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Doublesex establishes sexual dimorphism in the *Drosophila* central nervous system in an isoform-dependent manner by directing cell number

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ABSTRACT

doublesex (*dsx*) encodes sex-specific transcription factors (DSX^F in females and DSX^M in males) that act at the bottom of the *Drosophila* somatic sex determination hierarchy. *dsx*, which is conserved among diverse taxa, is responsible for directing all aspects of *Drosophila* somatic sexual differentiation outside the nervous system. The role of *dsx* in the nervous system remains minimally understood. Here, the mechanisms by which DSX acts to establish dimorphism in the central nervous system were examined. This study shows that the number of DSX-expressing cells in the central nervous system is sexually dimorphic during both pupal and adult stages. Additionally, the number of DSX-expressing cells depends on both the amount of DSX and the isoform present. One cluster of DSX-expressing neurons in the ventral nerve cord undergoes female-specific cell death that is DSX^F-dependent. Another DSX-expressing cluster in the posterior brain undergoes more cell divisions in males than in females. Additionally, early in development, DSX^M is present in a portion of the neural circuitry in which the male-specific product of *fruitless* (*fru*) is produced, in a region that has been shown to be critical for sex-specific behaviors. This study demonstrates that DSX^M and FRU^M expression patterns are established independent of each other in the regions of the central nervous system examined. In addition to the known role of *dsx* in establishing sexual dimorphism outside the central nervous system, the results demonstrate that DSX establishes sex-specific differences in neural circuitry by regulating the number of neurons using distinct mechanisms.

Introduction

Sex-specific differences in neural circuitry contribute to differences in male and female reproductive behaviors (reviewed in Ball and Balthazart, 2004; Simerly, 2002). In *Drosophila*, sex-specific reproductive behaviors are specified through a genetic regulatory cascade called the sex determination hierarchy (reviewed in Manoli et al., 2006). This hierarchy consists of a pre-mRNA splicing cascade that culminates in the production of sex-specific transcription factors. *doublesex* (*dsx*) is at the bottom of one branch of the sex hierarchy and has been shown to specify all aspects of sex-specific development outside the nervous system (Hildreth, 1965). *fruitless* (*fru*) is at the bottom of another branch of the sex hierarchy and has been shown to encode male-specific transcription factors [FRU^M, encoded by *fru P1* transcripts; (Ryner et al., 1996)] that underlie the potential for male courtship behaviors (reviewed in Manoli et al., 2006). Recent studies have shown that both *fru* and *dsx* collaborate in the central nervous system (CNS) to bring about the potential for one step in the male courtship ritual, the production of courtship song (Rideout et al., 2007). Despite this progress, the mechanisms by which *dsx* establishes differences in neural circuitry are largely unknown.

dsx encodes both male (DSX^M) and female (DSX^F) transcription factors (reviewed in Christiansen et al., 2002). DSX isoforms share a common amino terminal region that contains the DNA binding domain, but differ in their carboxyl terminal region [Fig. 1A; (Burtis and Baker, 1989)]. *dsx* specifies nearly all aspects of *Drosophila* somatic sex determination outside the nervous system, as *dsx* null animals display an intersexual phenotype (Hildreth, 1965). Furthermore, if DSX^M is the only DSX isoform produced in chromosomally XX animals, these animals look almost identical to wild type males (hereafter called pseudomales), suggesting that *dsx* is sufficient to specify most aspects of sex-specific somatic development (Duncan and Kaufman, 1975).

The role of *dsx* in directing sex-specific nervous system development and physiology has been more difficult to examine, given that

Abbreviations: APF, after pupal formation; BrdU, Bromodeoxyuridine; CNS, central nervous system; *dsx*, *doublesex*; *fru*, *fruitless*; *ix*, *intersex*; pC1 and pC2, posterior cells 1 and 2; PrMs, pro- and meso-thoracic ganglion region; TN1, thoracic neurons 1; TUNEL, terminal uridine deoxynucleotidyl transferase; VNC, ventral nerve cord; wpp, white pre-pupal.

