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Immunohistochemical analysis of a novel dehydroepiandrosterone sulfotransferase-like protein in *Drosophila* neural circuits

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Abstract

Sulfotransferase (ST)-catalyzed sulfation plays an important role in various neuronal functions such as homeostasis of catecholamine neurotransmitters and hormones. *Drosophila* is a popular model for the study of memory and behavioral manifestations because it is able to mimic the intricate neuroregulation and recognition in humans. However, there has been no evidence indicating that cytosolic ST(s) is(are) present in *Drosophila*. The aim of this study is to investigate whether or not cytosolic ST(s) is(are) expressed in the *Drosophila* nervous system. Immunoblot analysis demonstrated the presence of dehydroepiandrosterone (DHEA) ST-like protein in *Drosophila* brain and a sensitive fluorometric assay revealed its sulfating activity toward DHEA. Immunohistochemical staining demonstrated this DHEA ST-like protein to be abundant in specific neurons as well as in several bundles of nerve fibers in *Drosophila*. Clarification of a possible link between ST and a neurotransmitter-mediated effect may eventually aid in designing approaches for alleviating neuronal disorders in humans.

Keywords: Sulfation; *Drosophila*; DHEA ST; Memory; Neuropharmacology

Cytosolic STs are enzymes that catalyze the transfer of a sulfuryl group from the universal sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a variety of endogenous and exogenous compounds, such as steroids, amines, and various xenobiotic chemicals [1]. The reaction, usually referred to as "sulfation", occurs in many prokaryotic and vertebrate species and plays an important role in numerous biological processes including hormone regulation, homeostasis of neurotransmitters, as well as transport and metabolism of steroids in circulation. The sulfation of steroids decreases their biological activity, rendering them incapable of binding and activating steroid receptors. These sulfated steroids, nevertheless, may serve as prohormones, which can be reactivated by desulfation [2]. Although most steroids are synthesized in steriodogenic organs, a few, such as progesterone (PROG), pregnenolone (PREG), and DHEA, are produced *de novo* in the central nervous system (CNS) and peripheral nervous system (PNS) [3]. These neurosteroids regulate specific gene expression and protein synthesis, cellular development, neuroendocrine functioning, and behavioral pattern [4]. Dehydroepiandrosterone sulfotransferase (DHEA ST) catalyzes the sulfation of DHEA, converting it to dehydroepiandrosterone sulfate (DHEAS) [5]. Both DHEA and DHEAS are critical precursors for the production of several types of androgens and estrogens. DHEA can also be metabolized to form testosterone, estradiol, and androstenediol [6]. DHEA and DHEAS are involved in

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numerous neurophysiological processes, such as increasing neuronal excitability, and enhancing neural plasticity and neuroprotective properties. Previous studies have demonstrated that the concentrations of DHEA and DHEAS in blood decrease markedly with age in humans, and have been proposed to be the neuromodulators involved in age-related cognitive decline [7]. These findings have led to the hypothesis that elevated concentrations of steroids may influence both physical and cognitive aging.

Drosophila melanogaster is a popular animal model for pathological and neuropharmacological research. At the molecular level, the nervous system of the *Drosophila* can mimic the intricate neuroregulation of the neuronal network in humans. In *Drosophila*, several types of carbohydrate STs have been cloned and characterized, and the physical functions and biological regulations modulated by the sulfate conjugates have been extensively investigated [8]. The sulfation of hexuronate and glucosamine units, usually *N*-acetylgalactosamine (GlaNAc) or *N*-acetylglucosamine (GlcNAc), has been shown to be essential for development and embryogenesis, as well as differentiation and neuronal functions [9]. The recent studies have demonstrated that sulfation is a critical regulator for developmental and neuronal functions in *Drosophila*, however, there has been no evidence indicating that cytosolic ST(s) is(are) present in this important animal model. In the present work, we used a specific DHEA ST antibody to determine the existence and distribution of DHEA ST-like protein in the nervous system of *Drosophila*. Moreover, we investigated and confirmed the presence of DHEA-sulfating activity of in *Drosophila* brain extracts by employing a continuous fluorometric assay.

