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The molecular and immunochemical expression of innexins in the yellow fever mosquito, *Aedes aegypti*: insights into putative life stage- and tissue-specific functions of gap junctions

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Abstract

Gap junctions (GJ) mediate direct intercellular communication by forming channels through which certain small molecules and/or ions can pass. Connexins, the proteins that form vertebrate GJ, are well studied and known to contribute to neuronal, muscular and epithelial physiology. Innexins, the GJ proteins of insects, have only recently received much investigative attention and many of their physiological roles remain to be determined. Here we characterize the molecular expression of six innexin (Inx) genes in the yellow fever mosquito *Aedes aegypti* (*AeInx1*, *AeInx2*, *AeInx3*, *AeInx4*, *AeInx7*, and *AeInx8*) and the immunochemical expression of one innexin protein, *AeInx3*, in the alimentary canal. We detected the expression of no less than four innexin genes in each mosquito life stage (larva, pupa, adult) and tissue/body region from adult males and females (midgut, Malpighian tubules, hindgut, head, carcass, gonads), suggesting a remarkable potential molecular diversity of GJ in mosquitoes. Moreover, the expression patterns of some innexins were life stage and/or tissue specific, suggestive of potential functional specializations. Cloning of the four full-length cDNAs expressed in the Malpighian tubules of adult females (*AeInx1*, *AeInx2*, *AeInx3*, and *AeInx7*) revealed evidence for 1) alternative splicing of *AeInx1* and *AeInx3* transcripts, and 2) putative *N*-glycosylation of *AeInx3* and *AeInx7*. Finally, immunohistochemistry of *AeInx3* in the alimentary canal of larval and adult female mosquitoes confirmed localization of this innexin to the intercellular regions of Malpighian tubule and hindgut epithelial cells, suggesting that it is an important component of GJ in these tissues.

Keywords

RT-PCR; molecular cloning; gene structure; protein localization

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1. Introduction

Gap junctions (GJ) are intercellular channels that allow for direct communication between adjacent cells via the transport of small molecules and ions (Cao et al., 1998). GJ are formed when a hemichannel from one cell docks with a hemichannel in an adjacent cell (Phelan and Starich 2001; Johnson et al. 2012; Scemes et al. 2009). In arthropods, the hemichannels of GJ are hexamers composed of ‘innexin’ protein subunits, whereas in mammals and other vertebrates the hemichannels are hexamers composed ‘connexin’ protein subunits (Phelan et al., 1998; Swenson et al., 1989). Although innexin and connexin genes are evolutionarily distinct and encode proteins that share nominal amino acid homology, their encoded proteins have convergently evolved many structural and functional features (Abascal and Zardoya, 2013).

In vertebrates, GJ play important roles in embryogenesis, neurophysiology, muscle physiology, epithelial physiology, and endocrinology (Bosco et al. 2011). In arthropods, the physiological roles of GJ have not yet been fully elucidated, but they appear to play important roles in development, reproduction, and epithelial physiology. In *Drosophila melanogaster*, innexin 1 (*ogre*) is critical to the development of retinal neurons (Curtin et al. 2002), and innexin 3 is necessary for dorsal closure in embryogenesis (Giuliani et al., 2013). Moreover, innexin 4 (*zero population growth* or *zpg*) is involved with functional gonad formation in males of both *D. melanogaster* flies and *Anopheles gambiae* mosquitoes (Magnusson et al., 2011; Tazuke et al., 2002), and is necessary for early germ cell differentiation in female *D. melanogaster* (Gilboa et al., 2003). Furthermore, innexins are likely responsible for the functional coupling of epithelial cells in the renal (Malpighian) tubules of mosquitoes (Beyenbach and Piermarini, 2011; Piermarini and Calkins, 2013; Weng et al., 2008).

The goal of this study was to describe the post-embryonic expression of the six putative innexin (*AeInx*) genes in the yellow fever mosquito *A. aegypti* (Weng et al. 2008) and to localize the expression of one innexin protein (*AeInx3*) in the alimentary canal. We hypothesized that innexins would exhibit different patterns of mRNA expression throughout the mosquito life cycle and among various tissues, providing insights into the putative functional roles of these genes in mosquitoes. Furthermore, we hypothesized that the immunoreactivity of *AeInx3* would concentrate near the intercellular regions of epithelial cells of the alimentary canal where it would potentially form GJ.

2. Materials and Methods

2.1 Mosquitoes

Eggs of *Aedes aegypti* were obtained through the Malaria Research and Reference Reagent Resource Center (MR4) as part of the BEI Resources Repository (Liverpool strain; LVP-IB12 F19, deposited by M.Q. Benedict). Mosquitoes were reared as described in Piermarini et al. (2011) in an environmental chamber set at 28°C and 80% relative humidity with a 12 h:12 h light:dark cycle.

2.2 Dissections

Adult and larval mosquitoes were anesthetized on ice, and then grasped with forceps (Dumont #5; Fine Science Tools, Inc., Foster City, CA) either at the thorax to remove the legs and heads (adults) or one segment posterior to the head (larvae) to remove the head. The insect was then submerged in a mosquito Ringer solution consisting of the following in mM: 150 NaCl, 3.4 KCl, 1.7 CaCl₂, 1.8 NaHCO₃, 1 MgCl₂, 5 Glucose, 25 HEPES. The pH of the Ringer solution was adjusted to 7.1 with NaOH before use and the desired osmolality was verified (330 ± 5 mOsm/kg) with a vapor pressure osmometer (Wescor, Logan, UT). The second to last abdominal segment was grasped with forceps and gently pulled away from the rest of the abdomen, bringing with it the alimentary canal and gonads. Tissues of interest were isolated and placed into separate 1.5 ml sterile microcentrifuge tubes (USA Scientific; Orlando, FL) containing the mosquito Ringer solution on ice. For whole insect samples, dissection was unnecessary. Once all mosquito samples were collected, the Ringer solution was aspirated and replaced with TRIzol® (Life Technologies, Carlsbad, CA) before storing at -80°C .

