Expression and modulation of neuroligin and neurexin in the olfactory organ of the cotton leaf worm Spodoptera littoralis

Nicolas Durand, Thomas Chertemps, Francoise Bozzolan, Martine Maïbèche

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Durand N., Chertemps T., Bozzolan F. and Maïbèche M*.

Sorbonne Universités UPMC - Univ Paris 06, Institut d'Ecologie et des Sciences de l'Environnement de Paris, INRA, CNRS, IRD, UPEC, Département d'Ecologie Sensorielle, F-75252, Paris, France

* Correspondence: Dr Martine Maïbèche. Institut d'Ecologie et des Sciences de l'Environnement de Paris, Département d'Ecologie Sensorielle, 7 Quai St-Bernard, BC 1211, F-75005 Paris, France.

Tel: + 33 144276592; Fax: + 33 144276509; e-mail: martine.maibeche@upmc.fr

Running tittle: *antennal Carboxylesterase-Like Adhesion Molecules*

Abstract

Carboxylesterases are enzymes widely distributed within living organisms. In insects, they have been mainly involved in dietary metabolism and detoxification function. Interestingly, several members of this family called Carboxylesterase-Like Adhesion Molecules (CLAMs) have lost their catalytic properties and are mainly involved in neuro/developmental functions. CLAMs include gliotactins, neurotactins, glutactins, neuroligins. The latter have for binding partner the neurexin. In insects, the function of these proteins has been mainly studied in *Drosophila* central nervous system or neuromuscular junction. Some studies suggested a role of neuroligins and neurexin in sensory processing but CLAM expression within sensory systems has not been investigated. Here, we reported the identification of five putative CLAMs expressed in the olfactory system of the model pest insect *Spodoptera littoralis*. One neuroligin, *Slnlg4-yll* and its putative binding partner neurexin *SlnrxI* were the most expressed in the antennae and were surprisingly associated with olfactory sensilla. In addition, both transcripts were upregulated in male antennae after mating, known to modulate the sensitivity of the peripheral olfactory system in *S. littoralis*, suggesting that these molecules could be involved in sensory plasticity.

Keywords : neuroligins, neurexin, Carboxylesterase-Like Adhesion Molecules ; olfaction ; insect

Abbreviations : CLAM: Carboxylesterase-Like Adhesion Molecules; CCE: carboxylesterases; NLG: neuroligins; NRX: neurexin

Introduction

Carboxylesterases or CCEs (EC 3.1.1.1) belong to a multigene family of enzymes widely distributed in animals, plants and microorganisms. They are involved in various functions, from xenobiotic detoxification to development regulation and neurogenesis. In insects, CCEs are known to play a key role in insecticide resistance and detoxication of plant allelochemicals. Some CCEs have been involved in specific functions, such as acetylcholinesterase (AChE) or juvenile-hormone esterase (JHE) (reviewed in (Oakeshott et al. 2005)). Several others are proposed to have a function in olfaction as odorant-degrading esterases (reviewed in (Leal 2013)). However, the physiological role of most insect CCEs is unknown. Based on substrate specificity and sequence similarity, insect CCEs have been divided into eight subfamilies: four of them, the α-esterases, β-esterases, AChEs and JHEs account for the majority of the enzymatic active CCEs that use a catalytic mechanism based on a triad featuring a serine nucleophile residue (Oakeshott et al. 2005). Four other subfamilies, namely neuroligins, gliotactins, neurotactins and glutactins are considered to be non-catalytic proteins. These CCEs have lost one or more of the active site residues, but have gained new structural characteristics such as transmembrane and binding domains and are also called Carboxylesterase-Like Adhesion Molecules (CLAMs). They are mainly associated with the promotion of cell adhesion during neural development (reviewed in (Gilbert and Auld 2005; Johnson and Moore 2013)).

In insects, functional data on gliotactin (Auld et al. 1995), neurotactin (Barthalay et al. 1990) and glutactin (Olson et al. 1990) are only available for *Drosophila melanogaster* (review in (Oakeshott et al. 2005)). The transmembrane protein Gliotactin is involved in the formation of septate junction in epithelia (Schulte et al. 2003) and in the establishment and maintenance of the blood-nerve barrier in peripheral glia (Auld et al. 1995). Neurotactin is also a transmembrane

glycoprotein (Barthalay et al. 1990). It binds to the secreted protein Amalgam and the resulting complex is responsible for axon guidance in *Drosophila* embryo (Frémion et al. 2000). Glutactins are secreted proteins forming part of the basement membrane. They are abundant in the envelope of the central nervous system (CNS) (Olson et al. 1990), but precise information on their function is lacking.

Contrary to the other CLAMs, neuroligins (NLGs) and their principal binding partner neurexin (NRX) are present in all animal phyla. They have been mainly studied in mammals and implicated in various neuronal processes, including the differentiation, maturation, stabilization and plasticity of synapses. Mutations in NLGs and NRXs in humans have been linked to several cognitive diseases, in particular autism (review in (Bang and Owczarek 2013)). If most of the NLGs and NRXs from mammals were brain-specific proteins, some of them also exhibited a more ubiquitous expression pattern, such as in the vascular system, suggesting other functions in the peripheral tissues (review in (Bang and Owczarek 2013; Bottos et al. 2011)).

In insects, NLGs and NRXs have been characterized in the fruit fly and in a lesser extend in the honey bee *Apis mellifera*. NRXs and NLGs are known as trans-synaptic binding partners and are mostly expressed in the presynaptic and post-synaptic compartments, respectively (Knight et al. 2011). *D. melanogaster* possesses four *nlg* genes expressed at synapses from the CNS and/or the neuromuscular junctions (NMJs): *dmnlg1* (Banovic et al. 2010; Mozer and Sandstrom 2012) and *dmnlg2* (Chen et al. 2012; Sun et al. 2011) are required independently for synaptic growth and function. *dmnlg3* played a role in the NMJ development and synaptic maturation (Xing et al. 2014), whereas *dmnlg4* is expressed in clock neurons and is essential for sleep regulation (Li et al. 2013). Five NLGs were found in the bee, all of them are mostly expressed in the brain, except *Amnlg1* which is more abundant in peripheral tissues (Biswas et al. 2008). Two NRXs were identified in *Drosophila*: *nrxIV* is distantly homologous to vertebrate neurexins and is mainly

expressed in the septate junctions of glial and epithelial cells (Baumgartner et al. 1996) but also in the midline neurons (reviewed in (Sun and Xie 2012)). *NrxI* is highly expressed in the brain, including the adult antennal lobes and mushroom bodies (Li et al. 2007; Zeng et al. 2007), two structures involved in olfactory learning and integration of sensory information. Moreover, *NrxI* has been also involved in sleep regulation (Larkin et al. 2015).

Several studies suggested a link between NRX-NLG function and sensory processing in insects (reviewed in (Knight et al. 2011)). In *Drosophila*, *DmnrxI* is required for synapse formation and associative learning in larvae (Zeng et al. 2007). In the honeybee, sensory deprivation resulted in *Amnlg1* reduced expression, while associative scent training induced an increasing of *AmnrxI* and *Amnlgn3* expression (Biswas et al. 2010). *AmnrxI* and *Amnlg3* genes were mostly expressed in the mushroom body of the adult brain, a pattern consistent with a role in sensory signalling and cognitive processing. Several *Amnlg*/*nrx* genes were in addition expressed outside the brain, putatively in the peripheral nervous system but their expression in the bee olfactory system has not been studied.

