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Developmental neurogenetics of sexual dimorphism in *Aedes aegypti*

Running title: Sexually dimorphic mosquito developmental neurogenetics

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Abstract: Sexual dimorphism, a poorly understood but crucial aspect of vector mosquito biology, encompasses sex-specific physical, physiological, and behavioral traits related to mosquito reproduction. The study of mosquito sexual dimorphism has largely focused on analysis of the differences between adult female and male mosquitoes, particularly with respect to sex-specific behaviors related to disease transmission. However, sexually dimorphic behaviors are the products of differential gene expression that initiates during development and therefore must also be studied during development. Recent technical advancements are facilitating functional genetic studies in the dengue vector *Aedes aegypti*, an emerging model for mosquito development. These methodologies, many of which could be extended to other non-model insect species, are facilitating analysis of the development of sexual dimorphism in neural tissues, particularly the olfactory system. These studies are providing insight into the neurodevelopmental genetic basis for sexual dimorphism in vector mosquitoes.

Key Words: mosquito, nanoparticle, siRNA, brain, olfaction, doublesex, development, gene targeting

Sexual dimorphism, a critical aspect of pathogen transmission by vector mosquitoes:

Mosquitoes, including *Aedes aegypti*, which exhibits innate sexually dimorphic behaviors that contribute to the transmission of dengue, yellow fever, and chikungunya viruses, are excellent subjects for studies that examine the biological basis of sexual dimorphism. Genes that contribute to mosquito sexual dimorphism, including the development of neural circuitries that promote human host-seeking, female blood-feeding behavior, mating, and oviposition, may represent targets for vector control (Clemons, 2010a, Tomchaney et al., 2014). Unfortunately, knowledge concerning the extent of sexual dimorphisms in the structure of the central nervous system (CNS), the control of sex-specific behaviors by sexually dimorphic neurons, and the developmental genetic basis for sexually dimorphic behaviors is limited in all organisms, including insects (Kimura, 2011).

Research on the neurodevelopmental genetic basis for insect sexual dimorphism has largely been restricted to *Drosophila melanogaster*, a genetically-tractable—albeit highly derived— dipteran insect that displays innate sexually dimorphic behaviors. Although early studies suggested that few significant anatomical sexual dimorphisms exist in the *D. melanogaster* CNS, more recent investigations indicate that the *Drosophila* CNS has sexually distinct morphologies that originate during development (reviewed by Kimura, 2011). The availability of molecular markers and transgenic reporters to label particular *Drosophila* neurons greatly facilitated detection of sex-specific developmental differences. Sex-specific differences likely exist in the developing nervous systems of many other insects. However, given the lack of molecular markers for developing neurons in non-model species, comparable analyses have not yet been performed in most insects.

Mosquito genome projects (Holt et al., 2002; Nene et al., 2007; Arensburger et al., 2010; Neafsey et al., 2015) facilitated research in new facets of mosquito biology, including functional developmental genetics. Magnusson et al. (2011) assessed sex-specific transcriptomes throughout *Anopheles gambiae* development and characterized the functions of several testis- and ovary-specific genes during gonad development. Functional genetic analysis of nervous system development has been performed in *A. aegypti* (Clemons et al., 2011; Haugen et al., 2011; Sarro et al., 2013; Mysore et al., 2013, 2014a, 2014b), an emerging model for vector mosquito development studies (Clemons et al., 2010a). A recent functional genetic study explored the development of sexual dimorphism in the *A. aegypti* pupal nervous system (Tomchaney et al., 2014). Here, we review these findings and highlight possible future strategies and methodologies for dissecting the developmental neurogenetic basis for sexual dimorphism in *A. aegypti*, many of which may be applicable to other non-model arthropods.