2.3 Qualitative RT-PCR

Using an approach modified from Piermarini et al. (2013) and Rouhier and Piermarini (2014), qualitative RT-PCR was performed to assess *Aedes aegypti* innexin (*AeInx*) gene expression. In brief, total RNA was isolated from preserved tissues or whole insects using the method of Chomczynski and Sacchi (1987). The cDNA templates were synthesized using 1 µg of total RNA and GoScript Reverse Transcriptase (Promega, Madison, WI) with random hexamers, following the manufacturer's protocol. Primers for the RT-PCR reactions were designed against the six predicted innexin genes in the *A. aegypti* genome (i.e., *AeInx*, Table 1) using the SciTools-Real-Time PCR application on the Integrated DNA Technologies web site (www.idtdna.com). Note that since the original description of these genes in *A. aegypti* (Weng et al. 2008), one has been renamed. That is, the gene initially designated as 'passover' due to its homology to the *shakB/passover* gene in *D. melanogaster* is now named as innexin 8 (*Inx8*) to follow the nomenclature of Hasegawa and Turnbull (2014). The *AeInx* primers were designed to amplify a product of approximately 500 base pairs (bp). A 300 bp amplicon of the ribosomal protein S7 gene (*RPS7*) was used as the internal control (Piermarini et al. 2013). All PCR primers flanked at least one intron to potentially detect the amplification of genomic DNA.

All RT-PCR reactions were performed using GoTaq Green 2x MasterMix (Promega, Madison, WI). Each reaction consisted of the following: 25 µL GoTaq Green, 1 µL *AeInx* primer pair (10 µM), 1 µL *RPS7* primer pair (10 µM), 1 µL cDNA, 2 or 4 µL of 25 mM MgCl₂ and nuclease free water up to 50 µL. Reactions were individually optimized for MgCl₂ concentration; reactions for *AeInx1*, *AeInx3*, and *AeInx7* used 4 mM MgCl₂, whereas those for *AeInx2*, *AeInx4*, and *AeInx8* used 5 mM MgCl₂.

The amplification protocol consisted of a denaturation at 95° C for 5 min, followed by 50 cycles of 95°C for 30 s, 52°C or 58°C for 15 s, and 72°C for 30 s. Each reaction was performed in triplicate. *AeInx1* and *AeInx8* reactions used an annealing temperature of 52°C, whereas *AeInx2*, *AeInx3*, *AeInx4* and *AeInx7* used an annealing temperature of 58°C.

PCR products were separated by electrophoresis on a 1% agarose gel for 30 minutes at 100 mV, and visualized via ethidium bromide staining on a Gel Logic Imager (Kodak, Rochester, NY). The identities of all PCR products were confirmed by sequencing at the Molecular and Cellular Imaging Center at the Ohio Agricultural Research and Development Center (OARDC) of The Ohio State University, Wooster, OH.

In order to qualitatively assess the expression of the *AeInx* genes, we measured the optical densities of the *AeInx* bands and compared them to *RPS7*. For a given gel, the optical density of the *AeInx* and *RPS7* bands were quantified using ImageJ pixel counting software (NIH). For each *AeInx* band in a given tissue, the mean optical density for 3 replicates was compared to that of *RPS7* using a student's T-test. If the mean optical density of an *AeInx* band was significantly lower than the mean *RPS7* value, then it was categorized as 'weak' expression. If mean optical density of an *AeInx* band was not significantly different than the mean *RPS7* value, then it was categorized as 'moderate' expression. If mean optical density of an *AeInx* band was significantly greater than mean *RPS7* value, then it was categorized as 'strong' expression. If no detectable band was present in all 3 replicates, then it was categorized as 'not detectable'. The categorizations are graphically summarized in Figures 1–3.

2.4 Cloning

Malpighian tubules were dissected from adult female mosquitoes in Ringer solution and RNA was isolated from Malpighian tubules, as described above. The RNA was used as a template to generate 5' and 3' single-stranded cDNA libraries with the GeneRacer Kit (Invitrogen, Carlsbad, CA), as described previously (Piermarini et al., 2010). The resulting cDNA libraries were used for 5' and 3' rapid amplification of cDNA ends (RACE), respectively, as described previously (Piermarini et al., 2010).

RACE was performed for each innexin using one gene-specific primer (Table 2) and a generic 'Generacer' primer, according to the manufacturer's instructions. In brief, each RACE reaction consisted of 0.5 µL cDNA, 1.5 µL RACE primer, 0.5 µL gene specific primer, and 22.5 µL Platinum® PCR High Fidelity SuperMix (Invitrogen), and was subjected to a touchdown RT-PCR amplification, following the manufacturer's protocol.

Products from RACE reactions were ligated into a TOPO 4.1 plasmid (Invitrogen) and transformed into Premade Z-Competent™ *E. coli* Cells (Zymo Research, Irvine, CA). The *E. coli* cells were spread on a 2% agar LB media plate. After overnight growth, individual colonies were picked and grown up in liquid LB media at 37°C in a shaking incubator overnight. The plasmid DNA was extracted using a QIAGEN QIAprep® Spin Miniprep kit (Qiagen, Valencia, CA). Plasmid DNA was sequenced at the Molecular and Cellular Imaging Center at the Ohio Agricultural Research and Development Center of The Ohio State University, Wooster, OH. Full-length cDNA sequences were assembled from the RACE sequencing data using CLC Workbench 6 Software. The consensus sequences were built from five 5' and seven 3' RACE sequences for *AeInx1*, four 5' and nine 3' RACE sequences for *AeInx2*, six 5' and six 3' RACE sequences for *AeInx3*, and five 5' and three 3' RACE sequences for *AeInx7*. Consensus sequences were deposited into Genbank and assigned accession numbers as shown in Table 3.

2.5 Sequence Alignment

Deduced amino-acid sequences of the cloned innexins were aligned using ClustalW2 (European Bioinformatics Institute, Hinxton, Cambridgeshire, UK). Transmembrane domains were predicted using a Kyte-Doolittle hydrophobicity analyses (window size 19; Supplemental Figure 1) with supporting analysis from 1) Phobius prediction of transmembrane topology and signal peptide software (European Bioinformatics Institute) and 2) previous predicted membrane topologies for innexins of *Drosophila melanogaster* and *Bombyx morii* (Hong et al., 2009).

2.6 Anti-Inx3 Antibody

To raise and affinity purify a rabbit polyclonal antibody against an AeInx3 synthetic peptide, we hired 21st Century Biochemicals (Marlboro, MA). The peptide for antibody production and affinity purification corresponded to the COOH-terminal domain of the predicted AeInx3-A protein (L₃₇₄EMAPIYPEIGKYGKDTAI; Figure 5). Notably, the first 14 residues of this peptide are perfectly conserved between AeInx3-A and AeInx3-B, but the last six residues are only conserved AeInx3-A. Thus, the resulting antibody may have a higher affinity for the AeInx3-A vs. the AeInx3-B isoform.

