of W545 to solvent, through a subtle
796 conformational change in the GalNAc binding pocket region of D-III. In *M. sexta*, this
797 conformational change is hypothesised to be responsible for the marked increase in binding
798 affinity of the Cry1Ac oligomer to APN. Collectively, these data indicate that D-III functions
799 to bind both GalNAc and APN in a low affinity manner which can affect second-phase APN
800 binding, yet there are apparent species-specific differences which determine Cry1Ac
801 interaction with APN and toxicity, and an indication that Cry1Ac can retain toxicity even
802 when binding to APN and GalNAc is abolished – leaving the binding open to further
803 investigation

804 Domains II and III are not specifically linked to glycan interactions, yet a common
805 theme is apparent between mutational studies in all three domains; the binding to APN and
806 subsequent toxicity are not necessarily correlated. This could be explained by the presence of
807 alternative *in vivo* Cry1Ac receptors – such as cadherin-like receptors or ABC transporters -

that function independently of APN and could be potentially compensating for the lack of APN binding/activity. The exact model used may change the distribution/ concentration of APN and any potential alternative receptors. Furthermore, the exact experimental setup may play a significant role. If APN binding to D-III is the rate limiting step to binding to D-II, and D-II binding and membrane permeabilization is not abolished but slowed, and it may be possible to exert toxicity over a longer time course. A better understanding of the key residues in Cry1Ac required for binding to receptors, and the role of GalNAc in this binding, might enable improved engineering of both insect specificity and toxicity, as well as providing a valuable tool for identifying potential resistance-driving mutations.

***Cry1A binding to Cadherin-like receptors***

Vadlamudi *et al.*, purified and characterised the first cadherin-like receptor from *M. sexta* larvae, a 210 kDa protein termed BT-$R_1$ (Vadlamudi 1995). Sequence analysis showed a 30-60% similarity to the cadherin superfamily of proteins – a large family of transmembrane glycoproteins characterised by repeated calcium-binding domains. Since the discovery of BT-$R_1$, receptors with a highly similar domain organisation have been identified in an array of other lepidopteran species including *B. mori* (BtR175), *H. virescens* (HevCaLP), *O. nubilalis*, *L. dispar, P. xylostella* (PxCad)*, C. suppressalis* (CsCad), and *H. armigera* (HaCad) (Flannagan 2005; Gahan 2001; Morin 2003; Nagamatsu 1999; G. Wang 2005a; Xu 2005). Lepidopteran cadherin receptors are usually anchored to the apical membrane of the midgut epithelium via a single transmembrane domain and, unlike GPI-anchored receptors (such as APNs or ALPs), are not preferentially localised to glycolipid-enriched lipid microdomains (Midboe 2003; Zhuang 2002). Interestingly, Cry1Ab treatment of *M. sexta* microvilli membranes was shown to induce Bt-$R_1$ localisation to lipid microdomains – although this is likely due to Bt-$R_1$ remaining attached after toxin oligomerization and not due to a requirement for (glyco)lipid-facilitated binding (Bravo 2004).

There are significant data to show cadherin-like receptors function in determining Cry1A specificity and toxicity in lepidopteran larvae (Pigott and Ellar 2007) and lepidopteran and Drosophila-derived cell lines (Hua 2004; Keeton and Bulla 1997; X. Zhang 2005). Furthermore, expression of BT-$R_1$ and BtR175 in mammalian-derived cell lines can induce Cry1Ac toxicity (Dorsch 2002; Tsuda 2003), suggesting cadherin-like receptors alone may be enough to permit cytocidal action and no other 'insect-specific' features are required for action. The success of inducing Cry1A toxicity in cell lines through cadherin-like receptor expression alone may be due to the redundancy of glycosylation in specifying binding.