Identification of CLAMs within antennae has been done previously only in two species, *D. melanogaster* (Younus et al. 2014) and the blowfly *Calliphora stygia* (Leitch et al. 2015), through transcriptomic analysis, but without any detailed study of their expression patterns within this sensory tissue. The pest moth *Spodoptera littoralis* is a main model to study olfactory reception and its plasticity in insects. While the modulation of the peripheral olfactory system by circadian rhythms, physiological state, experience or mating has been well studied in this species (Bigot et al. 2011; Guerrieri et al. 2012; Martel et al. 2009; Merlin et al. 2007), the underlying mechanisms are still unknown. Using an EST library approach, we previously revealed an unsuspected diversity of 30 CCEs expressed in the antennae of this species (Durand et al. 2010b), including one glutactin-like (Durand et al. 2012). Among them, we functionally characterized

two active CCEs as odorant-degrading esterases (Durand et al. 2011; Durand et al. 2010a). We have now completed this repertoire with five new antennal CCEs belonging to the CLAMs. We identified single homologue of gliotactin and neurotactin, three homologues of neuroligin and one of neurexin I. We compared their expression profile in different adult tissues and, for the two genes abundantly expressed in the olfactory organ, *i.e Slnlg4-yll* and *SlnrxI*, we investigated whether their expression could vary in the antennae depending on the male mating status, known to trigger plasticity of the peripheral olfactory system. The involvement of NLG/NRX in synaptic plasticity makes them putative candidates for such a modulation.

Material and methods

Insects and tissue collection

Insects were reared on semi-artificial diet at 24°C, 60–70% relative humidity, and under a 16:8h light:dark (LD) photoperiod until emergence. Sexes were separated at pupal stage. Adults were kept under an inverted LD regime and provided with a 10% sucrose solution. Antennae and various tissues (proboscis, brain, leg, thorax and abdomen) from two day-old adults were dissected and stored at -80°C until RNA or protein extraction. For mating experiments, 15 one-day old insects of each sex were kept together for 24 hours into hermetically sealed boxes. Under these conditions, males started mating within a few minutes. Subsequently, male antennae and brains were then dissected 24 hours after mating. Control males were kept in the same conditions but without females.

RNA isolation and cDNA synthesis

Total RNAs were extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) then treated with DNase I (Roche, Basel, Switzerland) in accordance with the manufacturers' protocols and quantified with a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). Single-stranded cDNAs were synthesized from total RNAs (5 µg) from various tissues with Superscript II reverse transcriptase (Gibco BRL, Invitrogen) with the oligo(dT)18 primer according to the manufacturer's instructions. For 5’ and 3’-RACE PCR, antennal cDNAs were synthesized from 1 µg male antennal RNA at 42°C for 1.5 h using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) with 200 U of Superscript II, 3’-cDNA synthesis-primer and SMART II oligonucleotide.

Identification and cloning of Spodoptera littoralis CLAM sequences

Putative CLAM sequences were identified from *S. littoralis* antennal EST libraries (Jacquin-Joly et al. 2012; Legeai et al. 2011) by local TBLASTN analysis with the BioEdit Sequence Alignment Editor software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and using CLAM sequences from *B. mori* as queries. These libraries represented a total of 13,685 unigenes corresponding to 11,000-12,000 expressed genes, thus covering the majority of the transcripts in this species. ESTs were assembled and the missing regions of the corresponding transcripts were obtained by PCR or RACE-PCR using primers designed with AmplifX software v. 1.5.4 (Montpellier, France) in conserved regions of each gene (Table S1). The complete ORF were amplified from antennal cDNA with a high fidelity Taq DNA polymerase and sequenced. For missing 5' or 3' regions, 5'- and/or 3'-RACE-PCR (SMART RACE cDNA Amplification Kit, Clontech) were performed. For 5'-RACE, we used 2.5 µl of 5'-RACE-ready cDNA with specific reverse primers and Universal Primer Mix (UPM, Clontech) as anchor primer. 3'-RACE amplifications were carried out with UPM as reverse primer and specific forward primers. PCR were performed using hot start as follows: after 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55° and 3 min at 72 °C, then 10 min at 72 °C. The PCR products were purified (Nucleospin Extract II, Macherey-Nagel, Düren, Germany) and cloned into pCRII-TOPO plasmid (Invitrogen). After isolation (Nucleospin Plasmid, Macherey-Nagel), recombinant plasmids were sequenced (GATC Biotech, Marseille, France) and the overlapping sequences manually assembled to obtain full-length cDNAs. PDZ motifs were identified using the POW software (http://webservice.baderlab. org/domains/POW/).

Phylogenetic analysis

A dataset of 47 insect CLAM sequences was created, including sequences from *D. melanogaster, A. mellifera, T. castaneum and B. mori* and seven sequences from *S. littoralis*. Alignment was performed with CLUSTALW and used to create an entry file for phylogenetic analysis. An unrooted tree was calculated by the neighbor-joining method with Poisson correction of distances using the MEGA 6 program (Kumar et al. 2004) with default settings. Branch support was assessed by a bootstrap analysis based on 1000 replicates. Gene names were chosen according to previous CCE phylogenetic studies (Oakeshott et al. 2010; Teese et al. 2010; Tsubota and Shiotsuki 2010).

Expression analysis by RT-PCR and *quantitative RT-PCR (qPCR)*

Tissue distribution of *S. littoralis* CLAMs was first investigated by RT-PCR. Equal amounts of RNA (5 µg) extracted from male tissues and female antennae were reverse-transcribed. Primer pairs are indicated in Table S1. The ubiquitous ribosomal *Slrpl13* gene, which presents a constant expression in all tissues tested, was used to check the quality of the cDNAs. PCR products were loaded on 1% agarose gels and visualized using Gel Red (VWR, Radnor, PE, USA).

Amplification by qPCR of *Slnlg4-yll, SlnrxI, Slnrt, Slgli* and the reference gene *Slrpl13* was performed as described in detail in (Durand et al. 2010a) using the LightCycler 480® Real-Time PCR System (Roche). Each reaction was run in triplicate with at least three independent biological replicates. Data were analysed with LightCycler 480® Software (Roche). The crossing point values (Cp-values) were first determined for the reference genes with a run formed by the fivefold dilution series, the measuring points and three negative controls. The normalized expressions were thus calculated with Q-Gene software (Simon 2003) using *SlrpL13* as reference.

This gene has been already demonstrated as the best reference gene in these conditions (Durand et al. 2010a). Statistical analyses were performed with STATISTICA 7 (StatSoft Inc., Tulsa, OK, USA). A Mann−Whitney *U*-test was used for pair-wise comparisons.

In situ hybridization

For *slnlg4-yll* and *slnrxI*, cDNA fragments of respectively 748 bp and 565 bp were amplified by PCR using specific primers (Table S1), purified (Nucleospin Extract II, Macherey-Nagel) and cloned into pCRII-TOPO plasmid (Invitrogen), used as template for *in vitro* transcription to generate DIG-labeled RNA sense and antisense probes. The Pheromone-Binding protein *SlPBP1* (GenBank accession number EF396284), whose transcripts are highly expressed in male antennae, was used as positive control. Antennae from three-day-old male moths were embedded in Tissue Tek 186 medium™ compound (CellPath, Newtown Powys, UK). Cryosections (7 µm) were set in cell culture insert (Greiner Bio-one, Monroe, USA). Hybridization was conducted as described previously (Durand et al. 2010a). Pictures were acquired (Olympus BX61 microscope, ImagePro software) and digitalized using Adobe Photoshop® 7.0 (Adobe, USA).

Results

Sequence and phylogenetic analyses

The partial deduced gliotactin sequence (*Slgli;* Genbank accession number KP308209) consisted in 891 bp and contains a signal peptide and a part of the CCE domain, including a "HGG" version of the oxyanion hole. Despite several attempts using different primers and PCR conditions, we were unable to clone the 3' part of this gene, thus the expected transmembrane and intracellular domains, as deduced from alignment with its *B. mori* orthologue, were lacking.