Global and spatial analysis of sexually dimorphic gene expression in the developing *A. aegypti* nervous system:

Custom microarrays were used to examine global differences in female vs. male gene expression in the developing *A. aegypti* pupal head (Tomchaney et al., 2014). Head tissues were prepared 24 hrs after puparium formation, a critical period for nervous system development (Mysore et al., 2011, 2013, 2014a,b). At this time point, which follows periods of extensive proliferative activity and pupal histolysis, neuropils characteristic of the adult brain, including the antennal lobe, central complex, and optic lobe neuropils, have begun to form. Extensive neural process

outgrowth, targeting of higher order brain neurons, synapse formation, and arborization also occur, and the increased neuropil density of the adult is generated (Mysore et al., 2011). In total, 2,527 differentially expressed transcripts (DETs) were identified. Analysis of DETs indicated that dimorphic expression of genes linked to proteolysis, metabolism, catabolic and biosynthetic processes, ion transport, cell growth and proliferation underlie differences in developing *A. aegypti* males and females.

Sex-specific pupal brain spatial expression patterns were assessed for a subset of DETs (Figure 1; Tomchaney et al., 2014). These investigations were facilitated by the work of Mysore et al. (2011), who used cross-reactive *Drosophila* antibodies to establish the first set of molecular markers for the developing mosquito brain. Many of the antibodies work well in conjunction with a combined whole mount *in situ* hybridization/protein localization protocol (Haugen et al., 2010), which employs a detergent-treatment permeabilization step that has facilitated mRNA localization in many arthropod species (Patel et al., 2001; Duman-Scheel et al., 2002). The results obtained validated the microarray data and laid a foundation for future studies. For example, differential expression of the growth regulators *cyclin-dependent kinase 4/6* (*cdk4/6)* and *p53* (Figure 1C,I) may contribute to sexually dimorphic neurite outgrowth (Flannery et al., 2010; DiGiovanni et al., 2006). *p53* also controls apoptosis (reviewed by Sutcliffe et al., 2003), suggesting that this process may be regulated in a sex-specific manner in the developing brain. Differential expression of *synaptojanin (synj)* (Figure 1G), which regulates endocytosis at the *Drosophila* synapse (Verstreken et al., 2003), was also detected in *A. aegypti.* Furthermore, *geko,* which mediates *Drosophila* olfactory responses to ethanol (Shiraiwa et al., 2000) and is dimorphically expressed in *A. aegypti* (Figure 1E), is an interesting target for future functional studies. These expression studies, which detected sex-specific gene expression in the optic lobe, antennal lobe, and mushroom body (Figure 1; also confirmed in sectioned brains), suggested that sex-specific differences exist in the visual and olfactory systems and the processing of sensory information and invoked the question of how dimorphic gene expression is regulated in the developing mosquito nervous system.

Doublesex, a regulator of sex-specific gene expression in the developing mosquito brain:

The *D. melanogaster doublesex (dsx)* gene encodes a key terminal transcription factor in the sex-determination pathway (Kimura et al., 2005; Mellert et al., 2010). *Drosophila dsx* pre-mRNAs are spliced in a sex-specific manner (Ryner et al., 1996; Burtis and Baker, 1989), generating male (DsxM) and female (DsxF) proteins with a common N-terminus and DNA-binding domain, but distinct C-termini that differentially direct sex-specific gene expression (Christiansen et al., 2002; Camara et al., 2008). Male and female *dsx* splice variants were detected in *A. aegypti* (Salvemini et al., 2011), and analysis of their expression (Tomchaney et al., 2014) revealed sexually dimorphic *dsx* expression patterns in the *A. aegypti* antennal lobe and mushroom body (Figure 2). These sex-specific expression patterns differ from *D. melanogaster,* in which sexually dimorphic *dsx* expression was detected in only small subsets of neurons (Lee et al., 2002; Rideout et al., 2010). Moreover, *dsx* is expressed much more broadly in the *A. aegypti* female and male pupal brain. For example, *dsx* expression is not detected in the *D. melanogaster* pupal optic lobe, but sex-specific isoforms of *dsx* are expressed abundantly in *A. aegypti* pupal optic lobes (Figure 2). These results suggest that Dsx may play a more prominent role in the regulation of sex-specific neural development in *A. aegypti*. Furthermore, search of the *A.*