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there are no overt morphological differences between the sexes in the nervous system. Behavioral analyses on *dsx* mutants provide conflicting results. Initially, it was thought that *dsx* was unable to direct the nervous system to a male fate, given the observation that pseudomales do not display any male-specific behaviors (Taylor et al., 1994). However, it was also shown that *dsx* null males perform courtship in a quantitatively subnormal manner (Villella and Hall, 1996), and a population of abdominal neuroblasts undergoes more *dsx*-dependent divisions in males (Taylor and Truman, 1992). Recent studies suggest a key role for *dsx* in specifying reproductive behaviors, including a demonstration that DSX^M and FRU^M are co-expressed in subsets of neurons in the CNS (Billeter et al., 2006; Rideout et al., 2007), that DSX and FRU collaborate to bring about the potential for wing song (Rideout et al., 2007), and that animals that are transheterozygous for *dsx* and *fru P1* alleles show a reduction in male courtship behaviors (Shirangi et al., 2006).

DSX is expressed in a sexually dimorphic pattern in the adult CNS (Lee et al., 2002). In this study, the mechanisms responsible for generating this dimorphism were determined. DSX expression was examined during metamorphosis, in both males and females and the pattern is very similar to that which was previously described, with some differences (Lee et al., 2002). Additionally, this study shows that the DSX-expressing cell number is established during a small window of time during early stages of metamorphosis. DSX directs the number of DSX-expressing cells in the CNS in an isoform-specific and dose-dependent manner. The sexually dimorphic number of DSX-expressing cells in one region of the ventral nerve cord (VNC) is a result of DSX^F-dependent cell death that occurs during metamorphosis in females and not males. Additionally, a population of DSX-expressing neurons in the posterior brain of males undergoes more cell divisions than in females. Given that the sex-specific transcription factors DSX^M and FRU^M have overlapping expression patterns, we examine if these transcription factors are responsible for establishing differences in each other's expression patterns in the CNS, and find that they are not inter-dependent. Furthermore, in females, overlap between DSX^F and *fru P1*-expressing cells during development does not occur in the two regions examined that have a dimorphism of number of neurons. Taken together, this work demonstrates a role for DSX in forming sex-specific differences in cell number that underlie differences in neural circuitry in the CNS.

Materials and methods

Generation of polyclonal antibodies specific to DSX and FRU^M

We produced a recombinant glutathione *S*-transferase (GST) DSX fusion protein that contained ~100 amino acids that are common to both DSX isoforms, by PCR amplifying the region and cloning it into the pGEX 4T1 vector (GE healthcare). The PCR primers used are: 5′ CTCGAGCTCTTCGATTCGATTCGCCGGGAAGCCTCTTCAAT (Xho1 site is engineered at 5′ end) and 5′ GAATTCAGGTCATCGGGAACATCGGTGATCACTAGC (EcoR1 site is engineered at 5′ end). The GST-DSX fusion protein was produced in *E. coli*, purified and used to immunize a rat host (Josman, Napa Valley). The serum was affinity purified on a column containing the recombinant GST-DSX fusion protein covalently coupled to amino resin (Pierce). The FRU^M antibody was generated as described in Lee et al. (2000), and was shown to be specific to FRU^M.

Immunohistochemistry

Whole mount immunochemistry experiments were performed as previously described (Lee et al., 2000). The primary polyclonal DSX antibody was diluted 1:50–1:100 in TNT (0.1 M Tris–HCl, 0.3 M NaCl, 0.5% Triton X-100, pH 7.4). Secondary antibodies were purchased from Molecular Probes and used at recommended dilutions. Confocal microscopy was performed on a Zeiss Pascal. ELAV antibody was obtained from the Iowa hybridoma bank. Cell counts of FRU^M-expressing cells in 5–7-day old *dsx* null and wild type males were performed blindly by two independent members of the lab.

TUNEL assay for cell death

The Apoalert DNA Fragmentation Assay kit (Clontech) was used with modifications (as described in Firth et al., 2005).