Unlike APN, there are no reports of sugars acting as binding competitors with Cry1Ac to cadherin-like receptors. Further indication that glycosylation is not required comes from a study showing that the shortest fragment of Bt-$R_1$ that binds Cry1A toxins is a non-glycosylated 169 aa ectodomain fragment that is also capable of inhibiting toxicity (Dorsch 2002). To the best of our knowledge, the current literature does not report glycosylation to play a significant role in cadherin-like receptor binding, although N- and O-linked glycosylation sites are present on all identified lepidopteran cadherin-like receptors (Shao 2018).

***Cry1A binding to ALP receptors***

Selection of a Cry1Ac resistant strain of *H. virescens* allowed for comparison of midgut epithelium proteins between susceptible (YHD2) and resistant (YHD2-B) larvae (Jurat-Fuentes 2002; Jurat-Fuentes and Adang 2004). After observing reduced Cry1Ac binding to YHD2-B BBMVs, based on the rationale that GalNAc forms part of the Cry1Ac receptor, the authors investigated levels of SBA binding to BBMVs and indeed observed reduced SBA binding to YHD2-B resistant larvae – initially indicative of altered glycosylation (Jurat-Fuentes 2002). Further characterisation of YHD2-B BBMVs identified a 68-kDa glycoprotein as a GPI-anchored alkaline phosphatase - HvALP. Digestion of BBMV proteins with PNG-F to release N-terminal oligosaccharides, eliminated SBA binding to HvALP, confirming the presence of N-linked oligosaccharides with terminal GalNAc residues. Addition of Cry1Ac abolished SBA binding to HvALP, indicating competitive binding of both proteins for the same N-linked GalNAc residues on HvALP. Correlating with reduced Cry1Ac binding, Cry1Ac-resistant BBMVs also demonstrated a reduction in expression and a 3-fold decrease in activity of HvALP – suggesting the resistance was not due to altered glycosylation or recognition of GalNAc, but instead due to a reduction in HvALP protein expression – although the authors did not perform oligosaccharide analysis resistance through altered glycosyl interactions cannot be completely ruled out (Jurat-Fuentes and Adang 2004). In a parallel with the work described above, Nina *et al.*, described two ALPs cloned from *H. armigera* (HaALPs) that specifically bind Cry1Ac via N-linked GalNAc. Whether GalNAc binding on ALP is required for Cry1Ac toxicity is still open for debate – indeed GalNAc addition to *H. armigera* BBMVs inhibits permiabilisation (Rodrigo-Simon 2008), however, whether this is directly through ALP and the relevance to *in vivo* activity is yet to be determined.

**Cry5B and Cry14A**

Cry5B is the best characterised of the Cry5 subfamily of six phylogenetically related proteins (Cry5Aa, Cry5B, Cry12A, Cry13A, Cry14A and Cry21A) that may demonstrate nematocidal and/or insecticidal activity (Wei 2003). Consistent with the mode of 3D Cry protein insecticidal toxin actions, susceptible nematodes fed with nematocidal Bt strains experience dose-dependent lethality associated with reduced feeding activity, inhibited development and intestinal damage. To date, both Cry5B and Cry14A nematocidal activity is shown to be dependent, at least in part, on glycolipids (Griffitts 2001; Griffitts 2003; Griffitts 2005; Marroquin 2000).