Materials and methods

Preparation of recombinant STs. Recombinant human phenol-preferring phenol sulfotransferase (*h*P-PST), human catecholamine-preferring phenol sulfotransferase (*h*M-PST), and *h*DHEA ST were cloned into an expression vector, pGEX-2TK and transformed into *Escherichia coli* BL21 (DE3). These STs were expressed in the form of GST-fusion protein and purified by glutathione (GSH)-bound sepharose. The methods of expression and purification of these three STs were described previously [10]. Recombinant rat phenol sulfotransferase (*r*PST) was cloned into expression vector pET3c and transformed into *E. coli* BL21 (DE3). Briefly, DEAE, hydroxyapatite, and size-exclusion chromatography were used to purify the *r*PST [11]. Putative *Drosophila* cytosolic ST, *dm*CG5431, was found on the Flybase database (CG5431; http://flybase.bio.indiana.edu/). By using RT-PCR, a full length cDNA encoding *dm*CG5431 was cloned into pET-41b and transformed into *E. coli* BL21 (DE3). GST-fusion and His-tagged ST were expressed and purified by GSH-bound sepharose and Ni-chelating column. All purified STs were in homogeneous form and determined by SDS–PAGE.

Indirected enzyme-linked immunosorbent assay (IELISA). Approximately 1 μg of the recombinant STs, *h*P-PST, *h*M-PST, *h*DHEA ST, *r*PST, and *dm*CG5431 was coated on each well of an ELISA plate for screening using *h*DHEA ST antibody. Following washes with PBS, 50 μl of serially diluted solutions of *h*DHEA ST antibody were added to individual wells and incubated for 1 h. Afterwards, each well was washed three times with PBST (PBS containing 0.1% BSA and 0.05% Tween-20). Bound antibodies were then detected using a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) for 30 min in PBST. Finally, each well was washed and developed with 0.04% 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) containing 0.01% H_2O_2 in PBS. For denatured conformation analysis, all steps were the same except that the recombinant STs were treated with 2% β-ME and heating prior to being coated on the wells.

Preparation of Drosophila brains homogenates. The *Drosophila* heads were freshly isolated by liquid nitrogen freezing and harvested through a sieve to separate the heads from the bodies. Approximately 0.2 g of frozen brain samples were homogenized using a mortar and pestle, dissolved in a lysis buffer (2 mM sucrose plus 3 mM β-ME, 0.2% Triton X-100 and 0.5% protease inhibitor cocktail in 10 mM HEPES buffer, pH 7.4) and then centrifuged to remove cell debris. The homogenate was centrifuged twice at 15,000 rpm for 20 min at 4 °C. The supernatant was collected and the total protein concentration was estimated.

Immunoblot analysis. Approximately 5 μg of five recombinant STs, *h*P-PST, *h*M-PST, *h*DHEA ST, *r*PST, and *dm*CG5431, respectively, were loaded onto individual wells of a 12% SDS–PAGE for electrophoresis according to the method of Laemmli [12]. After electrophoresis, the separated proteins were electroblotted onto a nitrocellulose membrane and blocked with 5% skimmed milk for 1 h. The membrane was incubated with *h*DHEA ST antibody for 1 h and washed three times with PBST for 5 min. The membrane was then immersed in PBST containing antibody against rabbit IgG conjugated with HRP for 1 h. The bound antibodies were detected with an ECL Western blotting reagents for chemiluminescent detection. The native immunoblot analysis procedure was similar to that described previously with minor modifications. All buffer solutions were free from the addition of SDS. Similarly, approximately 300 μg of *Drosophila* brain proteins and 20 μg of purified hDHEA ST were used for analyzing the protein expression of DHEA ST-like protein by native and SDS immunoblot. The steps were similar to those described previously.

Determination of sulfating activity of DHEA in Drosophila brain. DHEA-sulfating activity of *Drosophila* brain was determined by the continuous fluorometric assay developed by Chen et al. [13]. By using DHEA as substrate, the activity of DHEA ST was determined by monitoring the fluorescence intensity of MU. The standard assay mixture had a final volume of 1 ml, and contained 100 mM potassium phosphate buffer (pH 7.0), 5 mM β-ME, 20 μM PAPS, 4 mM MUS, 5 μM DHEA and 3.2 mU K65ER68G, the recombinant β-form of PST. For use in the assay, a partially purified DHEA ST-like protein fraction, located by cross-reactivity with antibody against *h*DHEA ST, was prepared from *Drosophila* brain homogenates by using native gel electrophoresis. The intensity of MU was monitored using a spectrofluorometer (Hitachi F-4500, Japan).