2.7 Western Blotting

Whole animals for Western blot analysis were lysed in 10% ringer solution. Protein concentration was measured using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL) and then an equal volume of urea lysis buffer was added to the sample as well as an appropriate volume of a 5x Laemmli sample buffer (Laemmli, 1970; Piermarini et al., 2010). Proteins were separated via denaturing sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) through a 6% stacking/12% resolving gel at 125 mV for 2.5 hours in an XCell SureLock mini cell electrophoresis box (Invitrogen). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad) in the XCell II Blot module (Invitrogen) at 35 mV for 2 hours. Transfer of protein was confirmed by staining the PVDF membrane with Ponceau S. Membranes were then blocked for 2 hours in 5% nonfat dry milk (Nash Finch Co., Minneapolis MN) in Tris Buffered Saline with 0.1% Tween 20 (TBST) at room temperature on a rocking incubator. Membranes were incubated overnight with the affinity-purified anti-AeInx3 antibody (0.475 µg/ml) diluted in the milk solution at 4° C. The membrane was washed 3 times in TBST for 5 minutes each. The membrane was then incubated in secondary antibody, horseradish peroxidase-conjugated goat anti rabbit IgG (Thermo Scientific) diluted 1:20,000 at room temperature for 2 hours. After an additional 3 washes in TBST the membrane was incubated in SuperSignal West Dura (Thermo Scientific) for 5 minutes, transferred to clean cellophane wrap and imaged using a My ECL imager (Model 62236X, Thermo scientific).

2.8 Whole Mount Immunolabeling

Tissues for whole mount immunohistochemistry were dissected from adult or larval mosquitoes as described in section 2.2. After dissection, tissues of interest were transferred to a clean 1.5 ml centrifuge tube and fixed for 45 minutes in 1 ml of a fixative solution containing 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in

phosphate-buffered saline (PBS). Tissues were washed for 5 minutes with 1 ml of PBS three times, followed by two 10-minute washes of 0.1% TritonX 100 (Fisher Scientific, Pittsburgh, PA) in PBS and three additional 5-minute washes with 0.1% Tween 20 (Fisher Scientific) in PBS (PBST). Tissues were blocked for 1.5 hours at room temperature with 10% normal goat serum (Thermo Scientific, Rockford, IL) supplemented with casein (Vector Laboratories, Burlingame, CA) in PBST. Tissues were incubated with the rabbit anti-AeInx3 primary antibody (2.3 µg/ml) diluted in PBST supplemented with casein overnight at 4° C.

On the following day, tissues were washed three times with PBST (5 min each) and then incubated in a 1:600 dilution of Dylight 488 IgG goat anti-rabbit secondary antibody (Thermo Scientific) in PBST supplemented with casein for 1.5 hours at room temperature. Tissues were finally incubated with DAPI (Thermo Scientific, Rockford, IL) at a 1:5000 dilution. Peptide blocked tissue preparations followed the same procedure with the exception that the primary antibody was pre-incubated for 1 hour at 37 °C with the immunogenic peptide in 10-fold excess. Tissues were imaged using a Leica TCS SPS II confocal microscopy system along with the Leica Microsystems LAS AF software (Leica Microsystems, Buffalo Grove, IL) at the Molecular and Cellular Imaging Center of the Ohio Agricultural Research and Development Center (Wooster, OH).

3. Results

3.1 Patterns of AeInx expression

To describe the expression of the six predicted *AeInx* genes across developmental stages we conducted RT-PCR using gene-specific primers for each *AeInx* and an internal positive control gene (*RPS7*). As explained in the Materials and Methods section, we qualitatively categorized the expression of each innexin relative to *RPS7* as not detectable, weak, moderate, or strong.

Figure 1 shows that larvae (4th instar) are characterized by moderate or strong expression of *AeInx1*, *AeInx2*, *AeInx3* and *AeInx7*, and weak expression of *AeInx8* (expression of *AeInx4* is not detectable). Pupae exhibit a similar pattern of *AeInx* expression as larvae, but are distinguished from larvae by strong expression of *AeInx2* and moderate expression of *AeInx8* (Figure 1). Adult males are characterized by strong expression of *AeInx2* and moderate expression of *AeInx1*, *AeInx3* and *AeInx7* (expression of *AeInx4* and *AeInx8* are not detectable). On the other hand, adult females are characterized by the strong expression of *AeInx1*, *AeInx2*, *AeInx3*, and *AeInx7*, and moderate expression of *AeInx4* (expression of *AeInx8* is not detectable).

Figure 2 compares the expression of *AeInx* genes among three epithelial tissues of the alimentary canal (Malpighian tubules, midgut, and hindgut) in adult female and male mosquitoes. Common to all three tissues examined for both sexes is the expression of *AeInx2*, *AeInx3*, and *AeInx7* at moderate or strong levels; one exception is the adult female midgut, which expresses *AeInx3* weakly. The expression of *AeInx1* is weak in all three tissues of females, but is moderate in those of males. The expression of *AeInx4* is not detectable in all three tissues of females and the midgut of males, but is present at weak and moderate levels in the Malpighian tubules and hindgut, respectively, of males. Moreover,

the expression of *AeInx8* is not detectable in the Malpighian tubules and midgut of females and males, but is weak in the hindgut of males and females.

Figure 3 compares the expression of *AeInx* genes among the head, carcass, and gonads (ovaries or testes). Common to all of these tissues in both sexes is the moderate or strong expression of *AeInx1*, *AeInx2*, and *AeInx3*. Notably, the expression of *AeInx4* is not detectable in the head of females, but is moderate in those of males, and expressed at moderate or strong levels in the other tissues with the exception of the male carcass where it is weak. The expression of *AeInx7* is weak and moderate in the head of females and males, respectively, while it is strong in the carcass of both females and males. *AeInx7* expression is not detectable in gonads of females (ovaries), and is weakly expressed in those of males (testes). *AeInx8* is expressed at weak (females) or moderate (males) levels in all three tissues, with the exception of the testes where its expression is not detectable.