aegypti genome sequence uncovered 732 Dsx consensus binding sites, most of which are associated with genes that group under gene ontology terms linked to neurological processes or neural development, particularly the sensory system and sensory development, and 48 of which flank dimorphically expressed genes identified in the pupal head microarray experiments (Tomchaney et al., 2014). Together, these analyses support the hypothesis that Dsx is a regulator of sexually dimorphic gene expression in the *A. aegypti* nervous system and the development of sexually dimorphic traits in mosquitoes. This hypothesis was examined through functional genetic characterization of *dsx* in *A. aegypti*.

Functional analysis of sex-specific genes in the mosquito nervous system:

Small interfering RNAs (siRNAs), 20-25 nucleotide long double-stranded RNA (dsRNA) molecules that interfere with expression of genes complementary in nucleotide sequence, can be used to silence genes during *A. aegypti* development. In comparison to 400-600 bp dsRNAs, custom siRNAs are produced commercially en masse and are more readily designed to be both gene and species-specific. The time at which gene silencing initiates can be managed through control of siRNA delivery. This advantage facilitates the study of embryonic lethal genes during post-embryonic stages of development; it also permits identification of the critical periods in which loss of gene function generates phenotypes of interest, information that may inform the design of control strategies (Clemons et al., 2010b; Zhang et al., 2015).

Microinjected siRNA (Clemons et al., 2010b) can be used to target *A. aegypti* developmental genes (Clemons et al., 2011; Haugen et al., 2011; Nguyen et al., 2013; Sarro et al., 2013; Tomchaney et al., 2014). siRNA can also be delivered to *A. aegypti* larvae via chitosan nanoparticles (Mysore et al., 2013, 2014a,b) that are mixed with larval food and orally ingested by larvae, and which may promote the stability and cellular uptake of interfering RNA (Zhang et al., 2010). This technique, for which detailed methodology is available (Zhang et al., 2015), is relatively inexpensive, requires little equipment and labor, facilitates high-throughput analysis of multiple phenotypes including behavioral analyses (Zhang et al., 2010, 2015; Mysore et al., 2013, 2014a,b) , and could likely be adapted for gene silencing studies in other insect species. Furthermore, chitosan, a non-toxic and biodegradable polymer (Dass and Choong, 2008), could potentially be utilized in the field.

siRNA-mediated silencing facilitated analysis of the function of *dsx* during *A. aegypti* development. siRNAs corresponding to different target sequences in *Aae dsx* exon 2, which is common to male and female splice variants (Salvemini et al., 2011), were injected into pupae (Tomchaney et al., 2014). The *p53*, *synaptojanin*, *geko*, *rab6*, and *cyclin dependent kinase 4/6* genes are flanked by Dsx binding sites. The sex-specific pupal brain expression patterns of these genes were disrupted by silencing of *dsx* (Fig. 1), indicating that Dsx is required for sexually dimorphic gene expression in the developing *A. aegypti* CNS (Tomchaney et al., 2014). Analysis of the impact of developmental silencing of *dsx* on adult phenotypes will facilitate analysis of adult female morphological, physiological, and behavioral characters that result from loss of *dsx* function during *A. aegypti* development. In particular, it will be interesting to assess the impact of *dsx* silencing on the structure and function of the olfactory system.