The complete neurotactin (*Slnrt,* KP308208) consisted of 2238 bp. The deduced protein contained a predicted N-terminal extracellular domain, a transmembrane domain (amino-acids 221-243) and a C-terminal intracellular domain. The catalytic serine is replaced by an arginine residue. Unlike *Drosophila* neurotactin, but as its *B. mori* orthologue, SlNRT deduced protein did not contain the triad's glutamate and histidine residues, nor the three LRE (Leu-Arg-Glu) adhesion motifs involved in protein-protein interactions.

Three sequences of putative *nlg* genes were named according to their presumptive orthologs in other insect neuroligins, especially *B. mori*: *neuroligin 1* (*Slnlg1*, KP308205), *neuroligin 4 y-linked-like* (*Slnlg4-yll,* KP308206) and *neuroligin 5* (*Slnlg5,* KP308207). The three *nlg* sequences exhibited the predicted extracellular, transmembrane and cytosolic domains found in insect neuroligins. *Slnlg4-yll* and *Slnlg5* were full-length cDNAs with coding sequences containing respectively 3,129 and 2,307 bp (Fig. S1 and S2). They both retained putative C-terminal PDZ motif involved in protein-protein interactions and possessed a putative signal peptide at their N-terminus. *Slnlg1* partial sequence contained 4,132 bp and alignment with its *B. mori* homologue (Tsubota and Shiotsuki 2010) revealed that the sequence lacks both the signal peptide and the first part of the CCE domain. As for *Slgli*, we were unable to verify the 5' part of

this gene. *Slnlg1* partial sequence included a very long intracellular domain composed of 716 residues, which seemed by far the longest one found in insect neuroligins.

The complete coding sequence of *SlnrxI* (KP308210) presented all the characteristic features of insect NRXs (Fig. S3) and more precisely of the isoform A (Biswas et al. 2008). SlNRXI N-terminal region corresponded to the large extracellular portion and contained six laminin-G like separated by three EGF (Epidermal Growth Factor) domains. The short intracellular C-terminal part presented a putative PDZ motif "KEWYV" known to bind to various pre-synaptic proteins (Knight et al. 2011). SlNRX1 deduced protein presented 86% of amino acid identity with *B mori* NRX1 (isoform X2, XP_012544067.1).

Phylogenetic analysis of the inferred protein sequences with *D. melanogaster, A. mellifera, Tribolium castaneum and B. mori* NLGs was shown in Fig. 1. So far, only lepidopteran species possess six NLGs that include two *NLG4* forms. The NLG4 deduced sequence from *S. littoralis* identified here was 83.3% identical to BmNLG4-YLL (also named BmCCE030c) and only 48% identical to BmNLG4-XLL, allowing to clearly assign this sequence to the NLG4-YLL clade.

Tissue-related expression of S. littoralis CLAMs

To determine if the CLAM sequences isolated from the antennal transcriptome were expressed throughout the body or if they presented a more restricted expression pattern, their tissue-related distribution was first qualitatively determined by RT-PCR in various adult male tissues, including antennae of both sexes (Fig. 2). Most of them presented an ubiquitous expression pattern throughout the body. *Slnlg1* and *Slnlg5* were faintly expressed in the tested tissues. *SlnrxI* was amplified in antennae, proboscis and brain but faintly detected in thorax and abdomen. *Slnlg1* and *Slnlg5* expression levels were low and despite several attempts using different sets of primers, we were unable to quantify the expression of these transcripts by qPCR

suggesting very low levels of transcription, even in the brain. However, the expression levels of *Slnlg4-yll*, *SlnrxI, Slnrt* and *Slgli* were sufficient for quantification by qPCR (Fig. 3). *Slnlg4-yll* highest transcription levels were found in the brain, then in abdomen, proboscis and antennae. *SlnrxI* expression levels were high in antennae and brain but barely detected in the other tissues tested (Fig 3A). Levels of expression of *Slnrt* and *Slgli* were 10^2 lower compared to *Slnlg4-yll* and *SlnrxI* (Fig 3B). *Slnrt* was mostly expressed in the brain, whereas *Slgli* expression levels were barely detectable in all the tissues tested. For all the studied genes, no sexual dimorphism was observed within the antennae (Fig. 3). In adult moths, such a sexual dimorphism is usually seen in male antennae for olfactory genes involved in female sex pheromone detection (*i.e.* pheromone receptors, Pheromone-Binding Proteins or PBPs), and in female antennae for genes involved in host plant detection (Poivet et al. 2013), thus absence of such a dimorphism rather suggested a role of the corresponding genes in common functions.

Localization of Slnlg4-yll and SlnrxI expression within antennae

Within the male antennae, the cellular localization of *Slnlg4-yll* and *Slnrx1* transcripts was studied by *in situ* hybridization (Fig. 4). The anatomy of the antennal segment is depicted in Fig 4A. Olfactory sensilla are cuticular structures with an aqueous lumen surrounding the dendrites of the olfactory receptors neurons (ORN). Accessory cells surround the ORN soma. ORNs project their axons to the antennal lobes were the first synapses occur. On longitudinal sections, *Slnlg4-yll* and *SlnrxI* signals were restricted to the sensilla side, with no labelling on the scale side (Fig 4C, 4E). The olfactory hairs, which are tuned to the detection of odorants in insects, are indeed grouped on the ventral side whereas the dorsal side, without any olfactory function, only bears scales (Ljüngberg et al. 1993). Labelling was associated with cells located at the base of the olfactory hairs (Fig 4D, 4F), within the olfactory epithelium. A similar expression pattern was

observed for *SlPBP1* (Fig. 4B) supposed to be restricted to the olfactory epithelium and supposed to be associated with the accessory cells surrounding the olfactory receptor neurons (ORNs), as for other described PBPs (Steinbrecht et al. 1992). The labelling observed here did not allow a precise identification of the type of labelled cells (ORNs, accessory cells or epidermal cells). The expression levels of the four other CLAMs in antennae were too low to obtain any signal after *in situ* hybridization, as expected after RT-PCR and qPCR analyses.

Effect of mating on Slnlg4-yll and SlnrxI expression levels

To test whether *Slnlg4-yll* and *SlnrxI* expression could be modulated by the mating status of the males, levels of expression of the corresponding genes in antennae and brains were compared before and after mating (Table 1). After mating, the two transcripts were both significantly increased in the antennae, especially *Slnlg4-yll* (3.53 fold). In the brain, *Slnlg4-yll* transcripts showed no difference after mating, whereas *Slnrx1* remained stable.

Discussion

We isolated here the homologs of five putative CLAMs, one gliotactin, one neurotactin, three neuroligins and one of their binding-partner, neurexin I in the antennae of a model pest moth. Most insects possess only one gliotactin and one neurotactin, as revealed by genomic/transcriptomic analysis (Oakeshott et al. 2010; Ramsey et al. 2010) , suggesting that these two genes have been evolutionary well conserved in insect taxa. Unlike vertebrates which possess three neurexin genes, insects possess only one *nrxI* (Knight et al. 2011). However, in honeybee, alternative splicing of *AmnrxI* generates a diversity of variants (Biswas et al. 2008). The number of neuroligins varies from four in *D. melanogaster* up to six in *B. mori* (Tsubota and Shiotsuki 2010). In this latter species, two forms of *nlg4* were identified suggesting a recent duplication event that could be common to other Lepidopteran species, as *Danaus plexippus* (Zhan and Reppert 2013) and *Plutella xyllostella* (You et al. 2013) also possess two *nlg4* isoforms. In *S. littoralis* antennae, orthologs of *nlg1*, *nlg4-yll* and *nlg5* were identified. Together with the sequence of glutactin-like (Durand et al. 2012) already isolated in *S. littoralis*, this leads to six putative non-catalytic CCEs expressed in the olfactory organ of this moth.