3.2 *AeInx* cDNAs and Gene Structures

Our laboratory is especially interested in the putative roles of innexins in the synchronization and regulation of renal functions in adult female mosquitoes (Piermarini and Calkins 2013). Thus, we aimed to clone the *AeInx* cDNAs (and any splice variants) expressed in female Malpighian tubules (*AeInx1*, *AeInx2*, *AeInx3*, and *AeInx7*; Figure 2) and elucidate their respective gene structures.

Figure 4A shows the structure of the *AeInx1* gene, based on cloned cDNAs. *AeInx1* consists of six exons spanning ~34 kb on 'supercontig 1.1293' of the *A. aegypti* genome, and is expressed as two distinct splice variants in the Malpighian tubules: *AeInx1-A* and *AeInx1-B*. The *AeInx1-A* cDNA consists of 1,510 bp whereas the *AeInx1-B* cDNA consists of 1,040 bp. The variants differ in their 3' ends where *AeInx1-A* has a sixth exon not found in *AeInx1-B*. Notably, in *AeInx1-A*, the fifth exon is slightly truncated, resulting in a loss of the stop codon that occurs in *AeInx1-B*, which extends the open reading frame of *AeInx1-A* into exon 6 and lengthens its 3' UTR, compared to *AeInx1-B*. The predicted ORF of *AeInx1-A* consists of 1,092 bp encoding 364 amino acids. In contrast, the predicted ORF of *AeInx1-B* consists of 867 bp encoding 289 amino acids. Both predicted proteins are identical with the exception of the COOH terminal domain, which is 75 amino acids longer in *AeInx1-A* (Figure 5).

Figure 4B shows the structure of the *AeInx2* gene, based on the cloned cDNA. *AeInx2* consists of only two exons, which span ~7 kb of 'supercontig 1.293', ~20 kb downstream from *AeInx1*; we did not detect the expression of *AeInx2* splice variants. The *AeInx2* cDNA consists of 2,586 bp with a predicted ORF of 1,077 bp encoding 359 amino acids. The predicted 5'-UTR, ORF, and a partial 3'-UTR are all contained within exon 1, while exon 2 contains the remainder of the 3'-UTR.

Figure 4C shows the structure of the *AeInx3* gene, based on the cloned cDNAs. *AeInx3* consists of six exons spanning ~19kb of 'supercontig 1.560', and we detected the expression of two splice variants: *AeInx3-A* and *AeInx3-B*. The first exon of *AeInx3* resides ~15 kb upstream from the subsequent five exons (Figure 4C); the latter 5 exons reside within a 3 kb range. The *AeInx3-A* cDNA consists of 2,774 bp with a predicted ORF of 1,176 bp encoding

392 amino acids. The *AeInx3-B* cDNA is ~500 bp longer, consisting of 3,209 bp with a predicted ORF of 1,197 bp encoding 399 amino acids. While there is a 500 bp difference in the cDNAs, both predicted proteins are identical with the exception of the extreme COOH terminal domains (Figure 5). This is due to the retention of the intron between exons 5 and 6 in the 'A' variant and an extension of the sixth exon in the 'B' variant.

Figure 4D shows the structure of the *AeInx7* gene, based on the cloned cDNA. *AeInx7* consists of four exons spanning ~11 kb of 'supercontig 1.333', and we did not detect the expression of splice variants. The *AeInx7* cDNA consists of 1,350 bp and a predicted ORF of 1,221 bp encoding 407 amino acids, with relatively short 5' and 3' UTRs.

3.3 Predicted Innexin Proteins

Figure 6 shows an amino-acid sequence alignment of the deduced AeInx1-A, AeInx2, AeInx3-A, and AeInx7 proteins, based on the cloned cDNAs. All four innexins exhibit the expected, predicted membrane topology with relatively short and long cytosolic NH₂-terminal and COOH-terminal domains (NT and CT, respectively, in Figure 6) that flank a transmembrane domain containing 4 membrane-spanning segments (TM1-TM4 in Figure 6). Also notable are 1) the perfectly conserved innexin signature 'YYQWV' motif, located at the start of TM2 in all four of the innexins, and 2) the highly conserved extracellular loop (EL2 in Figure 6) between TM3 and TM4, which includes two cysteine residues ('*' in Figure 6) that are highly conserved across connexins and innexins (Barnes, 1994; Phelan and Starich, 2001). These cysteine residues play important roles in the docking of connexin hemichannels between the plasma membranes of neighboring cells (Bao et al., 2004). Overall, the proteins encoded by the different mosquito innexin cDNAs share only 30–50% amino-acid identity with each other, and share more identity with their respective orthologs in *Drosophila melanogaster* (Table 4).

A PROSCAN analysis of the AeInx amino-acid sequences identified a predicted *N*-glycosylation site in the second extracellular loop of both AeInx3 variants (N₂₂₉RSD), and the first extracellular loop of AeInx7 (N₈₄ESM). The PROSCAN analysis (npsa-pbil.ibcp.fr) also revealed putative phosphorylation sites (Table 5). AeInx1-A has one predicted phosphorylation site in the COOH-terminus, which is not present in AeInx1-B, due to the truncated COOH-terminus of AeInx1-B. AeInx2 has five putative phosphorylation sites, one of which is located in the NH₂-terminus; the other four reside in the C-terminus. AeInx3-A and AeInx3-B share the same five putative phosphorylation sites; one is in the intracellular loop and four are in the COOH-terminus. AeInx7 has the most putative phosphorylation sites with nine. Two reside in the intracellular loop and the remaining seven are in the COOH-terminus.

3.4 Immunochemical expression of AeInx3

Using an affinity-purified antibody, we characterized the immunochemical expression of AeInx3 in the alimentary canal of adult females and 4th instar larvae. The predicted molecular mass of AeInx3 is ~45 kDa. In crude lysates of adult female mosquitoes, the anti-AeInx3 antibody detected several bands, from as low as ~49 kDa to as high as >130 kDa

(Figure 7). The multiple bands suggest the presence of glycosylated and/or oligomeric forms of AeInx3.