Immunohistochemistry. The *Drosophila* brain was perfused with 4% paraformaldehyde for fixation and then penetrated with 30% sucrose. After washing with PBS, the brain was blocked with 1% BSA overnight at 4 °C to prevent nonspecific staining. The sample was then incubated with *h*DHEA ST antibody at 4 °C for 72 h, rinsed with washing buffer (containing 0.1% BSA and 0.2% Triton X-100 in PBS, pH 7.4) for 20 min three times, and then stained with biotinylated goat anti-rabbit secondary antibody. After an overnight incubation at 4 °C, the sample was washed with washing buffer for 20 min. Tertiary antibodies (streptavidin-Cy5 and biotinylated HRP) were also incubated with the brain sample overnight at 4 °C. Afterwards, the brain sample was mounted using FocusClear™ and examined by confocal laser scanning microscope photomicrographs. The *Drosophila* carries gene trap Gal4 (12423) and UAS-GFP to expresses GFP in the DPM neurons (green) was used to analyze the colocalization of DHEA-ST like protein and DPM neuron. Steps were same as mentioned above [14].

Results

Characterization of hDHEA ST antibody

The characterization of *h*DHEA ST antibody analyzed by IELISA and immunoblotting is shown in Fig. 1. The

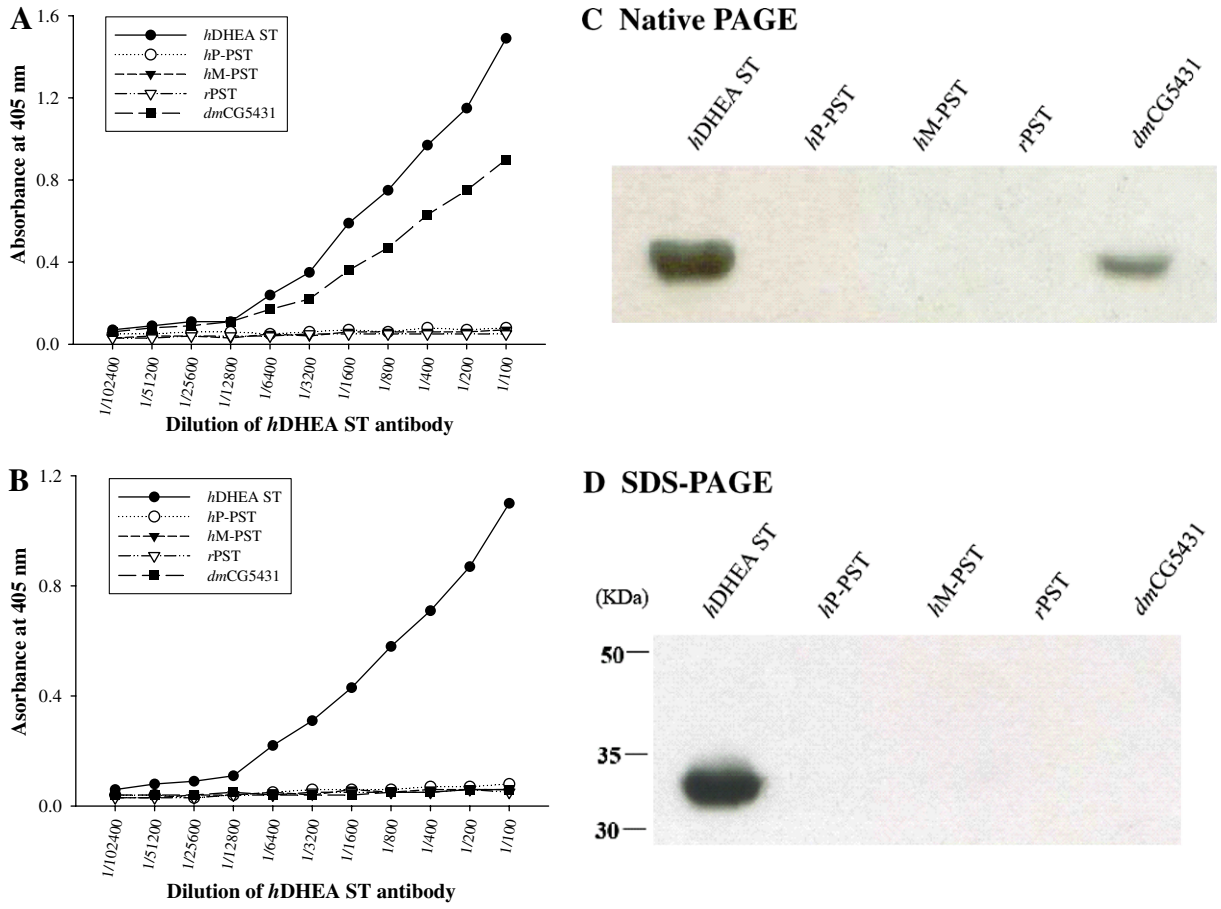

Fig. 1. The characterization of *h*DHEA ST antibody using recombinant STs and putative cytosolic ST of *Drosophila*. (A) The *h*DHEA ST antibody titre determined by IELISA using recombinant proteins in native conformation. (B) The *h*DHEA ST antibody titre determined by IELISA using recombinant proteins denatured by treatment with β-ME and heating. (C) Native immunoblot of the five recombinant proteins with *h*DHEA ST antibody. (D) SDS–PAGE immunoblot of the five recombinant proteins with *h*DHEA ST antibody. Each lane contained approximately 5 μg purified proteins.