Using forward genetics in *C. elegans*, Marroquin *et al.* identified five *bre* genes (for ***B****acillus*-toxin **re**sistant), four of which confer high levels of resistance to Cry5B induced toxicity and one (*bre-1*) that confers a significantly lower level (Marroquin 2000). In all resistant mutants, Cry5B toxin remained in the intestine and was not internalised into the gut cells indicating resistance via reduced 'receptor' binding. The first *bre* gene to be characterised was *Bre-5*, found to encode a β1,3-galactosyltransferase with strong sequence similarity to the *Drosophila brn* gene (required for glycolipid synthesis, see **Figure 4**) (Griffitts 2001). Successively, *bre-2*, *bre-3* and *bre-4* were characterised as encoding further glycolipid synthetic proteins; *bre-4* as a UDP-GalNAc:GlcNac β1-4-N-acetlygalactosaminyltransferase, *bre-2* encodes a β1,3 glycosyltransferase, and *bre-3* a putative glycosyltransferase homologous to *Drosophila egh* (see **Figure 4**) (Griffitts 2003; Griffitts 2005). Functional homology of *bre* genes to the *egh-brn* invertebrate-specific lipid glycosylation pathway was shown via TLC lipid analysis, demonstrating that *bre* mutants express no (*bre-3*, *bre-4*, & *bre-5*), or significantly reduced (*bre-2*) complex GSLs, yet have no change in N- or O-linked proteoglycan profiles. Specific binding of Cry5B to these *bre*-dependent complex GSLs alongside genetic epistasis-based experiments supported the proposal that *bre*-genes act consecutively (*bre-3*, *bre-4*, *bre-5*, *bre-2*) to synthesise a functional lipid-linked oligosaccharide receptor with terminal galactose residues (Griffitts 2005). In further support of GSLs as principal determinants for Cry toxicity, the *C. elegans* LEC-8 galectin (a ß-galactoside-binding protein) can compete with Cry5B for carbohydrate binding. Cry5B binding to *C. elegans* glycolipid-coated TLC plates was inhibited through the addition of recombinant LEC-8, and *C. elegans* LEC-8 deficient mutants were more susceptible to Cry5B, in comparison to wild type worms (Ideo 2009). *Bre* mutants also demonstrated a moderate resistance to Cry14A, a toxin with 34% sequence identity to Cry5B

in their protoxin forms and ~30% identity in the activated form. This relatively low level of amino acid identity suggests that other distantly related toxins may induce *bre*-mediated toxicity. However, the reduced resistance, compared to Cry5B, signifies that other Cry14A receptor(s) may compensate for the loss of the *bre*-mediated glycolipid (Griffitts 2001; Griffitts 2003).

Although identified in the same forward genetics screen as *bre 2-5*, *bre-1* mutants demonstrate substantially less Cry5B resistance (Barrows 2007; Marroquin 2000). *Bre-1* has since been identified as a GDP-mannose 4,6 dehydratase (GMD), an enzyme involved in a fucose salvage pathway. Unlike the *bre2 - 5* genes, it does not function in a glycolipid-specific manner, with *bre-1* defective mutants showing strikingly reduced levels of fucosylated N and O-linked proteoglycans as well as fucosylated glycolipids (Barrows 2007). This partial Cry5B resistance indicates that fucose is less critical for eliciting Cry5B binding than terminal galactose residues - as shown by competitive binding studies.

Interestingly, no obvious change in phenotype or lethality were observed in the *bre*-mutant *C. elegans*, apart from a small reduction in brood size in *bre-1* and *bre-3* worms (Barrows 2007). The nematode is apparently capable of surviving with reduced levels of GSLs and dramatically reduced fucose, which is perhaps surprising given the commonality of fucose in nematode glycans and the prevalence of detrimental phenotypes in mammalian GMD knockouts (Keeley 2019; Sturla 2001). This has implications for Cry resistance in nematodes, since they can tolerate changes in glycosylation while in *Drosophila,* the equivalent *brn* and *egh* mutants are lethal/sterile, suggesting a significantly lower tolerance to reduced *bre*-mediated glycosylation and an essential role for GSLs in insects. This contrast in phenotypes could suggest that insects, in contrast to nematodes, would be less able to achieve to Cry resistance via GSL alteration.

