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Review - Comparative mapping of selected structural determinants on the extracellular domains of cholinesterase-like cell-adhesion molecules

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Highlights
- Cell adhesion involves formation of homo- or heterophilic protein complexes
- A subset of cell-adhesion molecules shares an inactive cholinesterase-like ectodomain
- This subset is headed by the well-documented neuroligins, found in all animal phyla
- It also includes neurotactin, gliotactin and glutactin, found in invertebrates only
- The main functional and structural features of these molecules are compared

Abstract
Cell adhesion generally involves formation of homophilic or heterophilic protein complexes between two cells to form transcellular junctions. Neural cell-adhesion members of the α/β-hydrolase fold superfamily of proteins use their extracellular or soluble cholinesterase-like domain to bind cognate partners across cell membranes, as illustrated by the neuroligins. These cell-adhesion molecules currently comprise the synaptic organizers neuroligins found in all phyla, along with three proteins found only in invertebrates: the guidance molecule neurotactin, the glia-specific gliotactin, and the basement membrane protein glutactin. Although these proteins share a cholinesterase-like fold, they lack one or more residues composing the catalytic triad responsible for the enzymatic activity of the cholinesterases. Conversely, they are found in various subcellular localisations and display specific disulfide bonding and N-glycosylation patterns, along with individual surface determinants possibly associated with recognition and binding of protein partners. Formation of non-covalent dimers typical of the cholinesterases is documented for mammalian neuroligins, yet whether invertebrate neuroligins and their neurotactin, gliotactin and glutactin relatives also form dimers in physiological conditions is unknown. Here we provide a brief overview of the localization, function, evolution, and conserved versus individual structural determinants of these cholinesterase-like cell-adhesion proteins.

Keywords – Cell-adhesion molecule; cholinesterase-like domain; functional partnership; homology model; structural superfamily; surface determinants.

Abbreviations – AChE, acetylcholinesterase; ChE, cholinesterase (Hu, human; Mo, mouse; Dm, Drosophila melanogaster); GLIO, gliotactin, GLUT, glutactin; NLGN, neurexin; NRT, neurexin; NRXN, neurexin.
Introduction

Neuronal cell-adhesion generally involves formation of homo- or heterophilic protein complexes to form synaptic or non-synaptic cell contacts/junctions (Apóstolo & de Wit, 2019), and several superfamilies of cell-adhesion molecules have been characterized (de Wit & Ghosh, 2016). A particular subset of cell-adhesion proteins shares a characteristic cholinesterase (ChE) -like extracellular domain, despite significant sequence differences and absence of an enzymatic activity (Sussman et al., 1991; Krejci et al., 1991; Ollis et al., 1992) (Fig. 1, Table 1). This domain, which defines the large α/β hydrolase fold superfamily of proteins, is characterized by a central β-sheet core made of a tenth of parallel and usually one antiparallel β-strands, themselves connected by a related number of α-helices (Hotelier at al., 2004). In the enzymatically active members of the family, precise and conserved positioning of three catalytic residues is mandatory to form a functional nucleophile (Ser) / acid (His) / base (Asp or Glu) triad. A shell of secondary structural elements, mostly comprising α-helices and loops of variable sizes, wraps around this central core and defines less conserved surface determinants for, e.g., substrate attraction and guidance to the active center, binding of cofactors and partners, protein resistance to external factors, or cellular localization. A third level of specialization is sometimes achieved through insertion of additional, structurally and functionally unrelated domains which further extend the protein functionality (e.g., the flap in lipases), or add another function, or dictate a mode of attachment to membranes (Carr & Ollis, 2009; Marchot & Chatonnet, 2012).

The currently identified ChE-like cell-adhesion molecules comprise four members: the synaptic organisers neuroligins (NLGN), which are found in all animal phyla and for which several crystal structures are available, and the guidance molecule neurotactin1 (NRT; Hortsh et al., 1990), the glia-specific protein glutactin (GLIO; Auld et al., 1995) and the basement membrane protein glutactin (GLUT; Olson et al., 1990), which are found only in invertebrates. The NLGNs, GLIO and NRT are single-pass transmembrane proteins, albeit the first two are of type-I (intracellular C-terminus) while the latter is of type-II (extracellular C-terminus), whereas GLUT is a secreted protein found associated to the basement membrane of cells (for references, see below). These cell-adhesion proteins generally lack the Ser residue, and occasionally one of the other two residues, of the catalytic triad responsible for the enzymatic activity of the ChEs. Conversely, they display surface molecular determinants associated with specific partner recognition and binding.

Several reviews comparing the ChEs and their cell-adhesion relatives at the structural and functional levels were published before (Griffman et al., 1998; Grisaru et al., 1999; Scholl & Scheiffele, 2003; Gilbert & Auld, 2005) and after crystal structures of NLGNs provided in depth views of the structural peculiarities of a ChEL-like adhesion molecule (De Jaco et al., 2012; Bourne & Marchot, 2014, 2017). Here we describe the known subcellular localizations and functions of the ChE-like cell-
adhesion molecules NLGNs, NRT, GLIO and GLUT. We compare homology models of the NRT, GLIO and GLUT with experimental (crystal) structures of selected acetylcholinesterase (AChE) and NLGN proteins, in pointing to structural determinants such as their modified active center, disulfide bonding patterns, capacity for dimer formation, N-glycosylation and LRE motif patterns, and electrostatic surface potentials. We also assess their evolutionary relationships and review their known protein partners.