Drosophila strains

Drosophila were grown on standard media containing cornmeal, dextrose, and yeast at 25 °C. The wild type strain is Canton S. The *dsx* null genotype is transheterozygous for the *dsx* alleles dsx^{M+R15} and dsx^{D+R3}. Generally, flies homozygous for the ix^3 allele were not viable, although a small number of homozygous ix^3flies escaped to the adult stage. The loss-of-function *ix* genotype used here is transheterozygous for the ix^3 allele and a *Df(2R)en-B* deficiency. The *fru P1* null genotype is transheterozygous for the *fru* alleles fru^{P14} and fru^{4-40}. The *fru-P1-GAL4* driver was previously described (Stockinger et al., 2005). The UAS-DSX^F and UAS-DSX^M lines were provided by Gyunghee Lee. Genotypes of transgenic flies and flies mutant for cell death effectors are described in Supplemental Table 2; briefly, they include *Df(3L)XR38* and *Df (3L)H99* deficiencies (referred to as *XR38* and h*99*, respectively), described in Peterson et al. (2002), β *amyloid protein precursor-like* (*appl*)-Gal4, *tubulin*-Gal4, *actin*-Gal4, *elav*-Gal4, h*eat shock*-Gal4, UAS-*diap*, and UAS-*p35*. With the exception of the *XR38*, h*99*, *appl*-Gal4, UAS-*diap*, and UAS-*p35* strains, all were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, Indiana, United States). Pupal animals were sex-sorted at the white pre-pupal stage, and then aged at 25 °C.

BrdU labeling

Pupae were sex-sorted at the white pre-pupal stage and aged for 8 h. CNS tissues were dissected in cold PBS, incubated for 4 h in 15 μg/ml of BrdU in PBS at room temperature, washed once in PBS, and then fixed in 3% paraformaldehyde in PBS for 20 min at room temperature. Samples were washed three times for 5 min in PBS, three times for 5 min in TNT, and blocked for 1 h in 4% normal goat serum. To expose the BrdU antigen, samples were briefly boiled (4.5 min) at 100 °C. Samples were washed in PBS and incubated in mouse anti-BrdU (1:200 dilution, GE healthcare) and rat anti-DSX (1:50 dilution) in TNT overnight at 4 °C on a rocker. Samples were washed six times for 15 min each with TNT and then incubated in secondary anti-rat and anti-mouse antibodies (Molecular Probes) diluted 1:500 in TNT overnight at 4 °C. Samples were washed six times in TNT for 15 min each and mounted in VectaShield before imaging.

Statistical analyses

All cell counts are represented as mean ± standard error of the mean. Statistical significance was calculated by unpaired, two-tailed Student *t*-tests and performed in excel. The resulting *P*-value is reported.

Results

DSX expression in the CNS is sexually dimorphic across development

To determine the mechanism that underlies the sexual dimorphism in the number of DSX-expressing cells, a polyclonal rat antiserum specific to a common portion of DSX was generated and DSX-expressing cells during development were analyzed (see Fig. 1A). The antibody is specific to DSX, as signal is detected in wild type animals, but not in *dsx* null animals (Supplemental Figs. 1A and B). Using antibodies against DSX and the nuclear, neuron-specific ELAV protein (Robinow and White, 1991), we demonstrate that nearly all DSX-expressing cells detected here are neuronal and that DSX is localized to the nucleus (Supplemental Fig. 2). When DSX is over-expressed using a constitutive promoter, signal is detected throughout the brain using the DSX antibody (Supplemental Fig. 1C).

Previous studies have shown that DSX is present in the CNS and sexually dimorphic at the adult stage, but not at the 48-hour after puparium formation (APF) stage (Lee et al., 2002). DSX expression 48 hours APF was examined here and a sexual dimorphism in several regions of the CNS was observed. To determine how the dimorphism in DSX-expressing cells is both established and maintained, the number of DSX-expressing cells in the CNS was quantified in 48-hour APF pupae and 0–24-hour adults (see Tables 1 and 2). At 48-hour APF, the combined number of cells for pC1 and pC2 (see Fig. 1 for description of nomenclature) is 88 ± 1.9 and 13 ± 0.8, in males and females, respectively (all cell counts are represented as the mean ± standard error of the mean). A sexual dimorphism in the TN1 cluster of the ventral nerve cord (VNC) was also observed, with 16 ± 1.3 and 0 ± 0 cells, in males and females, respectively (Fig. 1).

A similar overall pattern of DSX-expressing cells was observed in the 0–24-hour adult CNS, with a dimorphism in the pC1, pC2 and TN1 regions, as was observed in 48-hour APF pupae [Tables 1 and 2, Fig. 1, and previously described (Lee et al., 2002)]. Two DSX-expressing