Analysis of the *A. aegypti* olfactory system:

Mosquitoes, including *Aedes*, show robust olfactory-driven behaviors, a number of which are sexually dimorphic (Zwiebel and Takken, 2004; Bowen, 1996; Carey and Carlson, 2011). Olfaction in adult *A. aegypti* is mediated by elaborate olfactory appendages, antennae and maxillary palps that are adorned with many hair-like structures called sensilla. A great majority of these are sensory sensilla that house olfactory receptor neurons (ORNs) in which olfactory receptor (OR) proteins are embedded. A plethora of chemicals originating from blood meal host skin and breath, plant/nectar and oviposition sites are detected by these ORNs (Zwiebel and Takken, 2004; Bowen, 1996). Olfaction initiates with interactions between specific odorants and distinct subpopulations of ORs present in the dendritic membrane of ORNs. While all the antennal segments of females are adorned with olfactory sensilla, they are present only on the terminal two segments in males. All types of olfactory sensilla in *A. aegypti* display sexual dimorphism in numbers. The most abundant type, trichodea sensilla that detect the majority of volatile cues derived from plants (in addition to host derived odorants), are four times more prevalent in females (Syed and Leal, 2009, ; Liu et al., 2013). Another category of sensilla, grooved pegs that primarily detect host-derived odors and express a distinct family of ionotropic receptors (IRs), are also at least twice as prevalent in females. Maxillary palps, the "broad spectrum odorant detectors" (Syed and Leal 2007), have only one type of olfactory sensillum that is approximately twice more abundant in females (McIver, 1971). In absence of clear evidence in mosquitoes so far, it appears that sexually dimorphic behaviors potentially result from numerical differences in sensilla, and/or the relative proportion thereof, as has been recently reviewed for other blood-feeding insects (Syed, 2015). Sexual dimorphisms in the number and size of glomeruli in the antennal lobe of the *A. aegypti* brain have also been identified (Ignell et al., 2005).

It will be interesting to examine how developmental silencing of *dsx* or other sex-determination genes impacts the sex-specific structure and function of the adult olfactory system and olfactory-driven behaviors in *A. aegypti*. For example, scanning electron microscopy could be used to explore resulting numerical and morphometric structural anomalies of the olfactory sensilla. Maxillary palp sensilla house three ORNs that respond to carbon dioxide, 1-octen-3-ol, and acetophenone respectively in *Culex* (Syed and Leal, 2007), *Aedes* (Grant and O'Connell, 1996) and *Anopheles* (Lu et al., 2007). To date, studies in all three species have been conducted exclusively in females, and it remains an exciting avenue to explore sexual differences, especially after *dsx* manipulation, in males. Males are attracted to host odors, but likely differ from females in their response amplitude and dynamics to host chemostimuli. Sexual dimorphisms may particularly be expected at very close range and for landing responses, as well as in the male mating system which facilitates interception of females at the host (recently reviewed by Oliva et al., 2014). It is tempting to speculate that developmental differences will potentiate measureable neuroethological differences. A variety of behavioral assays can be employed to efficiently dissect the sexually dimorphic or isomorphic mosquito life behaviors mediated by odors: sugar feeding (Syed and Leal, 2008), host feeding (Sim et al., 2012), and oviposition (Laurence and Pickett, 1985). It is predicted that loss of *dsx* will disrupt some or all of these olfactory-driven behaviors that are critical to mosquito survival and reproduction. Ultimately, the overarching goal will be to identify and functionally characterize specific Dsx target genes that regulate sex-specific olfactory-driven behaviors.