1- Subcellular localizations and functions

1a- The neuroligins (UniProt ID for human proteins: NLGN1 Q8N2Q7; NLGN2 Q8NFZ4; NLGN3 Q9NZ94; NLGN4X Q8N0W4; NLGN4Y Q8NFZ3)

Rat NLN1 (UniProt ID: Q62765), a type-I cell membrane protein of 843 amino acid residues whose extracellular ChE-like domain comprises residues 45-639 (Fig. 1; Table 1), was initially identified by affinity chromatography as a post-synaptic neuronal receptor for the pre-synaptic neurexin-β1 (NRXN-β1) (Ichtchenko et al., 1995). Subsequently, three other NLGN genes were identified in rodents (Ichtchenko et al., 1996) and up to five in humans (Bolliger et al., 2001). In humans, the NLGN1 and NLGN2 genes are located on chromosomes 3 and 17, respectively, while both the NLGN3 and NLGN4X genes are on the X chromosome. The fifth, NLGN4Y ‘gene’, a NLGN4X allele located on the Y chromosome, encodes a NLGN4Y or NLGN5 protein that is 97% similar to NLGN4X (Bolliger et al., 2001). In mouse there is a more divergent NLGN4-like gene/allele, located on the pseudoautosomal region of the X chromosome (Bolliger et al., 2001, 2008; Maxeiner et al., 2020). NLN1 localizes at excitatory glutamatergic synapses (Song et al. 1999; Graf et al., 2004), NLN2 selectively at inhibitory GABAergic and excitatory cholinergic synapses (Varoqueaux et al., 2004; Dong et al., 2007; Takács et al., 2013), NLN3 at both excitatory glutamatergic and inhibitory GABAergic synapses (Budreck & Scheiffele, 2007; Földy et al., 2013), and NLN4X preferentially at glycineric inhibitory synapses (Hoon et al., 2011), where they selectively control synaptic connectivity. In the central nervous system, NLN4X is barely present (Bolliger et al., 2001) while NLN3 is also expressed by glial cells, where it functions in a non-cell-autonomous manner, consistent with a signaling role (Sakers & Eroglu, 2019). Outside the central nervous system, NLN1 has been found in the vascular system (Bottos et al., 2009), NLN2 in uterus (Kang et al., 2004), NLN3 in muscle and pancreas (Philibert et al., 2000), and NLN4X in heart, liver, skeletal muscle and pancreas (Bolliger et al., 2001).

Alternative mRNA splicing events result in the presence or absence of splice inserts in the extracellular domain of NLGNs 1-4 (two consecutive inserts A1/A2 in NLGNs 1-4 and one N-glycosylated insert B in rodent NLN1 (Fig. 1, green A1/blue A2 and orange B stretches), which form extra loops at the subunit surface and give rise to several isoforms with distinctive partnership properties (Boucard et al., 2005; Chih et al., 2006; Talebizadeh et al., 2006; Koehnke et al., 2010).
NLGN insert A2 is at the same position as a splicing or individual insert found in insect AChEs, while additional inserts, of which a long Gly-rich stretch most likely to be fully disordered, are found at other positions in *Drosophila* NLGN4 (but not NLGN1) and several other *Drosophila* proteins (Fig. 1).

The essential neurodevelopmental role of the NLGNs was highlighted by the identification of genetic aberrations associated with a diagnosis of autism spectrum disorders and intellectual disability (Südhof, 2017; Cao & Tabuchi, 2017). Since the early 2000’s, several single residue substitutions in the ChE-like domain of the NLGNs have been found to be associated with autism. The first missense variant, Arg451Cys, was identified in two affected brothers (Jamain et al., 2003). The resulting substitution, localized in the extracellular domain of NLGN3 (Fig. 1), was shown to alter trafficking of the mutant protein through the intracellular secretory pathway (Comoletti et al., 2004, Chih et al., 2004, De Jaco et al., 2006). Other rare variants were also found for the other NLGNs, of which most were linked to autism and related neurodevelopmental disorders (Laumonnier et al., 2004; Yan et al., 2005; Talebizadeh et al., 2006; Lawson-Yuen et al., 2008; Daoud et al., 2009; Zhang et al., 2009; Pampanos et al., 2009; Sun et al., 2011; Xu et al., 2014; Landini et al., 2016; Nakanishi et al., 2017; Quartier et al., 2019; Shillington et al., 2020; and others).

**1b- Neurotactin** (for Dm-NRT: CG9704; UniProt ID: P23654; FlyBase ID FBgn0004108)

NRT, a 846-residue transmembrane protein with a type-II topology and whose extracellular ChE-like domain comprises residues 347-846 (Fig. 1; Table 1), is found only in insects (de la Escalera et al., 1990). In the *Drosophila* nervous system it is expressed during development but not in adulthood. Functionally, NRT appear to be a heterophilic cell adhesion molecule that accumulates on the cell surface where it induces axonal outgrowth, guidance, and fasciculation (Barthalay et al., 1990; Speicher et al., 1998). The only known ligand for NRT is amalgam, a secreted protein member of the Ig superfamily (Frémion et al., 2000). Strikingly, only the first third of the extracellular domain of NRT, whose sequence homology with the ChEs or ChE-like proteins is higher than those of the second and third thirds (Fig. 1), was found to be necessary for both amalgam binding and cell adhesion (Frémion et al., 2000). This suggests that C-terminally truncated NRT still folds and undergoes intracellular trafficking and presentation at the cell membrane, to display an extracellular ‘lobe’ comprising half of the central β-sheet and surrounding surface loops. In contrast to the NLGNs, whose ChE-like domain is separated from the transmembrane domain by a stalk domain (Ichchenko et al., 1995) permitting flexible positioning in the synaptic cleft, in NRT the ChE-like domain starts right after the transmembrane domain.

**1c- Gliotactin** (for Dm-GLIO: CG3903; UniProt ID Q9NK80; FlyBase ID FBgn0001987)

GLIO, a single pass transmembrane protein of 956 residues and whose extracellular ChE-like domain comprises residues 136-695 (Fig. 1), is found in both nematodes and arthropods (Auld et al., 1995). In
Drosophila it is expressed in a wide range of epithelial-like tissues including peripheral glia, epidermis, hindgut, etc. At the cellular level, GLIO is localized to septate junctions, which are structurally equivalent to vertebrate tight junctions. Specifically, GLIO is uniquely localized to the tricellular junction, a specialized structure formed by the convergence of septate junctions in three neighboring cells (Schulte et al., 2003). The highest expression of GLIO during embryonic development is in the peripheral glia from stage 13, with an expression peak at stage 17 during the formation of the blood-nerve permeability barrier (Auld et al., 1995). In addition to permeability defects in the fly salivary glands, gut, and trachea, lack of GLIO in the embryo causes nearly complete paralysis due to the high potassium leak into the motor axons (Auld et al., 1995; Venema et al., 2004). GLIO appears to function as a heterophilic cell-adhesion molecule, although its ligand is unknown. The ChE-like domain of GLIO is separated from the transmembrane span by a 10-residue peptide, i.e., a length intermediate between those for the NLGNs and NRT.