**Cry2**

Like the Cry1 class of Bt proteins, Cry2 proteins are largely specific towards lepidopteran insects (Hernandez-Rodriguez 2008), with some Cry2A variants also exhibiting toxicity against mosquito species, including *Ae. aegypti*, *Culex quinquefasciatus*, *Anopheles stephensi*, and *An. gambiae* (Goje 2020; McNeil and Dean 2011; Misra 2002; Moar 1994; Ricoldi 2018; Sims 1997; Valtierra-de-Luis 2020). Whilst Cry2 is not reported to bind any APNs, ALPs, or CADs, functional Cry2A ABC receptor binding proteins have been identified - ABCC1 and ABCA2 from *H. armigera* (HaABCC1, HaABCA2) and ABCA2 from *B. mori* (BmABCA2) (L. Chen 2018a; Li 2020; J. Wang 2017a), *Pectinophora*

*gossypiella* (PgABCA2) (Fabrick 2021), and *Helicoverpa zea* (HzABCA2) (Fabrick 2022). The ABC transporter superfamily of proteins are responsible for the ATP-powered translocation of a diverse assortment of substrates across membranes. In common with shared physiological mechanisms observed with mammalian ABC transporters, insect ABC transporters have been functionally linked to lipid transport, and the transport of xenobiotics and their metabolites (Broehan 2013; Rees 2009).

Sequence analysis of HaABCC1 showed the presence of 14 potential N-glycosylation sites and 16 potential O-glycosylation sites throughout the entire protein (L. Chen 2018b). In HaABCA2, sequence analysis identified six potential N-glycosylation sites within the extracellular domain loops of transmembrane domain (TMD)-1 and TMD-2 (Tay 2015). One of these putative N-glycosylation sites is located within a 5 bp deletion mutation shown to confer Cry2Ab resistance in *H. armigera*. This deletion mutation introduces a stop codon within HaABCA2 TMD-2, leading to a protein truncation. Although the use of these these putative glycosylation sites is yet to be confirmed, it has been hypothesized that binding of Cry2A toxins to the glycosylated extracellular domain loops of ABCA2 may form the basis of toxin oligomerization and sequential pore formation (Tay 2015). Given that ABC transporters have been shown to exist as multi-protein complexes in the membrane, it may also be the case that other ABC-associated proteins are involved in Cry2A binding and pore-formation (Kaminski 2006). Other reported Cry2 receptors include the Se-V-ATPase subunit B from *S. exigua*, also predicted to contain several putative glycosylation sites (Qiu 2017a).

Cry2Ab has also been shown to interact with lipophorin glycolipids (Ma 2012b). As discussed previously, Ma *et al.* demonstrated that Cry1Ac addition to *G. mellonella* lipid particles induced aggregation, and sequesters Cry1Ac to the gut lumen, possibly increasing Cry toxin tolerance. Cry2Ab was also shown to aggregate following lipid particle interaction and, hence, the authors suggest a similar mechanism of toxin tolerance.

**Cry3**

The Cry3 class is the best characterised of the coleopteran-specific proteins, with a domain architecture consistent with other 3D Cry proteins. The lectin-like D-III of Cry3Aa was found to exhibit strong resemblance to the N-terminal cellulose binding domain ($CBD_{N1}$) of the bacterial *Cellulomonas fimi* 1,4-β-glucanase C (CenC) (Johnson 1996) (Burton 1999). The $CBD_{N1}$ domain of CenC has been shown to interact with cellulose, as well as cell oligosaccharides and β-1,4-linked oligomers of glucose (Tomme 1996) - with binding thought to occur via β-strands within a 5-stranded cleft which constitutes the $CBD_{N1}$ (Johnson 1996;