Figure 8 shows representative localizations of AeInx3 immunoreactivity in the alimentary canal of adult females. In the proximal end of the Malpighian tubules, where there are no stellate cells, AeInx3 immunoreactivity is prominent at the intercellular boundaries between principal cells (Figure 8A, arrow). Diffuse, punctate labeling in the cytoplasm is also present. In the distal end of the tubules, where stellate cells intercalate among principal cells, the AeInx3 immunoreactivity occurs in principal cells at the intercellular boundaries between principal cells (not shown), and as dense, punctate labeling in the cytoplasm of principal cells (Figure 8B). AeInx3 immunoreactivity is not detectable in the cytoplasm of stellate cells (Figure 8B, arrowhead), but is found in principal cells that neighbor stellate cells (Figure 8B, arrow).

In the midgut of adult females, no immunoreactivity is detectable in the epithelium (Figure 8C), but the tracheal tubes associated with the midgut exhibit detectable labeling (Figure 8D, arrow). In the hindgut, strong labeling of the rectum (Figure 8E, arrow) and ileum (Figure 8E, arrowhead; Figure 8F) occurs at the intercellular boundaries and within the cytoplasm of the epithelial cells. Supplemental Figure 2E shows the junction of the midgut and hindgut where the Malpighian tubules attach, which demonstrates that the relative overall immunoreactivities exhibited by these three tissues in adult females are consistent with the respective, relative mRNA expression patterns observed in Figure 2. Similar patterns of AeInx3 immunoreactivity and localization are observed in the respective isolated tissues from larvae (Supplemental Figure 2 A–C). Preabsorbing the anti-AeInx3 antibody with its antigenic peptide depleted the observed immunolabeling (e.g., Supplemental Figure 2F). In adult male tissues, AeInx3 immunoreactivity was similar to those of females (Data not shown).

4. Discussion

This study is the first to compare and characterize the expression of innexins throughout the mosquito life cycle and in isolated tissues of mosquitoes. As discussed below, our results reveal trends in innexin mRNA expression that shed light on the potential developmental and physiological roles innexins play in mosquitoes. However, it is important to emphasize that these trends do not reveal the actual physiological function of individual innexins, but rather provide us with a starting point for developing hypotheses on their putative roles.

4.1 General trends

AeInx1, *AeInx2*, *AeInx3*, and *AeInx7* mRNAs are expressed consistently across all life stages and sexes examined (Figure 1), which suggests that these innexins are the most abundantly expressed in *A. aegypti* and may contribute to essential house-keeping functions throughout the mosquito life cycle. In contrast, *AeInx4* and *AeInx8* are the least consistently expressed, suggesting potential life stage- and/or sex-specific roles. Studies examining innexin expression in *Bombyx mori* have demonstrated that *BmInx2*, *BmInx3* and *BmInx4* exhibit similar expression patterns throughout the life cycle as those reported here in the mosquito

(Hong et al., 2008, 2009). Thus, the developmental expression patterns of some innexins may be well conserved across holometabolous insects.

4.2 A potential role of *AelInx4* in reproduction

Adult females are the only life stage and sex in which the expression of *AelInx4* mRNA is detectable in the whole animal (Figure 1), suggesting a sex-specific function of this innexin. Our analysis of isolated tissues shows that this gene is expressed primarily in the carcass and ovaries of adult females (Figure 3). The carcass consists of the thoracic and abdominal body walls, which contain the flight musculature, its innervation, and the fat body. Given that the fat body plays a key role in vitellogenesis of female mosquitoes after a blood meal, the expression of *AelInx4* in the carcass may indicate a role of this innexin in fat body-mediated vitellogenesis. Consistent with this notion, the expression of *AelInx4* is weak in the carcass of males, which do not feed on blood or undergo vitellogenesis.

Our finding of *AelInx4* mRNA expression in the ovaries of females is consistent with previous studies, which have detected expression of *Inx4* in the ovaries of *D. melanogaster* and *B. mori* (Bohrmann and Zimmermann, 2008; Hong et al., 2008, 2009). Furthermore, the coexpression of *AelInx4* with *AelInx1*, *AelInx2*, and *AelInx3* in *A. aegypti* ovaries (Figure 3) mirrors that of *D. melanogaster* ovaries, which also express these innexins (Bohrmann and Zimmermann, 2008). Notably, in the ovaries of *D. melanogaster*, *Inx4* immunoreactivity colocalizes with that of *Inx2* where it may form connections between soma and germ-cell lines (Bohrmann and Zimmermann, 2008). Moreover, in the ovaries of *D. melanogaster*, *Inx4* and *Inx2* are necessary for 1) germ cell differentiation and survival, and 2) cyst and egg chamber formation (Gilboa et al., 2003; Mukai et al., 2011). Interestingly, *AelInx4* mRNA expression is also strong in the testes of males (Figure 3), but it must not be abundant enough for its detection in the whole animal (Figure 1). The finding of strong expression in the testes is consistent with previous functional genetic studies in *A. gambiae* and *D. melanogaster*, which have shown that the disruption of *Inx4* expression elicits a sterile male phenotype (Magnusson et al., 2011; Tazuke et al., 2002). Thus, *AelInx4* may play similar key roles in the reproductive biology of *A. aegypti*.

4.3 A potential role of *AelInx8* in development and neuromuscular function

AelInx8 mRNA expression is detectable at weak and moderate levels, in larval and pupal life stages, respectively, but it is not expressed at detectable levels in adults (Figure 1). This suggests that *AelInx8* has a post-embryological role in larval development and pupal metamorphosis. In pupal *D. melanogaster*, *Inx8* expression occurs in neuronal tissue and decreases within two days after eclosion into adults (Crompton et al., 1995). Thus, a similar phenomenon may occur in mosquitoes.

Interestingly, in adult mosquitoes, *AelInx8* mRNA expression is detectable consistently at weak or moderate levels in the hindgut, head, and carcass of both males and females, and in the ovaries of females (Figures 2–3), which suggests that *AelInx8* expression persists throughout the adult stages, but at levels that are not detectable in the whole animal. The expression of *AelInx8* in the head and carcass is expected as both contain neuronal tissues (e.g., ganglia, nerve cord), and *Inx8* orthologs of other insects (*D. melanogaster*,

Schistocerca gregaria) have been implicated in the connectivity of the giant fiber system and frontal ganglion (Anava et al., 2009; Krishnan et al., 1993).