1d- Glutactin (for Dm-GLUT: CG9280; UniProt ID P33438; FlyBase ID FBgn0001114)

GLUT, a secreted protein of 1026 residues whose extracellular ChE-like domain comprises residues 18-602 (Fig. 1), is only found in diptera (Olson et al., 1990). The C-terminal domain that follows is rich in Gln and Glu residues (44% of its composition, hence the name of the protein) and it is organized as semi-repetitive patterns of five residues suggesting the presence of several hydrophilic α-helices. In Drosophila, GLUT was identified as a sulfated, N- and O-glycosylated, strongly acidic calcium-binding protein important for structuring the segmented divisions of the fly. GLUT is found adjacent to sheets of epithelial cells in the basement membrane, lining segmentally spaced channels between segmental nerves in the abdominal segments. It is also found lining channels at the exact medial position between the central nervous system posterior commissures of one segment and the anterior commissures of the next segment. And it is also found the boundaries of segmentally arranged muscle cells where it is secreted at embryonic basement membranes, as part of the extracellular matrix (Olson et al., 1990). Ectopic expression of GLUT inhibits synapse formation by motor neurons that normally innervate muscle M12 (Inaki et al., 2007). The secreted character of GLUT led to suggest a hormone/semiochemical processing-like mode of action (Montella et al., 2012), yet its association with the basement membrane of the extracellular matrix of cells makes it appropriate for cell-adhesion.

1e- Evolution relationships of the ChEs and ChE-like molecules

The evolutionary tree generated from the sequence alignment shows that vertebrate NLGNs 1-4 most likely result from a double whole genome duplication event that occurred in the ancestor of this lineage (Fig. 2). In the arthropods, NLGNs 1-4 and GLIO result from duplications that occurred from the same gene present in the last common ancestor of the primitive invertebrates (protostomes) and the chordates, echinoderms and hemichordates (deuterostomes) (Lenfant et al., 2014). They may have
either conserved the initial function of their common NLGN ancestor or acquired glial and neuronal functions independently. This tree also reflects the limited sequence homology of the second and third thirds of the ChE-like domain of NRT with the other domains, and the correlation between glial NLGNs and GLIO (Fig. 1).

2- Common versus individual characteristics of the ChE-like cell-adhesion proteins
While the ChE enzymes contain a well-conserved catalytic triad that can hydrolyze substrates of various sizes and chemical structures, their ChE-like cell-adhesion relatives are catalytically inactive and display distinctive structural features likely to be related to their respective functions. Below we compare the protein sequences and either experimental (crystal) structures or homology models of the extracellular domains of selected representative members of the ChE and ChE-like subfamilies.

2a- Absence of a functional active center
The most prominent hallmark of the ChE-like cell-adhesion proteins is the lack of the Ser residue and occasionally one of the other two (Glu or His) residues of the catalytic triad responsible for the enzymatic activity of the ChEs (Fig. 1). Exceptions include Drosophila NLGN1, whose analysis through sequence alignment suggests retention of the Ser and Asp residues but substitution of the His residue by a Met; and GLUT, where both the Ser and Glu residues appear to be replaced by an Ala, while presence/absence of the His is unclear due to lower sequence identity in these regions.

Strikingly, in human and insect NLGNs a Ser residue replaces the third of those three Gly residues that in the ChEs form a typical triplet contributing to the oxyanion hole (Fig. 1). The same Ser substitution is found in insect AChEs resistant to insecticides (Weill et al., 2003), where the Ser side chain, located midway the active center gorge, sterically hinders ligand access to the catalytic Ser (Cheung et al., 2018). In the NLGNs, compared to the ChEs, the catalytic triad is modified and the active center pocket is reduced to a vestigial cavity, yet this new Ser side chain contributes coordinating a bound phosphate (PO4) trapped at the center of the subunit (Fabrichny et al., 2007). This observation may support, at least in part, the proposed correlation between exposure to ambient pesticides during pregnancy and early childhood as an environmental risk factor for autism (Bakian & Van Derslice 2019), in addition to the predominant genetic factors.

2b- A partially conserved disulfide-bonding pattern
Another distinctive feature of the ChE-like cell-adhesion molecules is the conservation of the first two of the three disulfide bonds found in the ChEs (Fig. 1). The first disulfide bond ties a large loop, which in the ChEs is named the Ω loop for its shape, and which forms the upper part of the active center gorge, essential for the high catalytic activity of these enzymes (Sussman et al., 1991). This loop also contributes part of the binding surface for the snake toxin and peptidic AChE inhibitor, fasciculin²
(Bourne et al., 1995; Harel et al., 1995). In the ChE-like adhesion members this loop is called Cys-loop because of its more variable shape (Fabrichny et al., 2007), and it was proposed to be important for heterologous partner recognition (Chatonnet et al., 2019). The second half-cystine in this first bond belongs to a conserved SEDCLYLN sequence, which is a signature motif of the ChE and ChE-like proteins and their carboxylesterase cousins. A consensus N-glycosylation sequence is often found just before or within the loop and shown to be occupied.

The second disulfide bond, which ties the base of a short loop protruding at the surface of the subunit, is the most conserved across the α/β-hydrolase fold superfamily of proteins (Fig. 1). The loop sequence is relatively variable, yet in the crystalline ChEs and NLGNGs this loop forms a typical helix-loop-helix motif. The loop often contains a consensus N-glycosylation sequence, found to be occupied in crystal structures.

The third disulfide bond, which in the ChEs stabilizes the C-terminal third of the subunit and insures proper positioning of the catalytic His residue, is both variable in its position and facultative in its presence (Fig. 1). A third bridge, along with the His residue, is present in the mammalian NLGNs, albeit at a distinct position that leads to a more flexible dimerization interface. In contrast it is absent, and occasionally the His as well, in insect NLGNGs and in GLIO and GLUT, albeit one of the Cys is retained in GLIO. This suggests that the third bridge, important for the functional integrity of the ChE enzymes, is less important than the other two for the structural integrity of the ChE-like proteins. From an evolutionary perspective, this also suggests that vertebrate NLGNs conserved some enzymatic activity for a longer time than insect NLGNGs.