Antennal-expressed CLAMs have been previously annotated after transcriptomic analysis only in *D. melanogaster* (Younus et al. 2014) and in the blowfly *Calliphora stygia* (Leitch et al. 2015). In these two diptera species, sequences of gliotactin and neurotactin, as well as of two neuroligins, *nlg3* and *nlg4*, were identified. However, the expression patterns of these genes were not studied. The diversity of the CLAMs expressed in the adult antennae of these three species raised the question of their possible function in this specialized organ. Insect antennae bear numerous olfactory sensilla tuned to various odorants. These structures consist of one or several ORNs surrounded by three accessory cells, namely the thecogen, trichogen and tormogen cells.

These cells delimit the sensillar lumen filled with sensillar lymph in which the outer part of the sensory dendrite projects (Keil and Steinbrecht 1987). Axons of all the ORNs are enveloped by glial cells and form the antennal nerve surrounded by hemolymph. The sensillar lymph and the hemolymph have different ionic compositions and septate junctions form a diffusion barrier between these two spaces (Keil and Steinbrecht 1987).

In *Drosophila*, gliotactin plays a main role in the formation of the glial-based blood-nerve barrier, allowing insulation between nerves and hemolymph (Auld et al. 1995). Electrophysiological analysis of gliotactin mutant embryos revealed that the functioning of the motor neurons was altered with a modification of the synaptic communication pattern, leading to uncoordinated movements (Auld et al. 1995). It has been proposed that gliotactins insulates peripheral motor and sensory axons against the high K^+ concentration of the hemolymph, which would disturb their excitability. We can suggest a similar role here for *Slgli* in the antennal septate junctions, participating in sealing off the sensillar lymph against the hemolymph space, a separation required for ORN functioning.

The NLG/NRX complex in vertebrates and invertebrates interacts within the synapse cleft and their intracellular domains bind with numerous PDZ-containing proteins, which in turn interact with transmitter receptors, ion channels and signalling proteins (Biswas et al. 2008; Knight et al. 2011). Interestingly, in the bee, *AmnrxI* and *Amnlgs* are also expressed outside the adult brain, and in particular, *Amnlg1* is more expressed in peripheral tissues than in brain, potentially with a role in the NMJ (Biswas 2008). Significant expression of *Amnlg1* gene in wings and legs suggested also a putative role in nerve endings responsive to sensory inputs (Biswas et al. 2008). We have here studied in more detail the expression pattern of *SlnrxI*, together with the most expressed neuroligin *Slnlg4-yll* within the antennae, showing that both transcripts were mainly expressed at the base of the olfactory sensilla. Surprisingly, such a

labelling restricted to the olfactory epithelium is usually observed for genes specifically involved in olfactory processes, such as olfactory receptors, odorant-binding proteins or odorant-degrading enzymes (Vogt 2005). However, as there are no synapses at this level of the antennae, this suggested that the *nrx/nlg* transcripts could be transported up to the axon parts of the ORNs which will form the antennal nerve. Indeed, RNA localization does not necessarily reflect the distribution of the proteins that can be distantly localized on axonal projections, as shown previously for *AmnrxI* (Biswas et al. 2008). Our result suggested also a putative presynaptic expression for *Slnlg4-yll*. If many studies showed that NLGs primarily function as postsynaptic adhesion molecules, several exceptions to this localization have been reported and in particular, in *Drosophila*, *dmnlg2* is required both at the pre and postsynaptic level for synapse development and function (Chen et al. 2012). As *in situ* hybridization was not conclusive with regard to cell type labelled in *S. littoralis* antennae, one can also hypothesize an expression of these molecules in non-neuronal cells of the sensilla. In vertebrates, NRX and NLGs have been described in non-neuronal cells, suggesting a broader function for this protein complex. They have been for example found in endothelial cells from blood vessels and involved in angiogenesis and in vessel tone control (Bottos et al. 2009; Bottos et al. 2011). In *Drosophila* photoreceptor cells, nrxI is involved in rhodopsin maturation via mediating retinoid transport (Tian et al. 2013), suggesting a non-synaptic mechanism for neurexin function in this sensory system.

In *Drosophila*, *nrxI* mutant larvae are impaired in associating odours with a reward and it has been proposed that *nrxI* may affect this associative learning by regulating synapse function (Zeng et al. 2007). In the honeybee, sensory deprivation and associative scent training modulated *AmnrxI*, *Amnlg1* and *Amnlgn3* expression levels in the brain (Biswas et al. 2010). These results suggested that *Amnlg/nrx* could be involved in synapse formation during adult neurogenesis, a process required for experience-and age-related plasticity. These *Amnlg/nrx* expression level

variations have been interpreted as a consequence of changes in neuronal wiring due to the modification of the olfactory environment (Biswas et al. 2010). However, variations of *nrx*/*nlg* in the bee olfactory system were not investigated, and more generally, nrx/nlg expression in insect sensory systems has not been studied, despite these systems are also able to sustain plasticity. In insects, various behaviours are modulated in response to physiological or environmental changes, such as experience, mating, age or time of the day (review in (Martel et al. 2009)). In most species, this behavioural plasticity is mainly sustained by a modulation of the central olfactory system (antennal lobes and mushroom bodies, see *e.g*. (Anton et al. 2015; Barrozo et al. 2011)). But as antennae are the first place of odorant detection, modulation can also take place at this peripheral level. In the case of *S. littoralis*, a nocturnal species, most behaviours rely on olfaction. Mating has been shown to modulate the olfactory-driven behaviours, with central and peripheral olfactory system modulations in both females (Martel et al. 2009) (Saveer et al. 2012) and males (Kromann et al. 2015). In male moths, mating temporary abolishes male attraction to females and host plant odours (Kromann et al. 2015). This behavioural modulation is correlated with a decrease of antennal and ORN sensitivity to the sex pheromone (Kromann et al. 2015) but the mechanisms underlying this peripheral plasticity are still unknown. As neurexin/neuroligins have been proposed to participate in sensory synaptic plasticity, we studied here their expression level variations within the antennae. The upregulation of *Slnlg4-yll* and *Slnrx1* in male antennae observed after mating suggest that these molecules could putatively play a role in peripheral olfactory system plasticity.

Further studies on the expression pattern of these genes within the olfactory organs of insects and their modulation by environmental and physiological cues would bring interesting information on the possible involvement of these molecules in antennal functioning and sensory plasticity.

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Disclosure

The authors declare no conflicts of interest.

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Legends of the figures

Figure 1: Diversity of antennal CLAMs in *S. littoralis.* Neighbor-joining tree of the antennal CLAMs from *S. littoralis* and various insects. *S. littoralis* sequences are in bold and red, *D. melanogaster* in blue, *A. mellifera* in green, *Tribolium castaneum* in black and *B. mori* in orange. GenBank accession numbers are indicated under brackets. Nodes with bootstrap values less than 50% were collapsed.

Figure 2: *S. littoralis* CLAMs present an ubiquitous expression pattern throughout the body. Qualitative analysis of *S. littoralis* CLAM expression throughout different body parts by RT-PCR.

Figure 3: Expression of *Slnlg4-yll, SlnrxI* (A) and *Slnrt*, *Slgli* (B) in *S. littoralis* tissues by quantitative PCR (qPCR). ♂ Ant: male antennae ; ♀ Ant: female antennae ; Prob: proboscis ; Tho: thorax ; Abd: abdomen. Data were obtained from triplicate experiments and are given as the mean +/- SD.

Figure 4: *Slnlg4-yll* and *Slnrx1* are associated with olfactory sensilla. Expression patterns of *Slnlg4-yll* (C, D) and *Slnrx1* (E, F) after *in situ* hybridization on longitudinal sections of *S. littoralis* male antennae. (A) Global view of an antennal segment, showing the disposition of the olfactory epithelium (1), the antennal lumen filled with hemolymph (2), the trachea (3), the antennal nerve (4), , and the epidermis (5). Arrowheads indicate some olfactory sensilla. (B) *SlPBP1* positive control. (D, F) Higher magnifications of olfactory epithelium showing labelling at the base of the olfactory sensilla. Scale bars: 25 μm.

Table 1 Expression levels of *Slnlg4-yll* and *SlnrxI* in male antennae and brains before and after mating, as measured by qPCR.