977 Kormos 2000). The structural correlation between Cry3 D-III and the $CBD_{N1}$ of CenC may
978 suggest a role for sugar moieties in Cry3 receptor binding.
979 Several studies have implicated CADs, ALPs, APNs, and ABCs as Cry3 binding
980 proteins and/or functional receptors – although less is known regarding the relevance of
981 glycosylation. In *Tenebrio molitor*, Cry3Aa has been shown to bind to a GPI-anchored ALP,
982 which is preferentially expressed in the BBMV of early instar larvae (Zuniga-Navarrete 2013).
983 In *D. virgifera virgifera* and *Chrysomela tremula*, ABCB1 has been identified as a functional
984 receptor for Cry3A (Niu 2020). Functional validation of the *D. virgifera virgifera* Cry3A
985 receptor (DvABCB1) was achieved through activated Cry3A addition to Sf9 or HEK293 cells,
986 both expressing DvABCB1. As the gut of *D. virgifera vigifera* is mildly acidic, this again
987 indicates the pH of cell studies does not need to replicate the gut environment for toxicity to
988 occur, in the presence of activated toxin. Sequence analysis of CtABCB1 predicts two putative
989 glycosylation sites on the extracellular loops of the transmembrane domains (Pauchet 2016).
990 Although the functional relevance of these sites is unknown, this is the first study to suggest
991 that glycosylation may be important for ABC receptors.
992
993 **Cry30Ca2**
994 Cry30Ca2 is produced by the Bt serovar. *jegathesan*, a mosquitocidal subspecies that shows
995 toxicity against *A. aegypti*, *A. stephensi*, *Culex pipiens*, and *C. quinquefasciatus* (Delecluse
996 1995; Kawalek 1995). Bioassays of the isolated Cry30Ca2 toxin indicate that this individual
997 protein is not toxic against *C. quinquefasciatus* and, hence, additional studies are required to
998 test its toxicity to other mosquitoes (Y. Sun 2013).
999 Using homology modelling, based upon Cry4Ba, Zhao *et al.* produced a three-
1000 dimensional model of the Cry30Ca2 structure consistent with that of other 3D Cry proteins
1001 (X. M. Zhao 2012). Dissimilar from the interaction of Cry1Ac with GalNAc, which occurs
1002 via Cry1Ac D-III, docking studies investigating the interaction of Cry30Ca2 with GalNAc
1003 highlighted a distinct, putative binding site within the apical loops of the Cry30Ca2 lectin-
1004 like D-II (residues I321 in loop 1, Q342, T343, Q345 in loop 2, Y393 in loop 3, which form 7
1005 hydrogen bonds with GalNAc) (X. M. Zhao 2012). Various studies have implicated the loop
1006 regions of Cry protein D-II in receptor binding, including Cry3Aa, which is shown to bind
1007 TmCad1 via D-II loop 1 (Zuniga-Navarrete 2015). Given the results of molecular docking
1008 studies and these structural similarities, a role for GalNAc containing carbohydrate moieties
1009 in Cry30Ca2 mosquitocidal activity has been suggested (X. M. Zhao 2012). However, the
1010 effect of GalNAc on the activity of Cry30Ca2 is yet to be investigated.

**Cry11a**

Bt serovar. *israelensis* (Bti) strains are highly toxic to a number of mosquito species and, as such, are used for the control of their populations in the field (Mittal 2003). One such Bti toxin is Cry11Aa, which displays toxicity against *Aedes* and *Culex* larvae and, to a lesser extent, *Anopheles* larvae (Otieno-Ayayo 2008). Cry11Aa has been identified to bind receptors in mosquito larvae that are in the same classes as Cry toxins that act against Lepidoptera, including APNs, ALPs and Cadherins.

Cry11Aa binding to an ALP is suggested to play a role in mediating toxicity in *Aedes* larvae (Fernandez 2006). Interestingly, the interaction between Cry11Aa and *A. aegypti* ALP1 (AaeALP1) was shown to be modulated by other proteins - namely C-type lectins and galectins - which both interfere with toxicity (Batool 2018; L. L. Zhang 2018). C-type lectins are a superfamily of proteins that have mannose and galactose type carbohydrate binding capabilities through conserved residues (G. D. Brown 2018). Galectins are a family of proteins that typically bind to β-galactoside carbohydrates (Modenutti 2019), although comparatively little is known about their carbohydrate binding properties and function in invertebrates compared to vertebrates (J. Yang 2011) (L. L. Zhang 2018). The *A. aegypti* C-type lectin-20 (CTL-20) can bind to both *Aedes* BBMVs and recombinant AaeALP1, in addition to binding to Cry11Aa itself. Further to this, CTL-20 has been shown to compete with Cry11Aa for binding to AaeALP1 suggesting that they bind AaeALP1 in the same region (Batool 2018). Similarly, galectin-14 has been shown to compete with Cry11Aa for binding to AaeALP1 and *Aedes* BBMVs, with modelled molecular docking indicating that Cry11Aa and Galectin-14 bind to ALP1 on two different, but overlapping, interfaces (L. L. Zhang 2018). Additionally, other galectins such as galectin-6 have also been shown to interfere with Cry11Aa toxicity (Hu 2020). There is some evidence that galectin-6 binds to molecules containing galactose-β1,4-fucose (Maduzia 2011; Takeuchi 2008), therefore, it is possible that Cry11Aa may be able to bind similar glycan moieties. These results draw comparison with the Cry5B data discussed previously, where LEC-8 competes for carbohydrate binding and suggests a role for glycan moieties in the interactions between Cry11Aa and ALP1. However, to the best of our knowledge, there are no studies which have directly investigated the involvement of glycan residues in this binding.