The ChE-like domain of NRT contains two additional Cys residues (positions 367 and 408, Fig. 1) belonging to two distinct surface loops, yet found to lay proximal to each other in our homology model (data not shown). This observation suggests presence of a non-conserved, fourth disulfide bond contributing to structural stabilization of the N-terminal lobe of the NRT extracellular domain.

2c- Determinants for dimer formation

Another hallmark of the ChE-like cell-adhesion molecules is the possible conservation of the two α-helices forming the four-helix bundle found at the dimer interface of all crystallized ChE species (Sussman et al., 1991; Bourne et al., 1995; Bourne et al., 1999; Harel et al., 2000; Nicolet et al., 2003; Dvir et al., 2010; Han et al., 2018) and structurally studied NLGNGs (Comoletti et al., 2003, 2006, 2007; Fabrichny et al., 2007; Araç et al., 2007; Koehnke et al., 2008) (Figs. 1, 3, 4). The first of these two α-helices, labeled α3(7,8) in the mouse AChE and human NLGN4X structures, is ~10 residues long and internal to the protein sequence (H1-helix in Fig. 1), while the second helix, labeled α10, is ~15-residues long and located at the subunit C-terminus (H2-helix in Fig. 1). In the ChEs, helix α10 is where the second Cys in the third disulfide bond resides. Human NLGN4X was shown to form the same non-covalent dimer of subunits as mouse AChE, albeit with hydrophobic interactions accounting
models for a larger part of the large interface area buried between the two subunits, an observation suggesting that NLGN dimers are even more stable than AChE dimers (Fabricnty et al., 2007). In fact, the NLGNs were reported be functional as homo- or heterodimers in physiological conditions, thereby expanding their repertoire of functions (Shipman & Nicoll, 2012; Poulopoulos et al., 2012). This ‘functional dimerization’ property is well illustrated by the structural organization of NLGN complexes with MDGA protein 1 (for abbreviations of complicated names, or little used, see the legend to Table 2), where each of the two elongated, multi-domain MDGA molecules wraps around and bridges the two subunits in the NLGN dimer (Gangwar et al., 2017; Kim et al., 2017; Elegheert et al., 2017; Thoumine & Marchot, 2017). In contrast, superimposition of the LNS6 domain in the long NRXNα molecule, which displays a L-shape with a flexible LNS5-LNS6 hinge (Miller et al., 2011; Chen et al., 2011), onto the LNS6 domain of each of the two short NRXNβ molecules bound to a NLGN dimer, suggested that the LNS4 domain in NRXNα could act as a secondary binder to the same subunit as already bound with LNS6.

Whether the invertebrate ChE-like molecules form dimers is not documented. However, sequence analysis do not reveal particular features that would drastically preclude subunit association, while our homology models suggest that despite their variable length and residue composition, the two canonical α-helices, α3(7,8) and α10, are conserved and appropriately positioned for bundle formation (Figs. 1, 3, 4). Formation of NRT dimers might either challenge or regulate its interaction with amalgam, itself shown to form a concentration-dependent dimer in vitro (Zeev-Ben-Mordehai et al, 2009a, 2009b). Contribution of GLIO to the architecture of tricellular junctions through direct or indirect interaction with the large, triple-repeat extracellular domain of the transmembrane protein, anakonda (Byri et al., 2015), may suggest the need for a trimeric, rather than dimeric, assembly of subunits.

2d- Diversified N-glycosylation patterns
Consistent with their euakryotic origin and extracellular localization, the extracellular domains of the ChE-like cell-adhesion molecules display a variable number of consensus sequences (aka sequons) for N-linked glycosylation (Fig. 1). Human NLGNs have two (conserved) to four of them (four in NLGN1, of which one in splice insert B; three in NLGN2; two in NLGN3; two in NLGN4X/Y). For the Drosophila proteins, one finds four to six of them in the NLGNs (five in NLGNs 1-2; six in NLGN3; four in NLGN4), six in NRT, eight in GLIO, and three in GLUT, pointing to an overall greater number of sites for the insect proteins. Only for recombinant NLGNs have actual N-glycosylation patterns been characterized, either in vitro for rat NLGN1 (Hoffman et al., 2004) or in cristallo for rat/mouse NLGN1 (Araç et al., 2007; Chen et al., 2008), mouse/rat/human NLGN2 (Koehnke et al., 2008; Gangwar et al., 2017; Kim et al., 2017), and human NLGN4X (Fabricnty et al., 2007).

Comparative mapping of these sites at the surface of the respective protein structures and models (Fig. 3) points to preferential clustering on the front face, i.e., the face of the subunit where in
AChE, the active center gorge opens (Ω-loop / Cys-loop face) and the inhibitory snake toxin fasciculin binds (Bourne et al., 1995; Harel et al., 1995). Fewer sites, including the one in splice insert B in rodent NLGN1 (see Fig. 1, but not shown in Fig. 3), are located on the back face, i.e., the opposite face of the subunit where in the NLGNs, NRXNβ1 or MDGA-Ig1 bind. Some of these sites appear to be fairly conserved in both the ChEs and ChE-like molecules, such as those located close to the N-terminus, just before the first Cys in disulfide bond 1 (exemplified by N102 in human NLGN4X, N115 in GLUT, N417 in NRT); within the surface loop tied by bond 2 (N296 in human AChE, N331 in Drosophila AChE); and close to the putative dimerization interface, just after the second Cys in bond 3b (N511 in human NLGN4, N595 in human AChE), while all were shown to be occupied, two features suggesting a structural or protective role. In NRT, five of the six sites seem to wave across the front face, from the N- to the C-terminal corners of the subunit (Fig. 3). Overall N-glycosylation of NRT was experimentally demonstrated in vitro, but not the exact pattern of site occupancy (de la Escalera et al., 1990), nor N-glycan contribution to amalgam binding.