Tissue	Reproductive status	Expression relative to *Slrpl13* (x 10^{-4})	
		Slnlg4-yll	***Slnrx1***
Antennae	Virgin	0.49 (± 0.14)	53.59 (± 7.20)
	Mated	1.73 (± 0.41) 3.53 fold ↑ **	93.00 (± 20.19) 1.73 fold ↑ **
Brain	Virgin	13.46 (± 2.13)	92.10 (± 10.68)
	Mated	11.40 (± 0.82) 0.84 fold ↓	94.16 (± 22.06) 1.02 fold →

The expression levels of *Slnlg4-yll* and *SlnrxI* were normalized to that of *Slrpl13* transcript, which was measured in the same cDNAs. Data were obtained from triplicate experiments and are given as the mean normalized expression +/- SD and fold changes. Stars indicate significant difference between the two conditions (**: $P < 0.01$).

Supplemental data

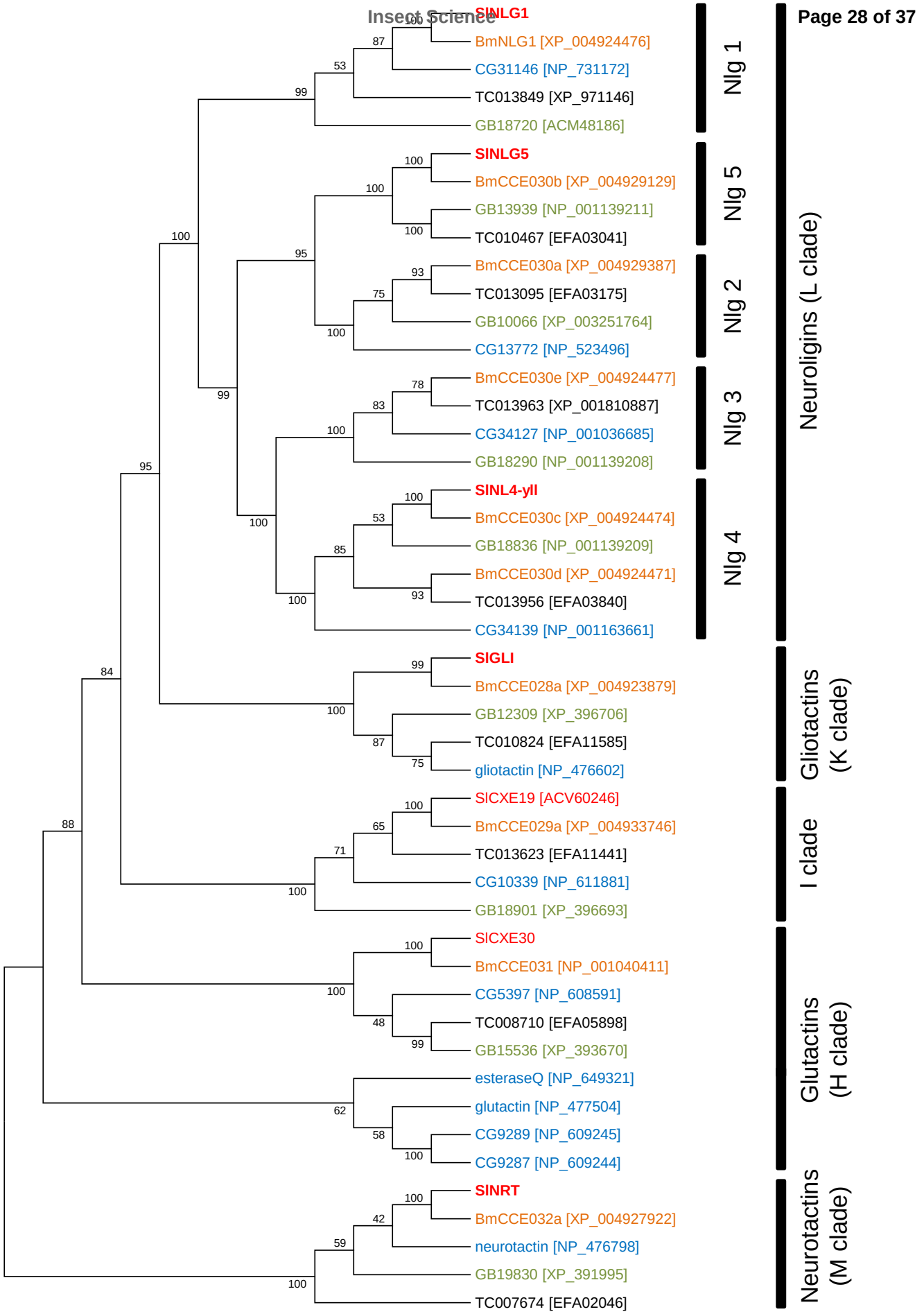
Figure S1: Alignment of *A. mellifera*, *B. mori* and *S. littoralis* neuroligins 4. The sequences of honeybee and silkworm were taken from NCBI (AmNLG4: NP_001139209; BmNLG4-Y-linked-like: XP_004924474; SlNLG4 KP308206). The alignment was performed using BioEdit with ClustalW. The signal peptide and transmembrane domain were predicted with SignalP 4.1 and TMHMM 2.0. Sequence depicted as the carboxyl/cholinesterase domain was found using the NCBI Conserved Domain Architecture Retrieval Tool. The oxyanion hole, nucleophilic elbow and critical elements of the catalytic triad are highlighted by light blue shading and the LER adhesion motif is in yellow. Asparagines predicted by NetNGlyc 1.0 to be N-glycosylated (jury 9/9) are highlighted in red. Serines predicted by NetPhos 2.0 to be phosphorylated (score > 0.99) are highlighted in purple. TMD: transmembrane domain; PDZ: PDZ (Postsynaptic density 95/Discs large/Zona occludens 1) binding motif; Am: *Apis mellifera*; Bm: *Bombyx mori*; Sl: *Spodoptera littoralis.*

Figure S2: Alignment of *A. mellifera*, *B. mori* and *S. littoralis* neuroligins 5. (AmNLG5: NP_001139211; BmNLG5: XP_004929129; SlNLG5: KP308207). Details as in figure S1.

Figure S3: Alignment of *A. mellifera*, *B. mori* and *S. littoralis* neurexin I.
(AmNrxI: ACM48184; BmNrxI: XP_004924111, SlNrxI: KP308210). The signal peptide and transmembrane domain were predicted with SignalP 4.1 and TMHMM 2.0. Sequence depicted as the Laminin G-like and EGF domains were found using the NCBI Conserved Domain Architecture Retrieval Tool except the second EGF domain that was identified in (Biswas et al. 2008). Asparagines predicted by NetNGlyc 1.0 to be N-glycosylated are highlighted in red.

Serines and threonines predicted by NetOGlyc 4.0 to be O-glycosylated are highlighted in green. Serines predicted by NetPhos 2.0 to be phosphorylated are highlighted in purple. EGF: Epidermial Growth Factor motif.