Perhaps the most extensive work looking into the role of glycoconjugates in Cry11Aa receptor binding has come from Chen *et al.* 2009, investigating the interactions between Cry11Aa and AaeAPN1 (J. Chen 2009a). This study identified AaeAPN1 as a Cry11Aa

binding partner through pulldown assays utilising biotinylated toxin performed on solubilised *A. aegypti* BBMV. The AaeAPN1 was cloned and expressed in both *E. coli* and Sf21 cells - significant in the context of investigating the role of glycoconjugates as *E. coli* do not naturally N- and O- glycosylate proteins (Du 2019). The glycosylation status of AaeAPN1 from BBMV was investigated through lectin blots (J. Chen 2009a) and demonstrated the native form of AaeAPN1 was detectable by WGA but not SBA, indicating AaeAPN1 contains N-acetylglucosamine moieties but not terminal N-acetylgalactosamine residues. In Sf21 cells, expression of a catalytically active form of AaeAPN1 did not render cells susceptible to Cry11Aa treatment. Although Sf21 cells have the ability to N- and O-glycosylate proteins (Davis and Wood 1995), the AaeAPN1 in these cells was not detected by WGA, SBA, ligand blot, or toxin pull down assays and the band detected by anti-APN1 antibody was smaller than expected – possibly due to differences in post translational modifications (J. Chen 2009a). The authors also hypothesised that alternative glycosylation in Sf21 cells could mask a glycan-independent binding site. Taken together these results may indicate that that glycosyl moieties are required for binding. However, Chen *et al*. 2009 also demonstrated, via dot blot and competitive ELISA, that a truncated AaeAPN1 fragment expressed in *E. coli* binds to Cry11Aa, suggesting that this interaction is be glycan-independent, due to the absence of N and O-glycosylation in *E. coli* – although this does not determine whether this binding is involved in mediating toxicity.

The sequence of an *Aedes* cadherin protein shown to bind to Cry11Aa has been sequenced and there are predicted N-glycosylation sites within the cadherin repeats, however, there has been no investigation so far into whether glycosylation is present and if it is required for this interaction (J. Chen 2009b).

**Cry4Ba**

Cry4Ba is also produced by Bti and is processed in the insect midgut to produce an active toxin of 65kDa (Angsuthanasombat 1991). Like Cry11Aa, Cry4Ba also targets *Aedes* and *Anopheles* mosquito larvae (Ben-Dov 2014; Otieno-Ayayo 2008), and is shown to target the same receptor classes as other 3D Cry toxins (APNs, ALPs and Cadherins) (Likitvivatanavong 2011; Saengwiman 2011).