2e- LRE motifs

The tripeptide leucine-arginine glutamate (LRE) was primarily identified as an adhesive site for slaminin, an extracellular matrix protein influencing cell differentiation, migration and adhesion in striated muscles and peripheral nerves (Durbeej, 2010). AChE was found to bind laminin using yeast two-hybrid screening and co-immunoprecipitation (Paraoanu & Layer, 2004, 2005). The positions of LRE motifs in ChEs, other enzyme relatives, and the ChE-like proteins were ranked using sequence comparison and 3D homology modeling based on AChE templates (no NLGN structures were available) and found to be distributed into four main sites at the surface of the ChE-like subunit (Johnson & Moore, 2013). Most AChEs and NLGNs along with NRT, GLIO and GLUT contain one to several complete or incomplete LRE motifs (Fig. 1), of which some indeed share similar spatial positions (Fig. 3). In mammalian NLGNs, one motif is conserved in helix α3(7,8), which contributes to the dimer interface (H1 helix in Fig. 1) (Figs. 3, 4). In human NLGN3, the Arg residue in this particular motif is the one whose Cys substitution results in intracellular retention of a misfolded protein (De Jaco et al., 2012). In turn, both the Arg and Glu residues in this motif form an integral part of the MDGA1-Ig2 binding site on human/rat NLGNs 1 and 2 (Kim et al., 2017; Gangwar et al., 2017; Elegheert et al., 2017). These two features offer insightful functional correlation for LRE motifs in ChE-like proteins. However, none of these motifs are involved in fasciculin binding to AChE nor NRXNβ1 binding to NLGNs (Fig. 3), and whether these motifs in the ChEs or the ChE-like cell-adhesion molecules bind laminin is not documented. Still, it is noteworthy that on NRT, the first LRE motif, located at the very N-terminal end of the subunit and surrounded by two putative N-glycosylation sites (of which one is likely to be occupied, see Fig. 1), belongs to that same lobe of the subunit as shown to be mandatory for both binding of amalgam and cell-adhesion.
2f- Calcium binding

Fasciculin binding to the peripheral anionic site of AChE, located at the entrance of the active center gorge (Fig. 3), is challenged by calcium (Karlsson et al., 1984; Marchot et al., 1993), and AChE is protected by magnesium against thermal denaturation (Millard et al., 2003). NRNX binding to NLGN is calcium-dependent (Ichtchenko et al., 1995, 1996; Comoletti et al., 2003). And GLUT binds calcium, preferably in presence of excess magnesium (Olson et al., 1990). Based on these observations, an in silico study pointed to putative calcium-binding motifs at the surface of ChE and ChE-like proteins (Tsigelny et al., 2000). However, no specifically bound calcium or magnesium was found in structures of AChE despite their presence in the crystallization liquors (P. Marchot, personal data), consistent with their low affinity for the peripheral site (Marchot et al., 1993), nor in a structure of NLGN4 crystallized in the presence of calcium (Fabrichny et al., 2007). In turn, calcium molecules found to be trapped at the NRXNβ1-NLGN1/4 complex interface were coordinated by NRXNβ1, with no direct interaction with the NLGN (Fabrichny et al., 2007; Araç et al., 2007; Chen et al., 2008; Leone et al., 2010). Calcium was found not to be required for MDGA binding to NLGN in solution nor in the crystal state (Kim et al., 2007; Gangwar et al., 2017; Elegheert et al., 2017).

2g- Electrostatic surface potentials

The AChE subunit is characterized by a markedly asymmetric distribution of charged residues at its surface, generating a negatively charged face, essentially contributed by the peripheral anionic site at the entrance of the active center gorge (and identified as the Ω-loop face, or ‘front face’ in Fig. 3), and a positively charged face on the opposite face of the subunit (identified as the ‘back face’ in Fig. 3) (Sussman et al., 1991; Ripoll et al., 1993) (Fig. 5). This electrostatic pattern produces a significant dipole moment, with a vector roughly oriented along the axis of the active-site gorge, and suggested to enhance long-distance attraction of cationic substrates toward the gorge entrance and their diffusion toward the active center (Silman & Sussman, 2008). The electronegative potential of the front face of AChE was also shown to be instrumental for long-range attraction of peptidic cationic non-competitive inhibitors (such as fasciculin, see Fig. 3) toward the peripheral anionic site of the enzyme (Bourne et al., 1995; Harel et al., 1995; Bourne et al., 2015). Early homology modeling of the extracellular domains of mouse NLGN1 and Drosophila NRT and GLIO, along with quantitative analysis of the electrostatic properties of their front face, evidenced a negatively charged annular patch similar to that found on torpedo AChE, and led to nicknaming these proteins ‘electrotactins’ (Botti et al., 1998). Later on, the crystal structure of human NLGN4X confirmed the electronegative character of the front face of the NLNG subunit, yet it also revealed the electronegative character of its opposite, back face (where NRXNβ1 binds, see Fig. 3), a feature generating an electronegative belt around the subunit and precluding formation of a dipole (Fabrichny et al., 2007; Leone et al., 2010) (Fig. 5).
To extend this analysis, we explored the electrostatic surface potentials of *Drosophila* AChE and rodent NLGNs 1 and 2, based on their experimental structures (Harel et al., 2000; Araç et al., 2007; Koehnke et al., 2008), and of *Drosophila* NRT based on our homology model, comparatively with those of mouse AChE and human NLGN4X, used as references (Fig. 5). Consistent with the situation with torpedo AChE (see above), on mouse AChE the electronegative surface potential is restricted to the front face of the subunit, while the back face is rather positively charged. In *Drosophila* AChE, the overall electronegative character of the front face is retained but not the electropositive character of the back face, which instead presents a well-delimited, strongly electronegative patch likely to alter the dipole vector and moment found in the torpedo and mouse enzymes. In NLGNs 1 and 2 the situation is similar to that found for human NLGN4X, albeit with a smaller, slightly eccentric electronegative patch on the front face of NLGN2 (data not shown). Of the three other ChE-like cell-adhesion molecules, GLUT resembles more mouse AChE for both faces of the subunit, a feature suggesting existence of a diplolar moment. In contrast, on GLIO the electronegative patch on the front face is smaller and restricted to the N-terminal part of the subunit, while the back face is frankly electropositive. It is noteworthy that on the front face, the electropositive surface area positive correlates well with the surface area devoid of N-glycan (Fig. 3). Finally, on NRT the electronegative patch on the front face appears to be split into two parts, while the back face resembles more those of mouse AChE and GLUT. However, whether these features are related to partner recognition by these three cell-adhesion molecules is unknown.