*h*DHEA ST antibody showed stronger affinity toward recombinant *h*DHEA ST and *dm*CG5431 in native conformations than the others co-tested (Fig. 1A). The dose–response curves showed that the *h*DHEA ST antibody was specific to *h*DHEA ST when the STs tested were first denatured by treating with β-ME and heating (Fig. 1B). The binding of *h*DHEA ST antibody to PST isoforms was barely discernible. The specificity of antibody for *h*DHEA ST was determined by probing five different types of recombinant STs by immunoblotting (Fig. 1C). The *h*DHEA ST antibody interacted with both *h*DHEA ST and *dm*CG5431 in their native forms, whereas neither *h*PST isoforms nor *r*PST was recognized. Furthermore, only *h*DHEA ST was identified by this specific antibody under the denatured conditions (Fig. 1D).

Expression of DHEA ST-like protein in Drosophila brains

As shown in Fig. 2, the immunoblot of *Drosophila* brain extracts was analyzed by *h*DHEA ST antibody. The DHEA ST-like protein expressed in soluble extracts of *Drosophila* brains was recognized in its native form (Fig. 2A). The SDS–PAGE immunoblot showed that only *h*DHEA ST (positive control) was recognized when the native conformation was disrupted (Fig. 2B).

Localization of DHEA ST-like protein in Drosophila brains

The distribution and relative abundance of DHEA ST-like protein in *Drosophila* brain are shown in Fig. 3. A total of six DHEA ST-like protein positive neurons were observed in the posterior section of *Drosophila* brain (Fig. 3A). Two DHEA ST-like protein positive neurons were also detected in the dorsal part of the brain (Fig. 3B). DHEA ST-like protein positive neuronal fibers, exhibiting the typical appearance of beaded nerve fibers, were seen throughout the entire *Drosophila* brain (Fig. 3C). Table 1 summarizes the relative localization and abundance of DHEA ST-like protein positive neurons and fibers. By using UAS-WGA as a trans-synaptic transmission marker and VAM-Gal4 as a driver, the colocalization of DHEA ST-like protein positive regions and DPM neuron were observed by transgenic fly that carries VAM-Gal4 and UAS-WGA and express WGA in the VAM neurons (Fig. 4).