Future functional genetic studies in *A. aegypti:*

Studies in *D. melanogaster* have demonstrated that Dsx and Fru function in the same neurons to establish neuronal wiring and behaviors (Rideout et al., 2007, 2010; Kimura et al., 2008). Neville et al. (2014) suggested that *Drosophila* Dsx and Fru act together, either in a physical complex or through co-regulation of target genes, to control sex-specific neural development. Although sex-specific Fru splice forms have been identified in *A. aegypti* (Salvemini et al., 2013), the expression patterns of these transcripts have not yet been assessed in the developing nervous system, and *fru* function, has not been characterized in mosquitoes. Given the likelihood of fertility defects in *dsx* loss of function animals and the lack of marked balancer chromosomes in mosquitoes, conditional siRNA-mediated gene silencing has proven to be an excellent strategy for analysis of *dsx* function, and this technique would likely permit analysis of *fru* function, as well as the functions of other components of the sex-specification pathway. The transcriptional targets of Dsx (Tomchaney et al., 2014) and Fru may also represent targets for vector control. It will also be interesting to characterize the functions of various ORs in males and females, particularly those that are known to be dimorphically expressed (Bohbot et al., 2007) and that may be direct or indirect targets of sex-specification genes. In addition to RNA interference, targeted mutagenesis is emerging as a viable option for assessing the function of these target genes.

Homing endonucleases, zinc-finger nucleases, and TALE nucleases (TALENs) have been used to generate heritable loss of function mutations in *A. aegypti* (Liesch et al., 2013; DeGennaro et al., 2013; Aryan et al., 2013a,b, 2014; McMeniman et al., 2014). DeGennaro et al. (2013) used zinc-finger nucleases to generate targeted mutations in the *A. aegypti orco* gene, which encodes the obligate co-receptor in the assembly and function of heteromeric OR/Orco complexes. Orco is crucial for discrimination between human vs. non-human hosts and for repulsion by volatile N,N-diethyl-meta-toluamide (DEET). Zinc-finger endonucleases were also used to target *AaegGr3*, which encodes a subunit of the heteromeric receptor complex required for carbon dioxide detection (McMeniman et al., 2014). CRISPR-Cas9 genome engineering was recently reported in *A. aegypti* (Kistler et al., 2015; Dong et al., 2015; Basu et al., 2015). This technology generates high levels of mutagenesis and is reportedly a cheaper, faster, and more flexible method for generating loss of function mutations. This technique, which is rapidly becoming the method of choice for mutagenesis studies in mosquitoes, will greatly facilitate interrogation of the adult *A. aegypti* olfactory system, olfactory development, and the development of sexually dimorphic traits in mosquitoes.

Despite substantial progress in mosquito genetic research, very few cis-regulatory elements (CREs), DNA sequences that control gene expression, have been identified in the mosquito genomes. This deficiency—a significant gap in basic knowledge of mosquito genetics—has resulted in a lack of drivers to manipulate or prevent gene expression in selected tissues at specific times. Such tools, which revolutionized research in genetic model organisms, would facilitate genetic studies and benefit all avenues of mosquito research, including analysis of neural development. Discovery of CRE drivers would also promote the development of transgenic insects for vector control, such as the female flightless mosquitoes generated with a flight muscle regulatory element (Fu et al., 2010; Wise deValdez et al., 2011). FAIRE-seq, formaldehyde-assisted isolation of regulatory elements paired with DNA sequencing (Simon et al., 2012), a powerful new approach for global biochemical isolation of CREs through their lack

of association with nucleosome proteins, will facilitate genome-wide discovery of putative *A. aegypti* CREs. Testing putative CREs in transgenic reporter assays will permit identification of gene drivers for the brain, olfactory system, and other tissues of vector importance. FAIRE-seq studies, as well as the use of other biochemical approaches (i.e. DNAse-seq) or computational approaches for the identification of insect CREs (Kazemian et al., 2014), will also facilitate analysis of gene regulatory networks in the developing nervous system. Moreover, since FAIRE assesses chromatin states, it is anticipated that FAIRE-seq might also be applied for epigenetic analysis of sexual dimorphism in *A. aegypti*, an exciting prospect.

Conclusions:

Recent technical advances are facilitating functional genetic studies in *A. aegypti*, an emerging model for vector mosquito development. These techniques are being used to study the development of sexual dimorphism in neural tissues, particularly the brain and olfactory system. Comparison of female vs. male transcriptomes and detailed spatial analysis of gene expression patterns are uncovering sexual dimorphisms in the developing nervous system. siRNA-mediated gene silencing studies and targeted mutagenesis studies with emerging CRISPR/Cas9 technology can be used to assess the functional contributions of various genes to the development of sexual dimorphism. These studies are providing insight into the neurodevelopmental genetic basis for sexual dimorphism in vector mosquitoes and may promote the elucidation of novel genetic targets for vector control strategies.