### 3- Extracellular binding partners of the AChEs and ChE-like cell-adhesion molecules

Peptidic ChE partners with a well-documented mode of binding and action comprise two types of molecules rich in β-strands: the natural snake toxins fasciculins and three non-natural monoclonal antibodies, all acting as non-competitive AChE inhibitors (Bourne et al., 1995, 2013, 2015; Harel et al., 1995; and references in them) (Table 2). The fasciculins and two of the antibodies are cationic molecules that bind the peripheral anionic site on the front face of the enzyme, thereby occluding the entrance to the active center gorge (Fig. 3). Other proposed peptidic partners, albeit not inhibitors, of AChEs comprise laminin, a large heterotrimeric glycoprotein and a major extracellular matrix component of the basal lamina (Paraoanu & Layer 2004, 2005), and the amyloid β-peptide, whose assembly into Alzheimer’s fibrils was shown to be accelerated by AChE (Inestrosa et al., 2008). Both were suggested to bind the peripheral anionic site of the enzyme. Structural analysis of mouse AChE pointed to resemblance of the amyloid β-peptide in its soluble, non-pathogenic state, with the short surface loop tied by the second disulfide bond and conserved among the ChE and ChE-like proteins (see above, and Fig. 1), thereby suggesting a mode of β-peptide nucleation by AChE to promote aggregation (Bourne et al., 1999).
Most of the identified or proposed partners of the ChE-like cell-adhesion molecules also encompass globular domains rich in β-strands, belonging to a large variety of structural families, and whose association in various combinations often forms elongated molecules (Bourne & Marchot 2014). The membrane-anchored NLGN partners, NRXNs α and β, have different extracellular domains. The long NRXNα extracellular domain comprises three ‘NRXN repeats’ made of one LNS, one EGF and one LNS domain, while the short NRXNs β contain only the C-terminal, sixth LNS domain (Ushkaryov et al., 1992) (Table 2). The membrane-anchored MDGAs, which belong to the immunoglobulin superfamily of proteins, comprise six Ig-like domains followed by one FN3-like domain and one MAM domain before the glycosylphosphatidylinositol anchor to the membrane (Litwack et al., 2004). In the NRNXs, only LNS6 appears to interact with NLGN, through loops forming the edge of the β-sandwich and the calcium cage (Fabrichny et al., 2007; Araç et al., 2007; Chen et al., 2008; Leone et al., 2010; Tanaka et al., 2012). For the MDGAs, these are the three consecutive Ig1-Ig2-Ig3 domains that interact with NLGN, with complementary contributions from both β-strands and intervening loops (Kim et al., 2017; Gangwar et al., 2017; Elegheert et al., 2017).

The NLGNs have also been proposed to interact extracellularly with other extracellular proteins or domains belonging to various structural families (Table 2). Selective interaction of NLGNs with the transmembrane PTPRT proteins was proposed to occur with varying affinities depending on the NLGN isoform (Lim et al., 2009). This interaction appears to regulate neuronal synapse formation, consistently with the role of several PTPRTs in the control of axonal outgrowth, guidance, and synapse formation in the central nervous system. Structurally, the PTPRT extracellular domain resembles that of MDGA besides a distinct organization (Alonso et al., 2004). Co-immunoprecipitation in heterologous cells also suggested that NLGN1-3 interact with TSP1 to mediate its synaptogenic effect, similar to that of NRXNs, on neuron development (Xu et al., 2010). TSP1 proteins are secreted molecules consisting of an LNS domain followed by a vWFC domain, three consecutive TSP1 repeats, three consecutive EGF-like domains, 13 Asp-rich calcium-binding type-3 repeats, and a L-type lectin-like domain (Tan et al., 2006; Misenheimer et al., 2000). Finally, the extracellular domain of NLGN1 was reported to specifically mediate cis interactions with the extracellular domain of the GluN1 subunit of the NMDA receptor (Budreck et al., 2013). This GluN1 extracellular domain consists of two large globular clamshell-like domains: an N-terminal domain involved in subunit assembly and an agonist-binding domain that binds glycine (Paoletti, 2011).

The NRT partner, amalgam, a secreted member of the immunoglobulin superfamily, is made of three different types of Ig-like domains (Frémion et al., 2000) (Table 2). Biophysical and low-resolution structural data in solution pointed to an elongated monomer at low concentration but a V-shaped, N-terminally coordinated dimer at high concentration (Zeev-Ben-Mordehai et al., 2009a, 2009b), similar to dimers formed by neuronal IgLON proteins (Ranaivovon et al., 2019). This observation led to propose that the two amalgam arms would bind the extracellular domains of two NRT molecules.
respectively anchored to facing membranes, thereby promoting neurotactin clustering in trans, and leading to cell adhesion and axon fasciculation in Drosophila (Zeev-Ben-Mordehai et al., 2009b).

The putative GLIO partner, anakonda, is a transmembrane protein whose extracellular domain is made of three repeats, each comprising a SRCR-like domain, a CUB or CUB-like domain and a single-stranded right-handed β-helix motif (Byri et al., 2015) (Table 2). The unusual tripartite organization of this domain was suggested to be correlated to requirement of anakonda for the formation of tricellular, but not bicellular junctions (Byri et al., 2015). Synergistic contribution by the four-transmembrane domain, proteolipid protein M6 was recently reported (Esmangart de Bournonville & Le Borgne, 2020; Wittek et al., 2020).

No specific binding partner for GLUT has been identified. However, protein-protein associations involving a large number of individual Drosophila proteins, examined using co-affinity purification coupled to mass spectrometry analysis, pointed to more than 30 potential interaction partners belonging to various protein types and families (Guruharsha et al., 2011). These data now need validation.