The finding of *AeInx8* mRNA expression in the hindgut, but not the midgut and Malpighian tubules (Figure 2) is intriguing, because these are all epithelial tissues involved with transepithelial ion and water transport. However, the hindgut is exceptional in that it undergoes spontaneous, peristaltic muscle contractions, even when isolated from the whole animal (Kwon and Pietrantonio, 2013; Messer and Brown, 1995). Thus, it is tempting to speculate that *AeInx8* may be playing a role in coordinating these peristaltic movements. Consistent with this notion, *AeInx8* mRNA is expressed in the ovaries (and not the testes) and the associated oviduct is known to undergo similar spontaneous contractions as the hindgut (Kwon and Pietrantonio, 2013; Messer and Brown, 1995). Thus, *AeInx8* may play an important role in the excretory and reproductive physiology of mosquitoes by modulating muscle function in the hindgut and oviduct.

4.4 A potential role of *AeInx7* in epithelial and neural function

AeInx7 mRNA is expressed throughout the alimentary canal (midgut, hindgut and Malpighian tubules) of both sexes (Figure 2). This expression pattern could indicate that *AeInx7* is important for coordinating the respective digestive and/or excretory functions that these tissues are responsible for, such as the diuretic processes of the Malpighian tubules, the release of digestive enzymes into the midgut, and the reabsorption of ions and water by the hindgut. *AeInx7* mRNA is also expressed in the adult head and carcass (Figure 3), which may indicate an important role of this innexin in the nervous system. Consistent with this notion, in *D. melanogaster*, *Inx7* is required for proper nervous system development during embryogenesis, and in memory formation by adult flies (Ostrowski et al. 2008; Wu et al. 2011).

4.5 The molecular potential for homomeric/heteromeric hemichannels and homotypic/heterotypic gap junctions

The molecular expression data of Figures 2–3 also reveals the potential types of GJ that can be formed in specific tissues. For example, in the ovaries, the potential is high for the presence of homotypic and heterotypic GJ consisting of homomeric and/or heteromeric hemichannels, because this tissue expresses mRNAs of *AeInx1*, *AeInx2*, *AeInx3*, *AeInx4*, and *AeInx8* (Figure 3). Likewise, the expression of no less than four innexin genes in each of the adult tissues examined suggests that the molecular composition of GJ in mosquitoes is complex. Thus, a highly integrative set of technical approaches that includes cellular localization of the encoded proteins using antibodies, functional characterization of the encoded proteins using a heterologous expression system (e.g., *Xenopus* oocytes), and knockdown of the mRNAs using RNA interference will likely be required to elucidate the precise functional roles of each innexin gene.

4.6 Cloned innexin cDNAs

This study is the first to clone innexin cDNAs and identify splice variants in the Malpighian tubules of any insect. For both *AeInx1* and *AeInx3*, the splice variation results in novel predicted proteins that differ at the extreme end of the cytosolic COOH-terminal domains.

This finding is notable, because in the connexins of vertebrates, the COOH-terminal domain appears to play important roles in membrane trafficking and voltage gating of GJ (L. Bao et al., 2007; Bao et al., 2004; Johnson et al., 2012). Thus, the alterations to the amino-acid sequences of AeInx1 and AeInx3 caused by alternative splicing may affect the surface membrane expression and activity of the encoded proteins. Alternate splicing has also been documented in the innexins of *D. melanogaster* (*DmInx*). In *DmInx2*, splicing occurs outside of the ORF in the 5'-UTR and thus does not change the predicted peptide sequence (Stebbins et al., 2000). However, in *DmInx8*, splicing affects the ORF leading to polypeptides with differing sequences of their NH₂-terminal domains, and these variants are expressed differentially in separate tissues (Crompton et al., 1995).

Both AeInx3 and AeInx7 are predicted to have glycosylation sites in their second and first extracellular loops, respectively. This finding is interesting because in pannexins (the innexin homologs of vertebrates) the second extracellular loop is glycosylated, which may prevent the formation of GJ, and instead lead to functional hemichannels in the plasma membrane (Scemes et al. 2009). Pannexin hemichannels are thought to play roles in ATP signaling, as well as in general cation transport (Scemes and Spray 2012; Scemes et al. 2009). Thus, AeInx3 and AeInx7 have the molecular potential to become glycosylated, which may allow them to take on other functional roles, besides that of GJ connectivity. Moreover, at least for AeInx3, Western blots in adult females reveal the appearance of multiple immunoreactive bands greater than the expected size (Figure 7), which is consistent the presence of glycosylated forms.

Nearly all of the cloned innexins were found to have at least one potential phosphorylation site, which suggests further putative regulatory mechanisms of innexin function in *A. aegypti*. The predicted AeInx1-A protein has only one predicted phosphorylation site in the COOH-terminus, and this site is lost in the predicted AeInx1-B protein as a result of alternative splicing. The phosphorylation of connexins can alter channel properties such as permeability and regulate protein trafficking (X. Bao et al., 2007; Lampe and Lau, 2004). Thus, there is potential for the two AeInx1 proteins to have differential intercellular transport and/or trafficking properties.

4.7 AeInx3 localization

Immunolabeling experiments in the alimentary canal with the anti-AeInx3 antibody revealed trends in AeInx3 immunoreactivity that mirror those of *AeInx3* mRNA expression, suggesting that AeInx3 protein levels follow mRNA levels. The immunoreactivity of AeInx3 was strongest in the hindgut, where it localized to the intercellular regions of epithelial cells in the ileum and rectum. This finding suggests that AeInx3 may play a role in coordinating the functions of the hindgut epithelial cells.

The immunoreactivity was next strongest in the Malpighian tubules, where it was prominent in principal cells of the proximal and distal segments. The labeling occurred at intercellular boundaries between principal cells and within discrete intracellular compartments of principal cells (perhaps in vesicles). The intercellular labeling presents strong evidence for a role of AeInx3 in mediating the known electrical coupling of principal cells (Masia et al., 2000; Weng et al., 2008). We did not find direct evidence for the expression of AeInx3

immunoreactivity in stellate cells, but in the distal segment we observed immunolabeling in principal cells that neighbor stellate cells (Figure 7B, arrow). Thus, *AeInx3* in principal cells has the potential to dock with opposing innexins in stellate cells and form heterotypic GJ. Additional experiments will be necessary to determine the identity of such innexin(s) in stellate cells, which would help support the hypothesis that stellate and principal cells are functionally coupled (Beyenbach and Piermarini, 2011; Piermarini and Calkins, 2013; Piermarini et al., 2010).