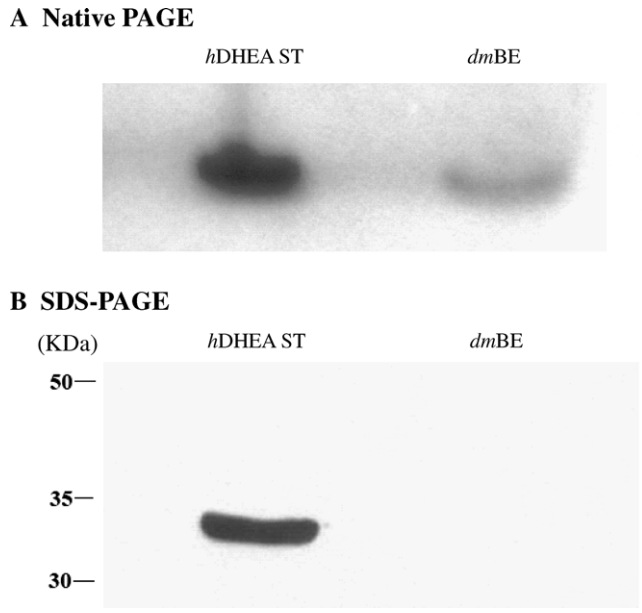

Fig. 2. The expression of DHEA ST-like protein in *Drosophila* brain extracts. (A) The native immunoblot of *Drosophila* brain extracts with *h*DHEA ST antibody. (B) SDS–PAGE immunoblot of *Drosophila* brain extracts and purified *h*DHEA ST with *h*DHEA ST. *dm*BE represented *Drosophila* brain extracts.

Determination of the DHEA-sulfating activity in Drosophila brains

The sulfating activity of DHEA in partially purified *Drosophila* brain extract was assessed using a continuous fluorometric assay (Table 2). The complete system (I) could detect not only sulfating but also desulfating activities due to the presence of MUS. The DHEA-sulfating activity could not be observed in the absence of PAPS, and therefore reaction condition II gave a background activity exhibited mainly by arylsulfatase. As a result, the specific activity and total activity of DHEA-sulfating activities in *Drosophila* brain were determined to be 57.7 ± 12.1 pmol/min/mg and 0.7 ± 0.2 nmol/min/g, respectively. It is to be noted hat a high level of arylsulfatase activity (specific activity and total activity, 319.1 ± 7.5 pmol/min/mg and 6.8 ± 0.1 nmol/min/g, respectively) in the fraction was detected as well.

Discussion

To date 23 structures of cytosolic STs have been solved on eleven different isoforms. Crystal structures for the ST1A1 (phenol ST), ST1A3 (catecholamine ST), ST1E1 (estrogen ST), ST1B1 (thyronine ST), ST2A1 (DHEA ST), two isoenzymes of ST2B1 (pregnenolone ST and cholesterol ST), ST4A1 (neuronal ST), and three subfamilies of ST1C have been characterized. Structure-based sequence alignments indicate that the PAPS binding site, and structural fold, is highly conversed, albeit the homology of the amino acid sequences between different ST isoforms is not high [15]. On the basis of the characteristic pattern of