4- Conclusion
ChE-like cell-adhesion molecules play a role during the development of multiple tissues within a wide range of metazoans. This small subset of the α/β-hydrolase superfamily of proteins shares an extracellular or soluble domain structurally related to the catalytic domain of the ChE enzymes, but they lost catalytic properties and acquired heterophilic partner recognition and association functions during evolution. This evolutionary divergence included variations in the disulfide bonding; in the presence, number and position of particular loops, N-glycans and repeat motifs and in the repartition of electrostatic charges at the surface of the molecule; and possibly in dimer formation. The NLGNs have been extensively studied at the functional and structural levels and several extracellular binding partners have been either identified and characterized, or proposed. A NRT binding partner has been identified and characterized; yet their mode of association and resulting mode of action remain unclear. For GLIO and GLUT, no bona fide endogenous ligands or receptors have been identified. Documenting and comparing the structure-function relationships of these partnerships is instrumental to understand how they work at the cellular level. Moreover, this knowledge is critical to expand our understanding of surface determinants for cell-adhesion and try to assign cell-adhesion functions to poorly characterized ChE-like proteins with an altered active-center machinery.

Footnotes
1 Not to be confused with the chemokine ‘fraktalkine’, aka chemokine (C-X3-C motif) ligand 1, occasionally named ‘neurotactin’ as well (see Hortsch, 1997).
2 Not to be confused with the membrane-linked cell-adhesion molecule ‘fasciclin’, of an Ig-related fold.
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Disclosure
The authors declare no conflict of interest.
Table 1: Calculated sequence identity (in parentheses: similarity) of the extracellular domains of acetylcholinesterase and cholinesterase-like proteins (%)

<table>
<thead>
<tr>
<th></th>
<th>Hu-NLGN4X</th>
<th>Dm-AChE</th>
<th>Mo-AChE</th>
<th>Dm-NRT</th>
<th>Dm-GLIO</th>
<th>Dm-GLUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu-NLGN4X</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dm-AChE</td>
<td>25.3 (50.7)</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mo-AChE</td>
<td>31.5 (58.1)</td>
<td>34.0 (60.4)</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dm-NRT</td>
<td>20.1 (44.0)</td>
<td>21.0 (46.6)</td>
<td>23.8 (47.5)</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dm-GLIO</td>
<td>27.3 (51.2)</td>
<td>22.1 (51.9)</td>
<td>27.9 (54.2)</td>
<td>24.4 (48.9)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Dm-GLUT</td>
<td>22.0 (46.1)</td>
<td>20.5 (47.6)</td>
<td>23.5 (50.8)</td>
<td>22.8 (45.8)</td>
<td>21.2 (51.7)</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 2: Identified and putative extracellular partners of the ChEs and ChE-like proteins, and their constitutive domains

<table>
<thead>
<tr>
<th>ChE or ChE-like protein</th>
<th>Partner</th>
<th>Domain type</th>
<th>Fold description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChEs</td>
<td>Fasciculins</td>
<td>Three-fingered toxin</td>
<td>Reticulated core, central β-sheet, three emerging loops</td>
</tr>
<tr>
<td></td>
<td>Antibodies</td>
<td>Immunoglobulin (Ig)</td>
<td>Two-layer sandwich of two β-sheets in a Greek key topology</td>
</tr>
<tr>
<td></td>
<td>Laminin</td>
<td>Laminin</td>
<td>Central α-chain with a varying number of globular regions; β- and γ-chains with α-helical and globular regions</td>
</tr>
<tr>
<td></td>
<td>Amyloid β-peptide</td>
<td>Helix-loop-helix</td>
<td>Disordered loop tied by two flanking α-helices</td>
</tr>
<tr>
<td>NLGNs</td>
<td>NRXNs β</td>
<td>LNS</td>
<td>β-Sandwich of 10-15 β-strands with a jelly-roll topology and canonical calcium binding sites located on one edge</td>
</tr>
<tr>
<td></td>
<td>NRXNs α</td>
<td>(*) 3x[LNS-EGF-LNS] (aka ‘NRXN repeats’)</td>
<td>LNS: see above; EGF: two-stranded β-sheets separated by a loop</td>
</tr>
<tr>
<td></td>
<td>MDGAs</td>
<td>(*) [6xlg-like]-FN3-MAM</td>
<td>Ig: see above; FN3: β-sandwich of two antiparallel β-sheets; MAM: compact β-sandwich of β-strands with a jelly-roll topology</td>
</tr>
<tr>
<td></td>
<td>PTPRT</td>
<td>(*) MAM-Ig-like-[4xFN3-like]</td>
<td>MAM, Ig, FN3: see above</td>
</tr>
<tr>
<td></td>
<td>TSP1</td>
<td>(*) LNS-vWFC-[3xTSP1]-[3xEGF]-[13xARCBT3R]-LTL</td>
<td>LNS, EGF: see above; vWFC: cystine knot (knottin) fold, i.e., compact core of 3-4 β-strands with alternating orientation, tied by 3 disulfides; TSP1: three β-strands with alternating orientation, stabilized by disulfides; ARCBT3R:</td>
</tr>
<tr>
<td>Abbreviations and names of domains - CUB, complement/UEGF/BMP1; EGF, Epidermal Growth Factor; FN3, FibroNectin type-III; Ig, Immunoglobuline; LNS, Laminin, Neurexin, Sex-hormone binding globulin; MAM, meprin, A-5 protein, and receptor protein-tyrosine phosphatase Mu; MDGA, Meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu [MAM] Domain-containing Glycosylphosphatidylinositol Anchor; PTPRT, Receptor-type tyrosine-protein phosphatase T; SRCR, scavenger-receptor; TSP1, thrombospondin-1; vWFC, von Willebrand factor type-C; LTL, L-type lectin-like; GluN1, GluN1 subunit of the N-Methyl-D-aspartate [NMDA] receptor. (*) From the N- to the C-terminal.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1 - Sequence alignment of selected ChE and ChE-like extracellular domains - Sequences in bold denote proteins for which a crystal structure is available. Residue numbering right to each sequence is that of UniProt, which includes the signal peptide. (For RCSB-PDB accession codes and UniProt entries, see below.) The N- and C-terminal extremities of the proteins, which display low sequence conservation, are not displayed. Only for Dm-NRT are two additional N-term residues included because they belong to a LRE motif, while only for Dm-NRT is the true C-terminal displayed as denoted by a double slash (///). Letter/color codes within the alignment: C letters on yellow and orange background, Cys residues involved (all proteins) and likely to be involved (NRT) in disulfide bonds, respectively; green LRE letters (or two of them only) on green background, putative motifs for partner recognition; blue NXS/T letters on grey background, consensus triplets for N-glycosylation (NB - a red N letter denotes experimentally demonstrated occupancy); red SEDCLYLN letters on orange background, conserved octapeptide signature in the ChE and ChE-like subfamilies; purple GGG letters on grey background, conserved triplet in ChE proteins – the star below the alignment points to the Gly to Ser substitution found in the NLGNs and the insecticide-resistant Ag-AChE mutant; red H letter on grey background, C-terminal boundary of the NRT sequence (His347-His482) required for amalgam binding and cell adhesion; red Y letters on grey background, predicted sulfo-Tyr residue; GXSXGX sequence on grey background, conserved hexapeptide signature of the α/β hydrolase fold superfamily; red S/E/H letters, catalytic triad residues in AChEs. Letter/color codes below the alignment: question marks on orange background, Cys residues proposed to form the non-conserved, N-terminal disulfide bond in NRT; 1/2/3ab letters on yellow background, Cys residues forming the conserved first and second and alternative third disulfide bridges; green/blue “A1/A2” stretches, splice inserts A1/A2 in human NLGNs 1-4 (NB - NLGN4X insert A2, found in low occurrence isoform 2, is displayed in lowercase letters and not included in the numbering (see * right to the sequence line); pink residues and ‘exon4-deletion’ note, splice insert in NLGN4X; orange “B” stretch, splice insert B in rodent NLGN1 (not found in human NLGN1); “aromatic” stretch, Trp/Tyr/Phe-rich insert also found in many insect proteins; “Gly-rich” stretch, Gly-rich insert also found in several Drosophila proteins (see below); “H1/H2-helix“ stretches, approx. coverage of helices α3(7,8) and α10 involved in dimer formation for the ChEs and NLGNs (NB – the red R letter in Hu-NLGN3 helix H1 denotes the position of the R451C substitution).