Table S1: List of the primers used for PCR, quantitative PCR (qPCR) and probe synthesis for *in situ* hybridization, indicating their sequences and annealing temperatures (Temp.).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TC013095 [EFA03175]
GB10066 [XP_003251764]
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BmCCE030e [XP_004924477]
TC013963 [XP_001810887]
CG34127 [NP_001036685]
GB18290 [NP_001139208]
SINL4-ylI
BmCCE030c [XP_004924474]
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BmCCE030d [XP_004924471]
TC013956 [EFA03840]
CG34139 [NP_001163661]
SIGLI
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TC010824 [EFA11585]
gliotactin [NP_476602]
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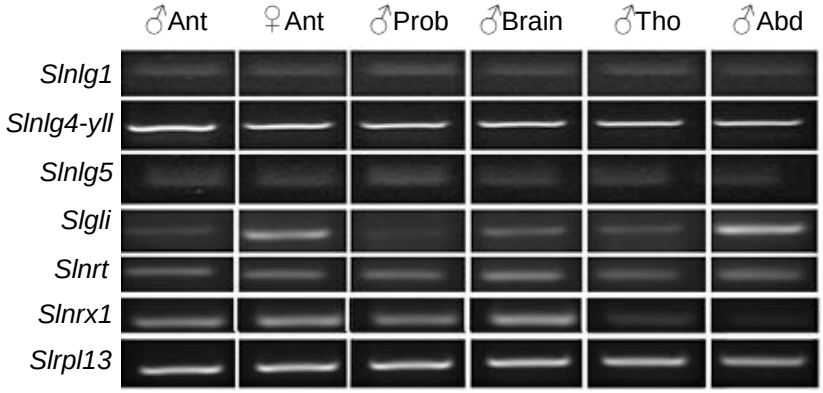
esteraseQ [NP_649321]
glutactin [NP_477504]
CG9289 [NP_609245]
CG9287 [NP_609244]
SINRT
BmCCE032a [XP_004927922]
neurotactin [NP_476798]
GB19830 [XP_391995]
TC007674 [EFA02046]
Nlg 1
Nlg 5
Nlg 2
Nlg 3
Nlg 4
Neuroligins (L clade)
Gliotactins (K clade)
I clade
Glutactins (H clade)
Neurotactins (M clade)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♀Ant
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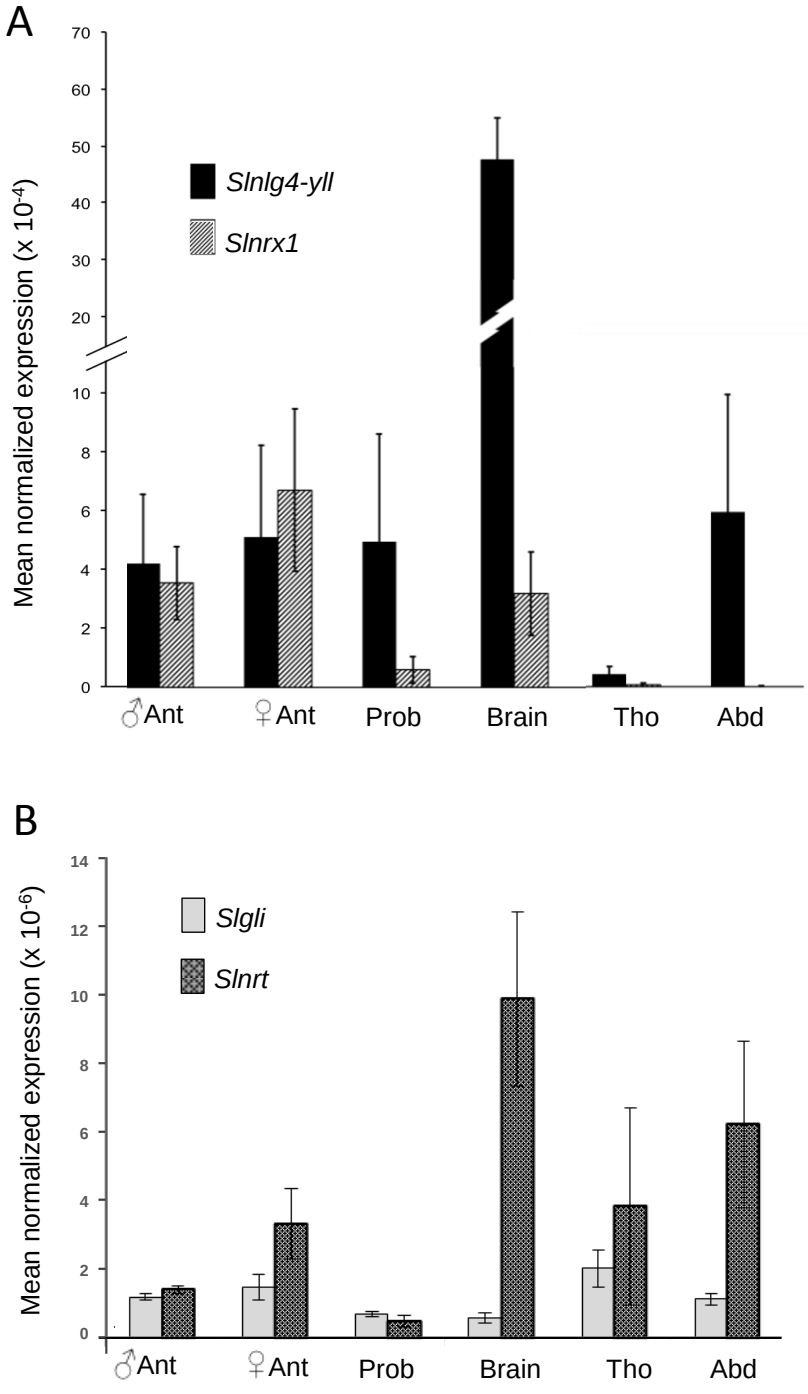
Slgli
Slnrt
Slnrx1
Slrpl13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Mean normalized expression (x 10-4)
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60
50
40
30
20
10
8
6
4
2
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Slnrx1
♂Ant
♀Ant
Prob
Brain
Tho
Abd
B
Mean normalized expression (x 10-6)
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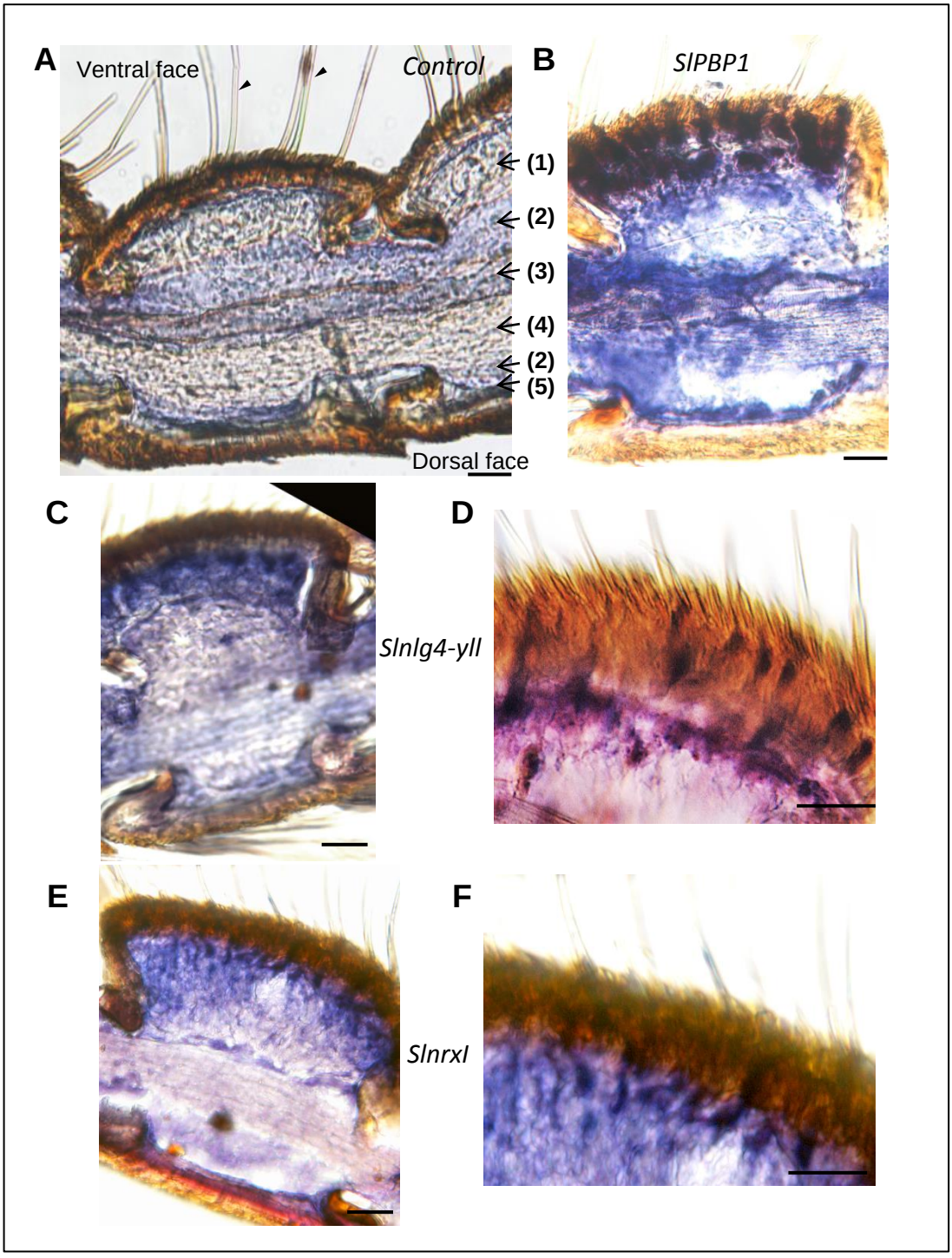
Prob
Brain
Tho
Abd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Ventral face
Control
(1)
(2)
(3)
(4)
(2)
(5)
Dorsal face
B
SlPBP1
C
Slnlg4-yll
D
E
SlnrxI
F