Conflicts of Interest: The authors declare that they have no competing interests.

Abbreviations: Central nervous system (CNS); differentially expressed transcripts (DETs); *doublesex (dsx)*; small interfering RNAs (siRNAs); double-stranded RNA (dsRNA); olfactory receptor neurons (ORNs); olfactory receptor (OR); ionotropic receptors (IRs); TALE nucleases (TALENs); formaldehyde-assisted isolation of regulatory elements paired with DNA sequencing (FAIRE-seq)

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Figure Legends:

Figure 1. Sex-specific gene expression in the *A. aegypti* pupal brain. The antennal lobe (AL), optic lobe (OPL), suboesophageal ganglion (SOG), and mushroom body (MB) regions are marked (red dots) in a whole brain labeled with anti-N-Cadherin (green in A, B) and TOTO nuclear stain (blue in **A, B**). These regions were assessed through confocal imaging following whole mount *in situ* hybridization and anti-HRP staining (**C-J**). Five merged Z-stacks (totaling 5 microns) of brain hemisegments (oriented dorsal upward in **C-J**) labeled through anti-HRP staining (center panels in **C-J**; green in overlays at right) and riboprobes corresponding to the indicated transcripts (left panels in **C-J**; red in overlays at right) are displayed. Differential expression of *cdk4/6* (**C, D**), *geko* (**E, F**), *synj* (**G, H**), and *p53* (**I, J**) is shown in 24 hr pupal brains of females (**C, E, G, I**) and males (**D, F, H, J**). *cdk4/6* is commonly expressed in the optic lobe in both sexes (white arrowheads in **C, D**), but additional *cdk4/6* expression is detected in the ventral suboesophageal ganglion of males (red arrowhead in **D**). *geko*, which is expressed in the optic lobe of both sexes (white arrowheads in **E, F**), is expressed in additional large cell bodies near the female midbrain and in the female antennal lobe (red arrowheads in **E**). Expression of *synj* is detected in the optic lobe (white/red arrowheads in **G, H**) and in a subset of midbrain neurons (yellow arrowheads in **G, H**). Sex-specific *synj* expression is detected in the optic lobe (red arrowheads in **G, H**), and midbrain levels of *synj* are generally higher in males (compare expression adjacent to yellow arrowheads in **G, H**). *p53* expression is detected in the suboesophageal ganglion and optic lobe of females (white arrowheads in I). *p53* expression is also detected in the male optic lobe (white arrowheads in **J**), but not in the subesophageal ganglion of males. Male-specific *p53*-expressing neurons are found adjacent to the antennal lobe (red arrowheads in **J**). This figure originally appeared in Tomchaney et al. (2014), which contains further information regarding experimental details.

Figure 2. Sex-specific expression patterns of *dsx* in the *A. aegypti* pupal brain. Expression of *dsx* was analyzed through *in situ* hybridization experiments performed on paraffin sections of female (**A-C**) and male (**D-F**) heads. 12 micron sections through different portions of the brain revealed the antennal lobe (al), lamina (la), and medulla (me) in brain hemisegments oriented

dorsal upward (**A-F)**. Expression of *dsx* is detected in the developing female and male visual systems (blue arrowheads in **A, B, D, E**). However, sex-specific expression of *dsx* is detected in the antennal lobe (marked by red dots in **C, F**) and mushroom bodies (red arrowheads in **A, D**). This figure originally appeared in Tomchaney et al. (2014), which provides further experimental details.