RCSB-PDB entries: Hu-NLGN4X, 3BE8; Hu-NLGN1, 3BIX for the rat homologue; Hu-NLGN2, 3BL8 for the mouse homologue; Dm-AChE, 1QO9; Ag-AChE1, 5X61; Hu-AChE, 1B41.

UniProt entries: Dm-NRT, P23654; Dm-GLU, P33438; Dm-GLIO, Q9NK80; Dm-NLGN1, Q9VIC7; Dm-NLGN4, Q9VDP5; Hu-NLGN4X, Q8N0W4 (variant, Q8N0W4-2); Hu-NLGN3, Q9NZ94; Hu-NLGN1, Q8N2Q7; Hu-NLGN2, Q8NFZ4; Dm-AChE, P07140; Ag-AChE2, Q7QFG0; Ag-AChE1, Q869C3; Hu-AChE, P22303.
Drosophila proteins with a Gly-rich stretch: GH07829p (gene Nlg4, UniProt entry B6IDZ4), GM23100 (Dsec\GM23100, B41E9), GD19341 (Dsim\GD19341, B4QS57), uncharacterized protein (Danai\GF17640, A0A0P8XXC1), GL12091 (Dper\GL12091, B4GL70), NLGN1 (DGUA_6G016471, A0A3B0KJP9).

Figure 2 - Evolutionary relationships of taxa - The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 3.37097384 is shown. The tree is drawn to scale, with branch lengths proportional to the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei & Kumar, 2000) and are in the unit of the number of amino acid residue differences per site. The analysis involved 13 protein sequences (ChE-like domains only). All positions with less than 70% site coverage (e.g., individual or splice inserts) were not considered for the alignment. That is, fewer than 30% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 503 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Figure 3 – Comparative mapping of selected surface determinants on the extracellular domains of mammalian and Drosophila AChE and ChE-like cell-adhesion molecules - Shown are experimental structures of human NLGN4X bound with NRXNβ1 (PDB ID: 2XB6, green NLGN4X molecular surface, brown NRXNβ1 backbone ribbon), mouse AChE bound with fasciculin (1MAH, grey AChE surface, gold fasciculin ribbon) and Drosophila AChE (1DX4, red surface), along with homology models of Drosophila GLIO (blue surface), GLUT (purple surface) and NRT (orange surface). All molecules are displayed in two orientations showing the front face (where the AChE active center gorge opens, as indicated for human and Drosophila AChEs) and the back face (180° rotation around a vertical axis) of the subunit. Sequence-predicted positions for Asn-linked glycans are highlighted in yellow and labeled. Surface LRE motifs are highlighted in white. All N- and C-termini are labeled. Homology modeling was carried out by submitting individual protein sequences to the Swiss Model server (https://swissmodel.expasy.org/). Selected templates were: for NRT, human liver carboxylesterase 1 (PDB ID: 5A7H; 24.5% sequence identity; 93% coverage); for GLUT, Manduca sexta juvenile hormone esterase (2FJ0; 25.9% sequence identity; 87% coverage; for GLIO, mouse NLGN2 (3BL8; 36.2% sequence identity; 74% coverage). Figure generated with PyMol (The PyMol Molecular Graphics System, version 2.2.3, Schrödinger, LLC). For NRT, GLIO and GLU the positions of the surface loops and determinants are likely to be biased by the selected template.
Figure 4 – Superimposition of the homology models of NRT, GLIO, GLUT with the experimental structure of human NLGN4X. The molecule backbones are displayed as ribbons (green NLGN4X, blue GLIO, purple GLUT, orange NRT). The N- and C-termini and helices α3(7,8) and α10 are labeled. The presence of the two helices α3(7,8) and α10 is compatible with dimer formation by all of these proteins.

Figure 5 – Electrostatic surface potentials of mammalian and Drosophila AChE and ChE-like cell-adhesion molecules. Shown are the same crystal structures of mammalian and Drosophila AChE and homology models of NLGN4X and Drosophila NRT, GLIO, GLUT, displayed in the same two orientations, as in Fig. 3. Surface potentials are expressed as a spectrum ranging from -3 kT/e (deep red) through 0 kT/e (white) to +3 kT/e (deep blue). The calculation used default parameters. Figure generated with PyMol (The PyMol Molecular Graphics System, version 2.2.3, Schrödinger, LLC). For NRT, GLIO and GLUT the positions of the surface loops and determinants are likely to be biased by the selected template.
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