The immunoreactivity was weakest in the midgut, where it was only found in the tracheal tubes attached to the gut, but not in the epithelium or surrounding muscle. The weak immunoreactivity is consistent with the weak expression of *AeInx3* mRNA detected by RT-PCR and suggests that the GJ of the midgut epithelia cells may have distinct functional properties from those of the hindgut and Malpighian tubules.

4.8 Summary

The present study sheds new light on the molecular expression of innexins and their potential functional roles in mosquitoes. We demonstrate patterns of life stage- and tissue-specific expression for six innexins of *A. aegypti* and clone the cDNAs for four of these innexins, including two splice variants. We also characterize the localization of *AeInx3* throughout the alimentary canal of adult females. Taken together, the data allow for the generation of hypotheses on the putative functions of these GJ in vivo, which will require testing in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Histogram summarizing the qualitative expression of each innexin in mosquito life stages and sexes. Numbers indicate the corresponding innexin gene (e.g., 1 = *AeInx1*). The qualitative score for each innexin was assigned based on the mean optical density of an *AeInx* PCR product relative to that of internal control gene *RPS7*, as described in the Materials and Methods.

Figure 2.

Histogram summarizing the qualitative expression of each innexin in tissues of the alimentary canal of adult female and male mosquitoes. Numbers indicate the corresponding innexin gene (e.g., 1 = *AeInx1*). The qualitative score for each innexin was assigned based on the mean optical density of an *AeInx* PCR product relative to that of internal control gene *RPS7*, as described in the Materials and Methods.

Figure 3.

Histogram summarizing the qualitative expression of each innexin in the head, carcass, and gonads. Numbers indicate the corresponding innexin gene (e.g., 1 = *AeInx1*). The qualitative score for each innexin was assigned based on the mean optical density of an *AeInx* PCR product relative to that of internal control gene *RPS7*, as described in the Materials and Methods.

Figure 4.

Mapping of the *AeInx1*, *AeInx2*, *AeInx3*, and *AeInx7* genes (A, B, C, and D respectively). The top drawing of each panel shows the gene structure based on the cloned cDNAs with the thick, horizontal orange bars representing exons and the thin black lines representing introns. The bottom drawings of each panel show cartoons of the exon composition(s) for cloned cDNAs from each gene. Cyan-shaded areas represent the open reading frames and red areas represent the untranslated regions. Splice variants are indicated as 'A' or 'B'.

Inx1 Splice Alignment

```

      10      20      30      40      50      60      70      80      90     100
Inx1-A MYKLLGGKLDYFPRQDIYDQSAVFLRHSFTALLLACSLIITATQVGGPISCIWNGIPRHVVNTFCWISSTFTMPDARKREVREVAHPGVANDFNDE
Inx1-B MYKLLGGKLDYFPRQDIYDQSAVFLRHSFTALLLACSLIITATQVGGPISCIWNGIPRHVVNTFCWISSTFTMPDARKREVREVAHPGVANDFNDE

      110     120     130     140     150     160     170     180     190     200
Inx1-A DAKKYTTYTYQWVCFLFQAACILPRFLWDATGGGLMRITVMGLNIGICREDERCAKKQALIEYVLKRLRRHKLIVLRVFACEALCFVNIILVQLWMNNR
Inx1-B DAKKYTTYTYQWVCFLFQAACILPRFLWDATGGGLMRITVMGLNIGICREDERCAKKQALIEYVLKRLRRHKLIVLRVFACEALCFVNIILVQLWMNNR

      210     220     230     240     250     260     270     280     290     300
Inx1-A FFDGSEFYSYGWVLFKFSEVPQDQRLDPVYVFRVTKCIFPKYGASGTIQKHSCLILPLINVNEKTYIFWFWFIILATMLFCLLIYRAMIVMPSPFRI
Inx1-B FFDGSEFYSYGWVLFKFSEVPQDQRLDPVYVFRVTKCIFPKYGASGTIQKHSCLILPLINVNEKTYIFWFWFIILATMLFCLLIYR-----

      310     320     330     340     350     360
Inx1-A RLLNTINRMVPREAAEAIQKTDLGDNWIIYMLGGNLDPPIFKDVVAELAKQIEPPHNGKQRL
Inx1-B -----

```

Inx3 Splice Alignment

```

      10      20      30      40      50      60      70      80      90     100
Inx3-A MAVFGLVSSVAGFIKVRYLIDKAIIDNMVFRCHYRLTSVFLCCVLVTANLLIGDPISCINDGAI PGHVINTYCWITITFTFLPGQHGRRPVGTSVAHSGL
Inx3-B MAVFGLVSSVAGFIKVRYLIDKAIIDNMVFRCHYRLTSVFLCCVLVTANLLIGDPISCINDGAI PGHVINTYCWITITFTFLPGQHGRRPVGTSVAHSGL

      110     120     130     140     150     160     170     180     190     200
Inx3-A GNEYNQERTYHSYQWVFMFFQGLLFTYMPHWIKNWEEGRMRMISDGLRGNMISPDERRKGRQSLAKYLESINTHNSYSFGFYICEALNFPNVIGN
Inx3-B GNEYNQERTYHSYQWVFMFFQGLLFTYMPHWIKNWEEGRMRMISDGLRGNMISPDERRKGRQSLAKYLESINTHNSYSFGFYICEALNFPNVIGN

      210     220     230     240     250     260     270     280     290     300
Inx3-A IFFVDKFLGGAFITYGSEVLKFSMDQENRSDPMIEIPRVTKCTFHKGASGSIQKHDALCVLALNILEKIYIFLNFWFILLSILSGLAILYSAIVM
Inx3-B IFFVDKFLGGAFITYGSEVLKFSMDQENRSDPMIEIPRVTKCTFHKGASGSIQKHDALCVLALNILEKIYIFLNFWFILLSILSGLAILYSAIVM

      310     320     330     340     350     360     370     380     390
Inx3-A MPTTREAAALKRRFRTPADNQIENLVRIQIGDFLMIHLLQGNINVTSYSEVLHSLMSRLNEDRTRDTPSAPSTLEMAPIYPIGKYQKDTAI-----
Inx3-B MPTTREAAALKRRFRTPADNQIENLVRIQIGDFLMIHLLQGNINVTSYSEVLHSLMSRLNEDRTRDTPSAPSTLEMAPIYPIGKYQKKAAMDQYEA

```

Figure 5.