Figure 1.TIF

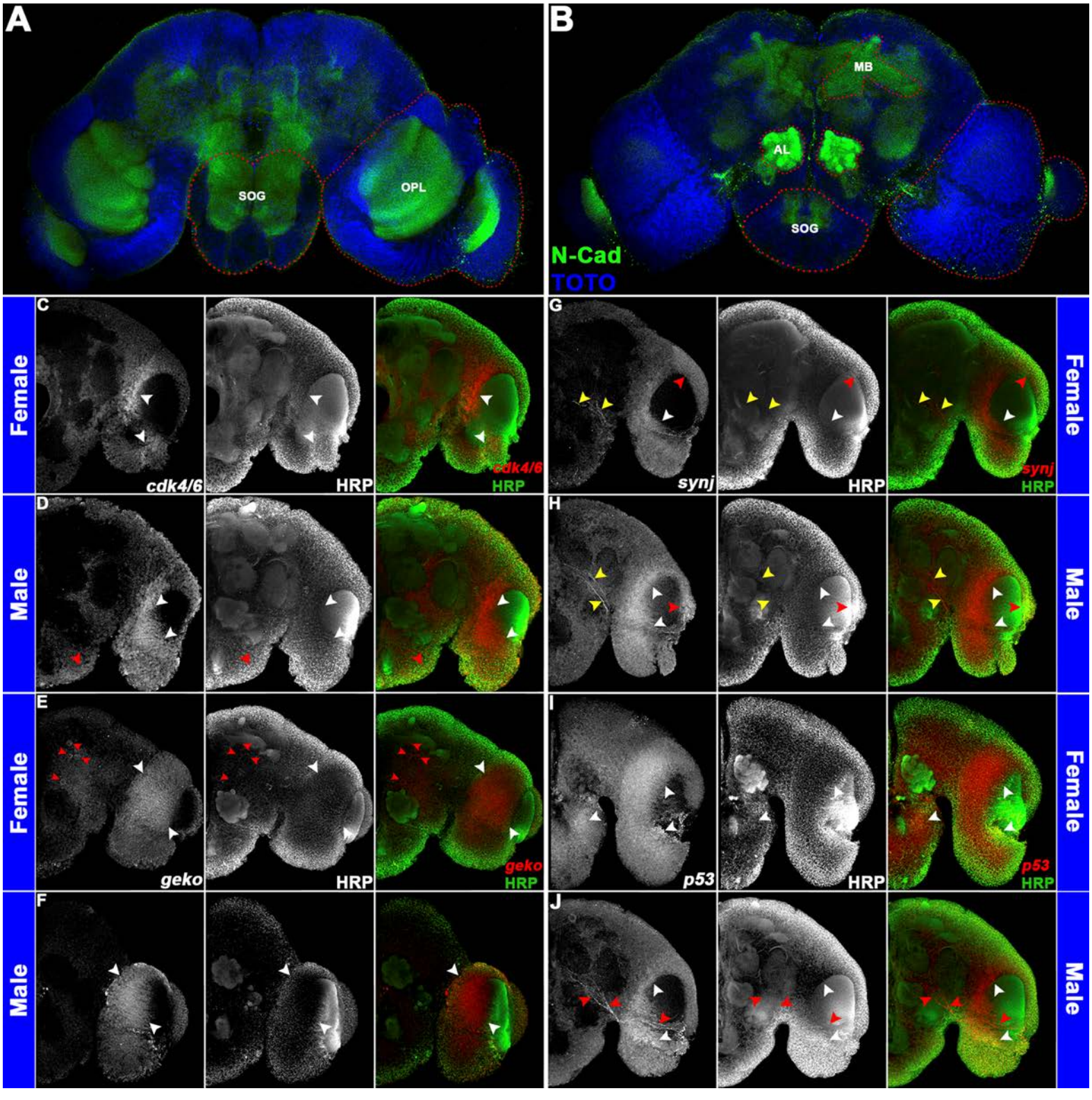
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Figure 2.TIF