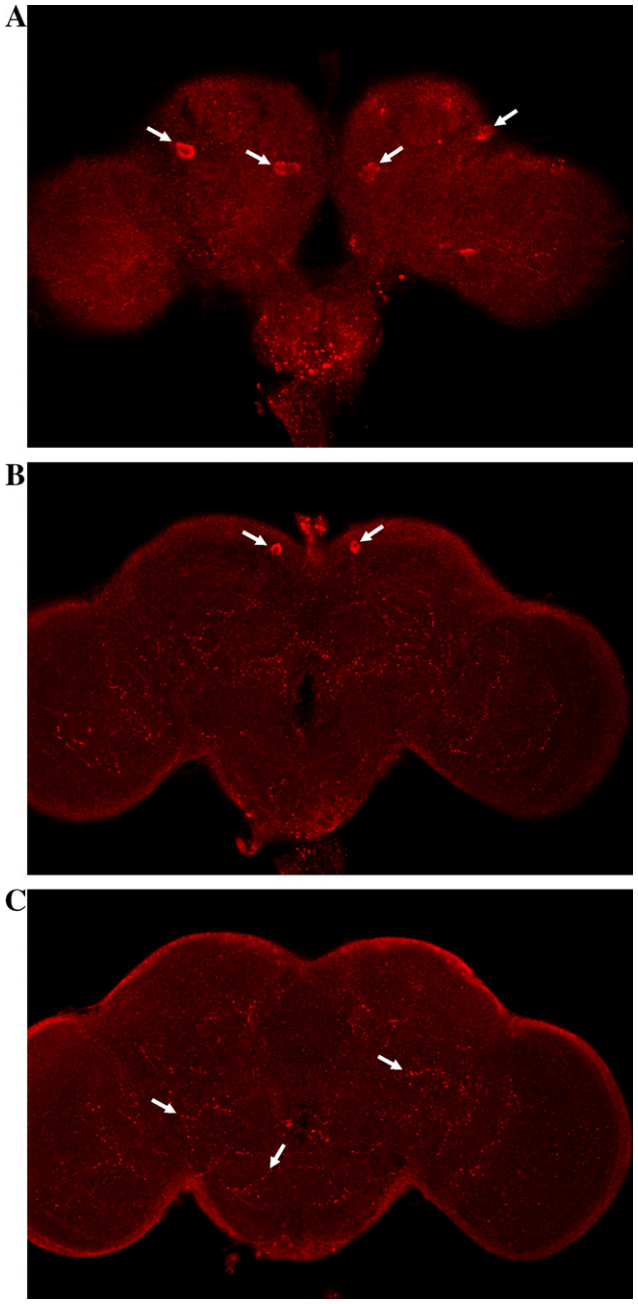

Fig. 3. Distribution of the DHEA ST-like protein in *Drosophila* brain as illustrated by confocal laser scanning microscope. (A) DHEA ST-like protein selectively expressed in posterior region of brain. (B) DHEA ST-like protein expressed in dorsal part of the brain. (C) Expression of DHEA ST-like protein in neural fibers throughout the entire *Drosophila* brain. Granular staining suggests fibers labeled. 200× magnification.

the STs, we demonstrated the possible presence of a cytosolic ST-like protein in *Drosophila* neural circuits by the specific recognition of the *h*DHEA ST antibody. The results obtained from immunoblot analysis and IELISA are close agreement with conserved nature of STs and indicate that STs may exert similar biological functions in various animals.

Drosophila is an excellent experimental model to systematically study the neuroregulative mechanisms in human

Table 1
Distribution and cellular colocalization of DHEA ST-like protein in *Drosophila* brain[a]

Brain region	Specific areas[b]	Neural cytoplasm[c]	Fibers[c]
Anterior brain			
	MB's vertical lobe	–	–
	Superior medial PR	–	–
	Median bundle	–	–
	MB satellite neuropil	–	–
	Anterior optic tubercle	–	+
	Ventrolateral PR	–	+
	MB's medial lobe	–	–
	Antennal lobe	–	–
	Antennal nerve	–	–
	Ventral body	–	–
Middle brain			
	Pedunculus	–	+
	Fan-shaped body	–	+
	Superior lateral PR	–	++
	Ventral body	–	++
	Superior medial PR	–	+
	Tritocerebrum	–	++
	Superior arch	–	+
	Antennoglomerular tract	–	++
	Ellipsoid body	–	+
	Nodulus	–	++
	Inferior lateral deutocerebrum	–	+
	Lo	–	++
	Medulla	–	+
Posterior brain			
	Lo	–	–
	LoP	–	++
	MB calyx	–	–
	PR bridge	+++	+
	Ocellar nerve bundle	–	–
	Inner antennocerebral tract	–	+
	Lateral horn	+++	–
	Posterior lateral fascicle	–	++
	SOG	–	+
	Axons of vertical cells of the LoP	–	++
	Axons of horizontal cells of the LoP	–	+
	SOG nerves	–	–

[a] The distribution of DHEA ST-like protein in *Drosophila* brain was investigated by the continuous sections of the confocal laser scanning microscope photomicrographs. Detailed procedures were mentioned in Materials and methods [14].

[b] Abbreviations: MB, mushroom body; PR, protocerebrum; Lo, lobula; LoP, lobula plate; SOG, subesophaeal ganglis; SOG nerves, roots of nerves from the fused subesophageal ganliea.