Table S1

Primer	**Purpose**	**Sequence 5'-3'**	**Temp** (°C)
SlNLG1-F	RT-PCR/ qPCR	AGGGACTGGATTGCCAATGA	60°C
SlNLG1-R	RT-PCR/ qPCR	AGAGCCAAGCGCTGAAGTA	60°C
SlNLG4-yll-F	RT-PCR / qPCR	ACTCACCGCCTTTGCAAGAGTTAC	60°C
SlNLG4-yll-R	RT-PCR / qPCR	GCAGAGTCAGATGGGATTAACCGT	60°C
SlNLG5-F	RT-PCR/ qPCR	TGTTCGCCGGCATCTACCT	60°C
SlNLG5-R	RT-PCR/ qPCR	TGCGCTTGAGCGTGCTCTT	60°C
SlNRT-F	RT-PCR/ qPCR	GAAGCAAATTGAGGCTGAGGA	60°C
SlNRT-R	RT-PCR/ qPCR	TTCACTTCACGGCCCATCT	60°C
SlGLI-F	RT-PCR/ qPCR	TGCCTAGTCCAGGTGACAGTGA	60°C
SlGLI-R	RT-PCR/ qPCR	ACCAGGGAATAAGGCATCTGGGTT	60°C
SlNrxI-F	RT-PCR / qPCR	TCCGAGGGCTGGAACCGACC	60°C
SlNrxI-R	RT-PCR / qPCR	AGTTCCTCAGTTTGCGTTATATGTTCAGG	60°C
SlNrxI-ORF-F1	ORF cloning	ATGCCCTCCGACGTCG	58°C
SlNrxI-ORF-R1	ORF cloning	TCAGGGTGGAAAAGTCCTCCT	58°C
SlNrxI-ORF-F2	ORF cloning	CGACACTTGGCATACGGTG	58°C
SlNrxI-ORF-R2	ORF cloning	CACGTACCACTCTTTGATGCC	58°C
SlNLG1-5'	3'RACE-PCR	TTGGCAATCCAGTCCCTCACTTTAGTGT	68°C
SlNLG1-3'	3'RACE-PCR	CACTAAAGTGAGGGACTGGATTGCCAATGA	68°C
SlNLG4-yll-5'	5'RACE-PCR	AACCCATCTGCGGGTAGCTCACGTTA	68°C
SlNLG5-5'	5'RACE-PCR	GGAGAGGTTACAGAAGTCGGCTCGTTGCT	68°C
SlNRT-3'	3'RACE-PCR	TGGAGAACATCGAGCCTGCGGAGTTG	68°C
SlNLG4-yll-HIS-F	*In situ* hybridization	CGACACAGCCCTAACGACTGTTGA	60°C
SlNLG4-yll-HIS-R	*In situ* hybridization	CACTCTCATTTCGTCAATGGCGGCT	60°C
SlNrxI-HIS-F	*In situ* hybridization	CGAAAACGCTTGGAGTCGGAACTA	54°C
SlNrxI-HIS-R	*In situ* hybridization	TCCAGACTGGACATGCATCCTTCA	54°C
SlPBPI-HIS-R	*In situ* hybridization	ATGGCGAAGGAAGTTGGACC	55°C
SlPBPI-HIS-f	*In situ* hybridization	CTCGTCGATCTTAGTGCGGA	55°C

Table S1: List of the primers used for PCR, quantitative PCR (qPCR) and probe synthesis for *in situ* hybridization, indicating their sequences and annealing temperatures (Temp.).