A cadherin Cry4Ba binding partner (AgCad1), expressed in *An. gambiae* BBMVs was predicted to be glycosylated, based upon the observed AgCad1 protein band having a slightly larger molecular weight than expected (Hua 2008). The same group also demonstrated that Cry4Ba displays limited binding on dot blots to an *E. coli* expressed truncated peptide from

AgCad1 (a CR11 membrane proximal extracellular domain peptide), suggesting that some binding is possible in the absence of glycosylation or other *in vivo* requirements. Similarly, Cry4Ba was shown to bind to a segment of the *An. gambiae* cadherin BT-$R_3$, expressed in *E. coli*, which consisted of the extracellular domain (EC) module 7 through to the membrane proximal EC domain (Ibrahim 2013). As this cadherin fragment was expressed in *E. coli* it is unlikely to be glycosylated and provides further evidence that glycosylation of cadherins is not required for Cry4Ba binding.

Multiple studies have implicated ALPs as binding partners for Cry4Ba (Bayyareddy 2009; Dechklar 2011; Jimenez 2012). Mutagenesis studies demonstrated Cry4Ba binding to ALP1, in part, through D-II loop II. Multiple Cry4B D-II mutants displayed reduced binding to ALP1 from BBMV and *E. coli*, and reduced toxicity to *Ae. aegypti* larvae. The results of this study suggest it is unlikely that receptor glycosylation is essential for interaction, as the mutated versions of Cry4Ba also display reduced binding to *E. coli* expressed ALP1 and *Ae. aegypti* BBMV (Jimenez 2012). Further to this, Thammasittirong *et al* 2011 showed that Cry4Ba binds to an *Ae. aegypti* ALP expressed in *E. coli* with high affinity, which they conclude supports the notion that Cry4Ba interactions with ALPs does not require glycosyl moieties as proteins expressed in *E. coli* are unlikely to be glycosylated. Finally, Buzdin *et al.* 2002 showed through ligand blots that addition of monosaccharides (mannose, glucose, galactose, galactosamine, N-acetylglucosamine and N-acetylgalactosamine, either individually or in mixtures) did not interfere with Cry4Ba binding to ALP which was prepared from *Ae. aegypti* BBMVs, with similar results shown for Cry11Aa binding to ALP. They also demonstrated that the addition of N-acetylglucosamine or N-acetylgalactosamine failed to elute ALP from Cry4Ba- and Cry11Aa- Sepharose (Buzdin 2002). APNs have also been identified as receptors for Cry4Ba (Saengwiman 2011). Sf9 cells expressing two *Ae. aegypti* APN isoforms (AaeAPN2778 and AaeAPN2783) displayed increased sensitivity to Cry4Ba and the toxin was shown to bind to APNs in Sf9 cells (Aroonkesorn 2015). The APNs expressed in these cells were thought not to be glycosylated, suggesting that the interaction between APNs and Cry4Ba is glycan independent.

Cry4Ba has been shown to interact directly with lipid bilayers, which is perhaps not surprising given the elucidation of GPI-anchored APN and ALP receptors. Thammasittirong *et al*. 2019 tested full length Cry4Ba and D-III-only binding to lipid bilayers and liposomes prepared from an artificial lipid mix containing phosphatidylethanolamine, phosphatidylcholine and cholesterol (but no glycans) (Thammasittirong 2019). They focused on D-III of Cry4Ba as it is shown to bind along the apical microvilli of the larval midgut of

*Ae. aegypti* (Chayaratanasin 2007). Domain III of Cry4Ba displayed tight binding to immobilised liposome membranes with a $K_D$ comparable to that of the full-length protein. However, unlike the full-length protein, the truncated D-III Cry4Ba fragment did not induce ion-channel formation in planar lipid bilayers or permeability of calcein dye-loaded liposomes, consistent with the role of this domain as a membrane anchor rather than having a role in pore formation (Thammasittirong 2019). The binding of Cry4Ba to lipids may suggest that, like other Cry proteins, it localises to lipid rafts – although whether glycolipid binding occurs, remains to be tested.