[c] The relative intensity of labeling was ranked by two independent observers. Ratings reflect mainly the density of DHEA ST-like protein labeled cell and fibers. Negative, –; weak, +; moderate, ++; strong, +++.

CNS. Many scientists have placed much effort into the molecular characterization and physical relevance of STs in this tiny creature, however, the biological significance of STs in *Drosophila* remains obscure. In general, sulfate conjugation is apparently involved in the metabolism of juvenile hormones and ecdysteroids in insects [16]. In *Prodenia eridania*, sulfate conjugation of ecdysteroids seems to play a critical role in embryonic development and puparium formation [17]. In the present study, the protein partially purified from *Drosophila* brain extracts was demonstrated to be capable of transferring a sulfuryl group from a sulfate donor, PAPS, to an analog of ecdysteroid, DHEA, and the result was consistent with that reported for *Mosquito*, *Aedes togoi* [18] suggested that sulfation in various insect species may exhibit similar biological functions in the metabolism of free hormones and post-stage embryogenesis. Earlier studies had also revealed the existence of cytosolic STs in flies of *Diptera*, *P. eridania* [19]. In addition to its activity on *p*-nitrophenol, the ST prepared from *P. eridania* gut was significantly active in the sulfation of the steroids, such as DHEA, oestrone, and insect moulting hormones α-ecdysone and 22,25-bisdeoxyecdysone. It appears logical to suggest that STs comprehensively regulate the biochemical transformations for the purpose of detoxication, others may have an important physiological implications in insects. Besides, it is noted that DHEA-sulfating activity of in *Drosophila* was significantly lower than that in rat and human [20,21]. This is to be expected because DHEA is a more common substrate for DHEA ST in mammalians than in insects. Furthermore, the validity of such comparison is always open to some question in consideration of differences in enzyme preparation and enzymatic assay. Nevertheless, it is noteworthy that the significant level of arylsulfatase activity was determined (Table 2). In agreement with these previous findings, the low or undetectable DHEA ST activities may be due to high levels of steroid sulfatase in soluble extracts, thereby interfering with the determination of DHEA ST *in vitro* [22].

Several neural regions of *Drosophila* brain were found to have DHEA ST-like immunoreactivity in this study. The immunoreactivity was selectively localized in the neurons of posterior and dorsal part of *Drosophila* brain, and nerve fibers indicating the relevant molecular and neuronal mechanism between this enzyme and its metabolites. In *Drosophila*, there are several types of projection neuron that forward information out of the antennal lobes. Medial and outer antennocerebral tracts (mACT and oACT, respectively) protrude into the ill-defined region and the lateral horn, while the others carry information along the inner antennocerebral tracts (iACT) to the mushroom body (MB). In our study, we observed an abundance of *Drosophila* DHEA ST-like protein expressed in the lateral horn and iACT (Table 1). The results implied that the DHEA ST-like protein may act as a neuromodulator of the ecdysteroids, which are involved in memory formation in *Drosophila*. The finding is also consistent with the result reported by Johnson et al [23]. The sulfate-conjugated steroid is essential to the process of memory retention and significantly enhanced the cognition and learning in rats. Basically, they act through γ-aminobutyric $acid_A$ ($GABA_A$) receptors, *N*-methyl-D-aspartate (NMDA)-type glutamatergic receptors and sigma receptors to induce excitatory cellular actions or inhibit cellular properties.