Figure S1: Neuroligin 4 Multiple Alignment

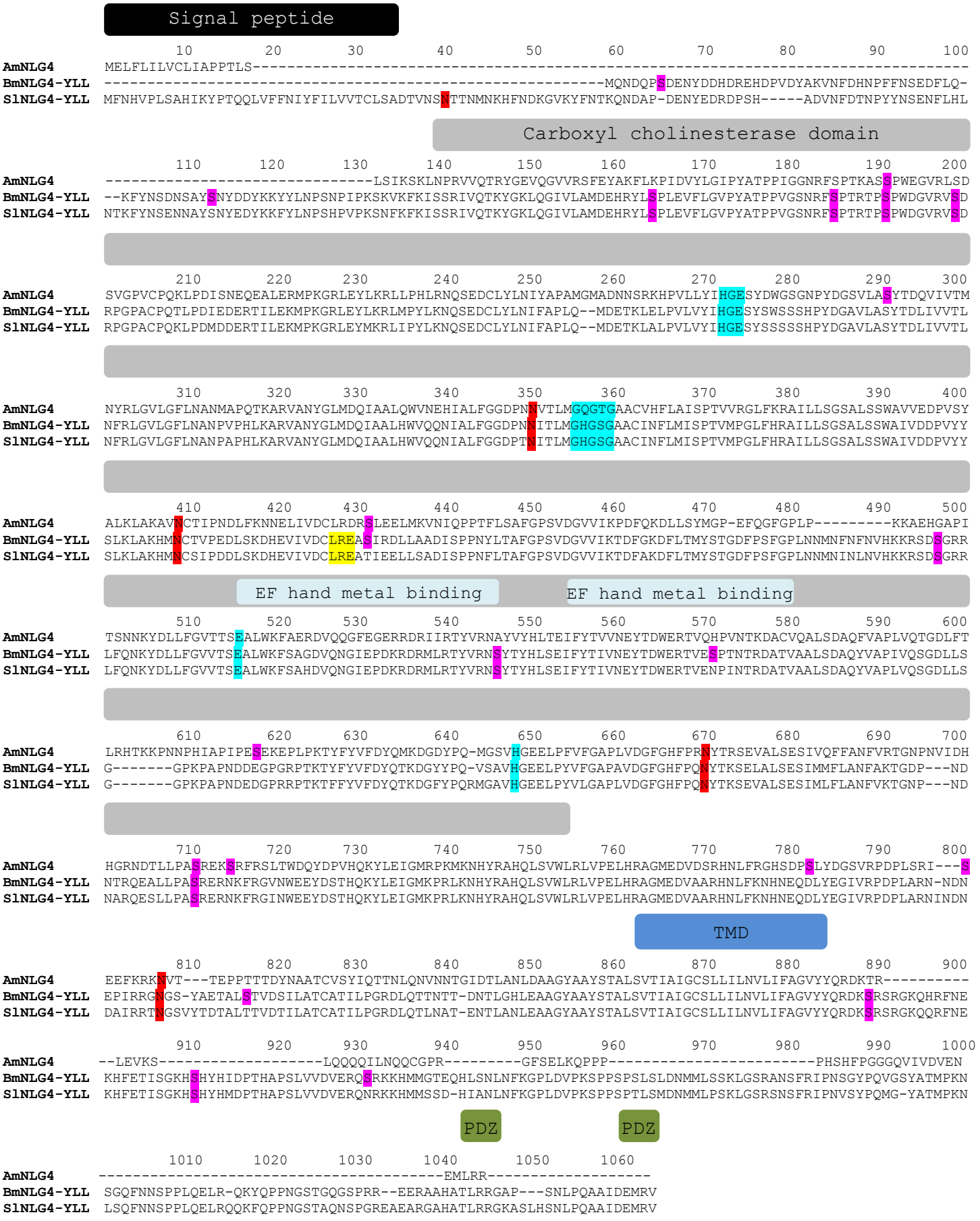
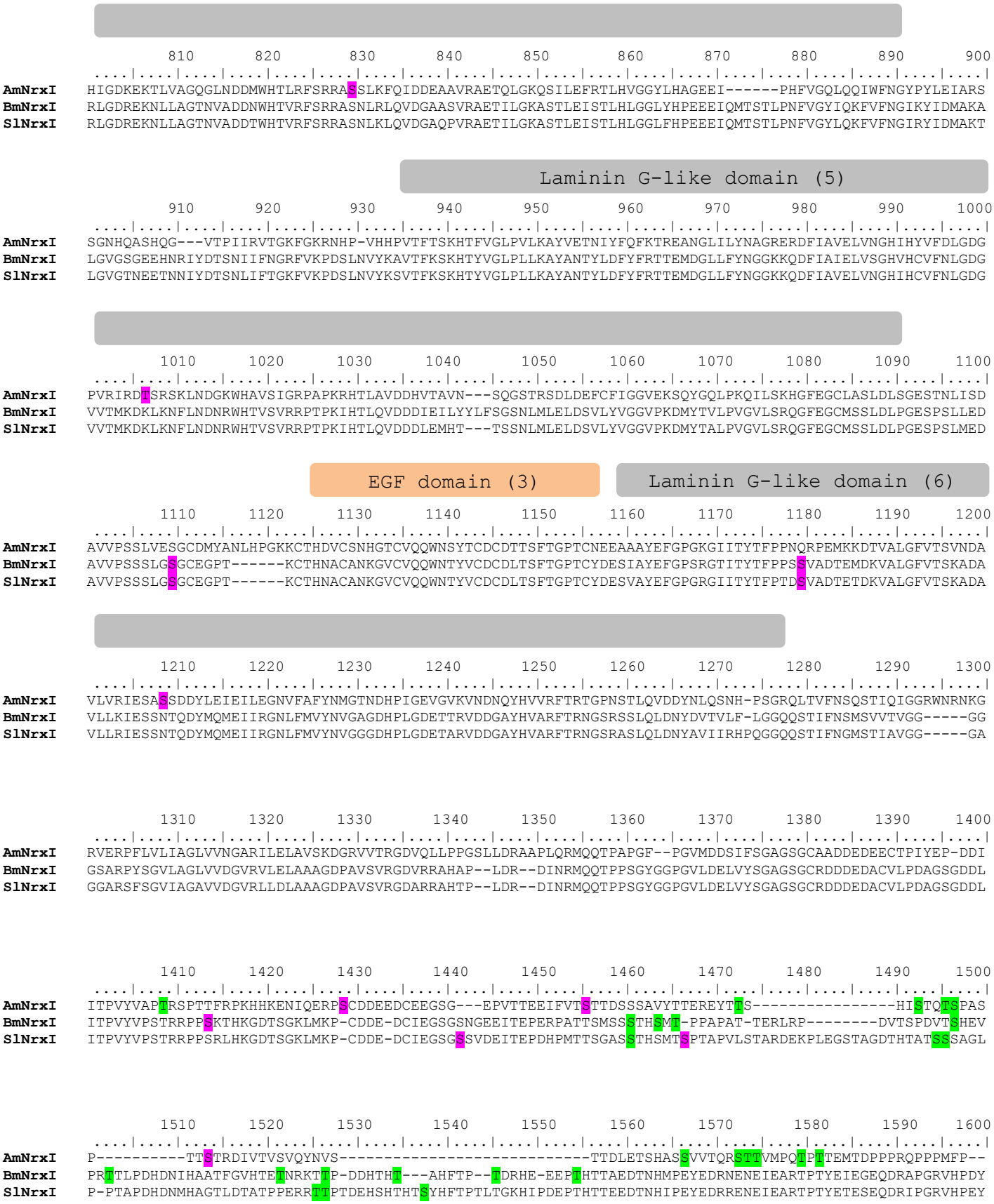
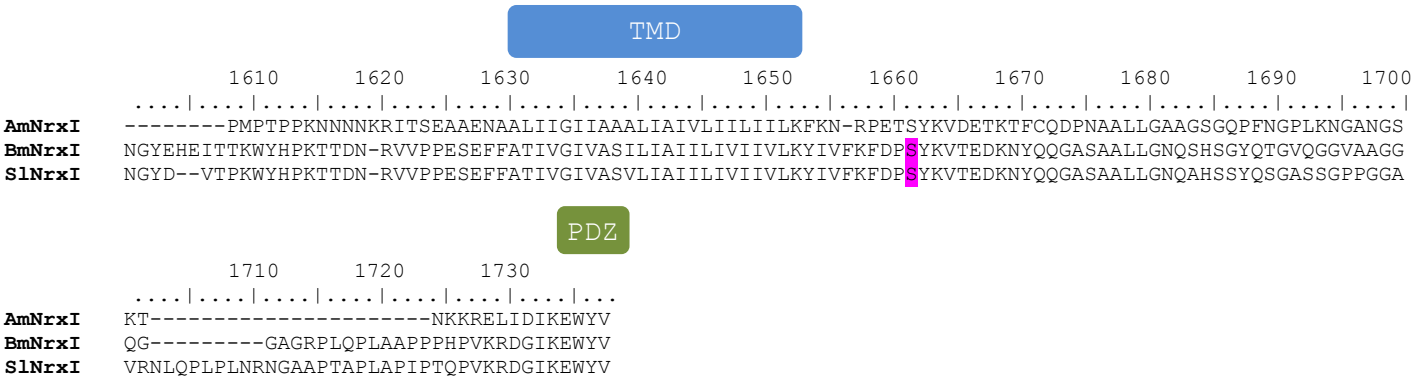


Figure S2: Neuroligin 5 Multiple Alignment

Figure S3: Neurexin I Multiple Alignment


Laminin G-like domain (5)
EGF domain (3)
Laminin G-like domain (6)
AmNrxI
BmNrxI
SlNrxI


TMD
1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
AmNrxI --------PMPTPPKNNNNKRITSEAAENAALIIGIIAAALIAIVLIILIILKFKN-RPETSYKVDETKTFCQDPNAALLGAAGSGQPFNGPLKNGANGS
BmNrxI NGYEHEITTKWYHPKTTDN-RVVPPESEFFATIVGIVASILIAIILIVIIVLKYIVFKFDPSYKVTEDKNYQQGASAALLGNQSHSGYQTGVQGGVAAGG
SlNrxI NGYD--VTPKWYHPKTTDN-RVVPPESEFFATIVGIVASVLIAIILIVIIVLKYIVFKFDPSYKVTEDKNYQQGASAALLGNQAHSSYQSGASSGPPGGA
PDZ
1710 1720 1730
AmNrxI KT----------NKKRELIDIKEWYV
BmNrxI QG---------GAGRPLQPLAAPPPHPVKRDGIKEWYV
SlNrxI VRNLQPLPLNRNGAAPTAPLAPIPTQPVKRDGIKEWYV