## Glycan binding in other bacterially-produced insecticidal toxins

There is also evidence that glycan binding could play an important role in the insecticidal action of other structural classes of bacterially-produced toxins, with lectin-like domains present in the Tpp family (D-I) (Colletier 2016), Vegetative insecticidal protein family (Vip3, D-IV and D-V) (Zheng 2020), mosquitocidal holotoxin (Mtx1Aa1) (Treiber 2008), and the membrane attack complex/perforin family (Mpf, C terminal domain) (Zaitseva 2019). Sugar binding appears to play a role with several members of the Tpp family, including Tpp78, Tpp80, and the Tpp1/Tpp2 binary complex. Several sugars – including chitotriose, N-acetylmuramic acid, chitobiose and N-acetylneuraminic acid – can reduce the mosquitocidal action of *Lysinibacillus sphaericus*-produced Tpp1/Tpp2 in *Culex* cell lines (Broadwell and Baumann 1987), and arabinose and fucose can reduce Tpp1 toxicity towards *Culex* larvae (Sharma 2018). Both galactose and GalNAc have recently been demonstrated to inhibit the activity of Bt-produced Tpp78 (Cao 2022) and Tpp80 (Best 2022) against their respective targets, rice planthoppers (*Laodelphax striatellus* and *Nilaparvata lugens*) and mosquitoes (*C. quinquefasciatus*, *Ae. aegypti*, and *An. gambiae*). The mosquitocidal Mtx1Aa1 contains 12-putative sugar binding domains across 4 ricin B-type lectin repeats, which are structurally related to Piersin – a cytotoxin that is reported to bind Gb3 and Gb4 glycolipids (Matsushima-Hibiya 2003). This is just a snapshot of the glycan-binding literature on other bacterial pesticidal proteins, and highlights glycan binding as an important mechanistic theme across bacterially-produced pesticidal proteins.

## Conclusions

Bt 3D Cry toxins are critical for progressing a sustainable approach to controlling pests of agriculture and vectors of human disease, with the development of field resistance threatening current effectiveness and progress. Understanding the mechanism of action is key

to understanding resistance and the potential development of new 3D Cry proteins. All known 3D-Cry proteins contain lectin-like domains, indicating a potential role for glycan-binding. For several Cry proteins, interaction with sugars, glycoproteins, glycolipids and competition by lectins has been demonstrated in receptor binding, but a role in toxicity is not always clear. For other members of the Cry family, these studies are absent, suggesting an important gap in our knowledge that should be addressed. While for some proteins, such as Cry4B, above, binding to protein receptors appears to be glycosylation independent, the potential carbohydrate-binding properties of D-II and D-III may play a role in binding to glycolipid moieties in the target cell membrane (as shown for Cry5B). The structural differences in glucoconjugates between insects, nematodes and mammals is a mechanistic explanation for target range that is independent of the protein receptor and may explain why the transfection of genes for such receptors does not always confer susceptibility to recipient cells. This effect will be mediated by both the specificity of the carbohydrate binding domains within the Cry proteins and the natural lipid composition of the transfected cells. Understanding the exact role of glycoconjugates can be a challenge due to the difficulty in replicating the *in vivo* environment of the gut target tissue – especially with many studies suggesting a complex coordination of binding components is required to elicit the full spectrum of toxicity. Indeed, the majority of model data comes from cell lines, which are not target-tissue specific and BBMV binding studies in which the concentrations of receptors and lipid microdomains do not necessarily accurately reflect the *in vivo* environment. In addition to normal development, glycan expression can be significantly altered by environmental pressures, such as temperature, infection, and dietary changes. This should be considered in terms of the development of Bt tolerance in target species – where changes in glycan binding profiles may be an indication of resistance as observed with nematocidal Cry5B. Despite these experimental complexities, it is clear that glycan moieties might be critical for exerting insecticidal and nematocidal activity, with glycan-moieties observed as primary receptors critical for activity, and in more additive roles that can affect the spectrum/ potency of activity. Despite many years of study of the Cry proteins, our understanding of their glycoconjugate interactions remains under-investigated and in its infancy. Application of the tools of glycobiology to the study of insecticidal proteins in future will help us to resolve the importance of these interactions.

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