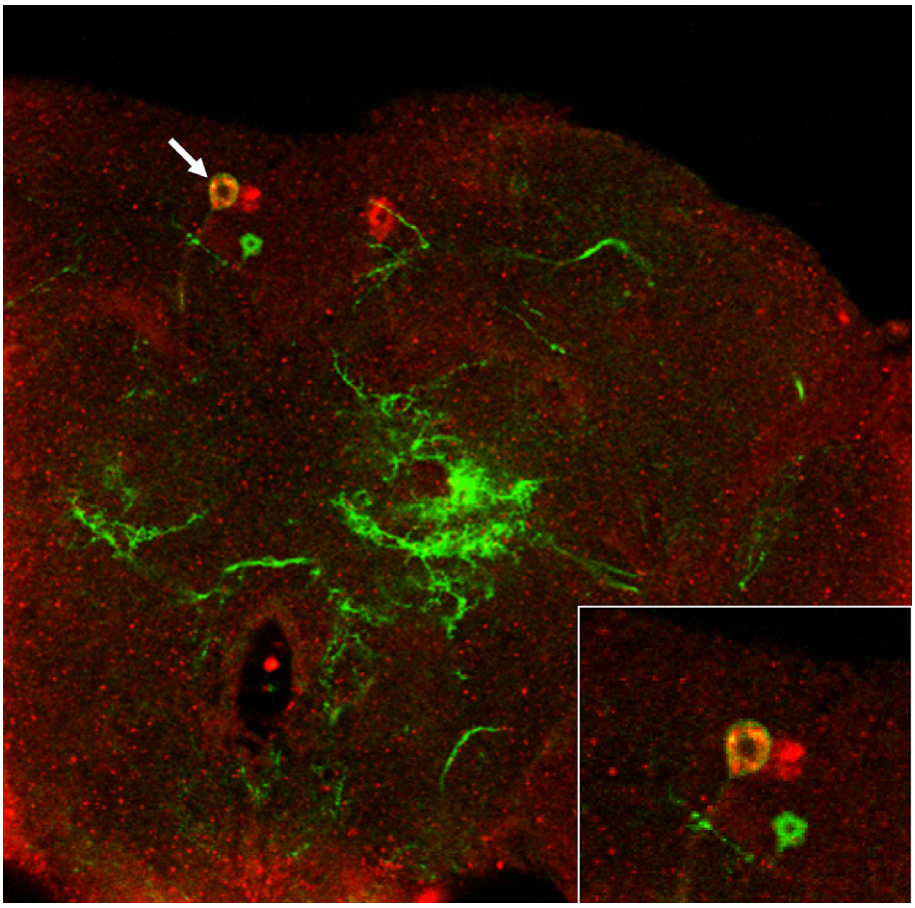

Fig. 4. Colocalization of the DHEA ST-like protein expression and DPM neuron in *Drosophila* brain. The arrowhead indicates the colocalization (orange) of DHEA ST-like protein (red) and DPM neuron (green) (400× magnification). *Right-lower*: the magnification of the colocalized region of DHEA ST-like protein and DPM neuron. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Sulfation and sulfatase activity in *Drosophila* brain[a]

Fraction			
Reaction conditions	Enzyme activity involved	Specific activity (pmol/min/mg)	Total activity (nmol/min/g)
I. Complete[b]	DHEA-sulfating protein + arylsulfatase	376.8 ± 9.5	7.5 ± 0.2
II. PAPS[c]	Arylsulfatase	319.1 ± 7.5	6.8 ± 0.1
I–II	DHEA-sulfating protein	57.7 ± 12.1	0.7 ± 0.2

[a] For use in the assay, a partially purified fraction which cross-react with antibody against *h*DHEA ST, was isolated from *Drosophila* brain homogenates by using native gel electrophoresis.

[b] Detailed procedures were described under *Coupled-enzyme assay for alcohol sulfotransferase (AST)* in Materials and method [13]. Specific activity referred to MU produced following the addition of extract whose protein concentration was determined by absorption at A_{280}. Total activity referred to MU produced with one gram of *Drosophila* brain extracts.

[c] AST activity was eliminated in the absence of PAPS [13].

Increasing evidences have suggested that DPM neuron may co-release *amnesiac* neuropeptide and acetylcholine in the *amnesiac* mutant flies [24]. Transgenic expression of the *amnesiac* gene in the DPM neurons rescues the *amnesiac* memory phenotype, establishing a possible route between DPM neuron function and *amnesiac*-dependent memory. Additionally, the paired conditioning of unconditioned stimulus (US) (electric shock) and conditioned stimulus (CS) (odor stimulus) increases odor-evoked calcium signals and synaptic release from DPM neurons [25]. These observations indicated that DPM neurons not only respond to the US pathway, but that they are also ''odor generalists'', responding to all odors that were tested. The colocalization of DHEA ST-like protein and DPM neuron implied that DHEA ST-like protein may not merely play the role as a neuroregulator in the process of odor-specific memory trace in *Drosophila* but also involve in the modulation of specific memorial and behavioral formations.

Acknowledgments

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