Alignments of the deduced amino-acid sequences encoded by the *AeInx1* and *AeInx3* mRNA splice variants. Differential splicing in *Inx1* results in identical proteins with the exception of a longer COOH-terminal domain in *AeInx1A*. Differential splicing in *Inx3* results in identical proteins for the first 386 amino acids, but differences in the identities of 5 of the last 6 mutually amino acids of *AeInx3-A* and a longer COOH-terminal domain in *AeInx3-B* indicated in grey.

Figure 6.

Amino-acid sequence alignment of the four innexins cloned from adult female Malpighian tubules. Black shaded regions indicate identical residues among the innexins, whereas gray shaded regions indicate similar residues among the innexins. The transmembrane regions as predicted by Kyte-Doolittle hydropathy plots (Supplemental Figure 1) in association with predicted membrane topologies of *D. melanogaster* and *B. mori* innexins (Hong et al., 2008). The key features of the proteins are labeled, NH₂-terminus (NT), transmembrane region (TM), conserved cysteine residues (*) extracellular loop (EL), intracellular loop (IL) and the COOH-terminus (CT).

Figure 7.
Western blot of AeInx3 immunoreactivity in a crude lysate of adult female mosquitoes.
Expected size of the predicted AeInx3 protein is ~45 kDa.

Figure 8.

Localization of AeInx3-immunoreactivity (green) in isolated tissues of the adult female alimentary canal. Nuclei are counterstained with DAPI (cyan). A) Proximal end of an isolated Malpighian tubule. The white arrow indicates AeInx3 localization to the intercellular membranes between principal cells. B) Distal, blind end of an isolated Malpighian tubule. Arrowhead indicates a stellate cell lacking AeInx3 signal and arrow indicates AeInx3 immunoreactivity in a neighboring principal cell. C) A midgut showing no detectable AeInx3 localization. D) A midgut showing immunoreactivity in associated tracheal tubes (arrow). E) Posterior hindgut showing the rectum (arrow) and distal end of ileum (arrowhead). F) Anterior hindgut showing the ileum; inset shows digital zoom of the region outlined by the white square.

Table 1

RT-PCR primers used to assess the expression of innexin genes in *A. aegypti*. Accession numbers are from Vectorbase (www.vectorbase.org).

Innexin	Accession number	Forward primer	Reverse primer
<i>AeInx1</i>	AAEL014846	CCGTATTCCGCCTCCACAATAG	ACCAAAGTGCTTCACATGCG
<i>AeInx2</i>	AAEL014227	AAGATCACGCCTGTCAGAAC	GTTGAACTTAGGGATTGGAATGTCC
<i>AeInx3</i>	AAEL011248	CAGCTACTCGTTCGGTTACTTC	TCACGTTGATGTTCTGTCCC
<i>AeInx4</i>	AAEL006726	TTCTGTTGGACACTGGGAAC	CCAGAGAATTATACGAAGTCCAGG
<i>AeInx7</i>	AAEL008588	CCAGGCAATACATAGGAGAGC	TCGCAGAGTACGTGTTTC
<i>AeInx8</i>	AAEL014227	GAAGTACCCTATCCAGGTGTTG	CCGCTGACACCATACTTGTAG

Table 2

Gene specific primers used for RACE..

Innexin	Forward primers (3'-RACE)	Reverse primers (5'-RACE)
<i>AeInx1</i>	1F: GTATTAACGAGAGGTAGGACGGGAA 2F: TTTATGGGATGCAACGGAAGGAGGA	1R: GGCGAGTTCCGCTACAACATCCTTGA 2R: GGGAATACGTAAACCATCGGATCTAA
<i>AeInx2</i>	1F: AGTCACGTCGACGGCCATGATGAAG 2F: CATCTGCTGTGCATAGAACCTAGAAA	1R: AAACGGTAGACCAATGATGCACCAG 2R: TCTTTGCCCTCAAATTTGAGAGAGA
<i>AeInx3</i>	1F: CGTGTTTCAGATTCGACGGACAAGACT 2F: CTCGGTAGCACACTCCGGTCTCGCC 3F: GGATCGCAACTCCGTAGAGGCACCC	1R: AGTGAATCGTAGAGGTACTTAGCGAC 2R: CAGGATGAACCAGAACCACAGGAAGC
<i>AeInx7</i>	1F: CGTCTTGTGGATGGTCTTCGTATGGT 2F: CCTCACCAGGAGTTGGACTAACATA	1R: ATTCCAGGACTAACCTTGCAGAAAA 2R: ATAAGTTCAGTTTCGTCAGCCTCAT 3R: CCAGTCCGAAAATGAAAGGCTCTCCG

Table 3

Genbank accession numbers for cloned innexin cDNAs and their splice variants.

Innexin cDNA	Accession number
<i>Inx1-A</i>	KJ736822
<i>Inx1-B</i>	KJ736823
<i>Inx2</i>	KJ736824
<i>Inx3-A</i>	KJ736825
<i>Inx3-B</i>	KJ736826
<i>Inx7</i>	KJ736827

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Table 4

Percent amino-acid identity of the cloned innexins and splice variants to each other and their closest ortholog in *D. melanogaster*.

	Inx1-A	Inx1-B	Inx2	Inx3-A	Inx3-B	Inx7
Inx1-A		100	45.1	41.8	41.8	33.2
Inx1-B	100		47.1	46.4	46.4	38.1
Inx2	45.1	47.1		46.0	46.0	39.3
Inx3-A	41.8	46.4	46.0		98.5	32.4
Inx3-B	41.8	46.4	46.0	98.5		31.6
Inx7	33.2	38.1	39.3	32.4	31.6	
<i>D. melanogaster</i>	72.4	78.2	81.8	68.0	67.5	48.3

Table 5

Phosphorylation sites as identified by PROSCAN analysis.

	Residue Location	Residue Identity
AeInx1A	297–299	SFR
AeInx1B	N/A	N/A
AeInx2	7–9	SVK
	310–313	SSK
	319–321	SLK
	335–342	KNIDPLIY
	350–352	SEHD
AeInx3A/B	157–160	SPDE
	303–305	TTR
	315–318	TAPD
	347–350	SYSE
	372–375	STLE
AeInx7	176–179	SELD
	150–157	RMVEVSRV
	319–321	SVK
	348–351	SFSD
	381–384	TRHE
	385–387	SSK
	386–388	SKK
	387–390	KKIS
	404–406	SEK