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Co-localization of Histamine and eGFP in the Central Nervous System from pHdc-5'-UTR-eGFP Transformants of *Drosophila melanogaster*

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Abstract

Histamine is a biogenic amine synthesized by the enzyme histidine decarboxylase (Hdc) and used as a neurotransmitter in the central nervous system of Drosophila melanogaster. *We are interested in understanding how tissue-specific expression of Hdc is controlled by examining the function of both the Hdc 5'-UTR (***U**n**t**ranslated **r**egion*) and 3'-UTR through the regulation of the expression of the reporter gene, eGFP. Initial studies reported here examine the function of the 5'-UTR of the Hdc gene by determining whether eGFP expression can be demonstrated in histaminergic cells of transformant flies containing the pHdc-5'-UTR-eGFP transgene. To determine if all cells expressing eGFP in these transformants are also histaminergic, examination of histamine-stained CNS preparations from various developmental stages of transformant flies was conducted and analyzed using fluorescence microscopy. Results indicate that many histaminergic cells appear to express eGFP as well. These results indicate that the 5'-UTR region of Hdc can induce expression of eGFP in centrally located histamine-containing neurons. Differences in the level of expression of eGFP observed between cell types and developmental stages suggest that the 3'-UTR of Hdc may still be required for complete expression. Since the co-localization of eGFP and histamine in cells has been achieved, new areas of research may now be conducted to investigate the function of histaminergic cells in culture, leading to a better understanding of the role that histamine cells play in the central nervous system.*

Introduction

Histamine has been shown to be an important neurotransmitter, a chemical that transmits signals between neurons, for photoreceptor cells and other sensory cells in *Drosophila melanogaster* (Sarthy, 1991; Melzig et al.,1996). The enzyme that catalyzes the decarboxylation of histamine, forming histamine in the central nervous system of *Drosophila melanogaster*, is histidine decarboxylase, with mutations in the Hdc gene having also been identified (Hdc; Burg et al., 1993). An earlier study indicated that a 9.4 kb genomic DNA fragment containing the Hdc gene could be used to restore Hdc function in mutant flies lacking Hdc function (Burg and Pak, 1995). Further studies also identified regions necessary for expression of Hdc in the central brain complex (Burg and Pak, 1995). More recently, the gene encoding the enhanced green fluorescent protein (eGFP) was inserted at the 5' end of the Hdc gene in *Drosophila melanogaster* using the pGreenPelican vector (Anderson and Burg, 2007) and transformed into flies, with one pHdc-5'-UTR-eGFP transgene being located on chromosome 2 and another on chromosome 3 (Miller and Burg, 2008). Transformants carrying the pHdc-5'-UTR-eGFP can therefore be used to determine whether the 5'-UTR region is sufficient for normal Hdc expression.

In addition to identifying cells that contain histamine, this type of labeling of histaminergic neurons may lead to a more thorough study of the regulation of Hdc itself. For example, not much is known concerning how the Hdc protein is regulated within a cell. As in other vertebrate HDC proteins, regions known as "PEST" regions have also been identified in both the "N"- and "C-" terminal regions of the Drosophila HDC protein. These regions may mediate cleavage of the HDC protein in Drosophila, as has been identified to occur in other species during the maturation of the HDC protein (Fleming and Wang, 2000). Due to this type of maturation, it has been difficult to purify or tag the protein with various epitopes, such as green fluorescent protein (GFP). Without the ability to purify or tag HDC, identifica-

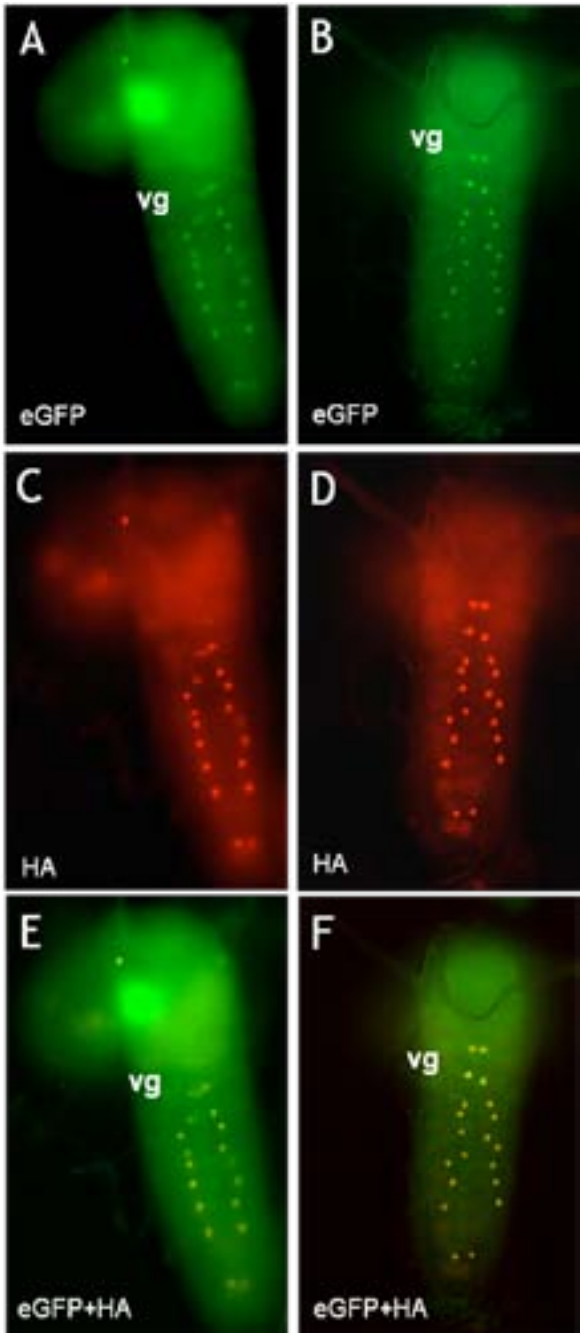

Figure 1: Whole-mount 2nd instar larval brains demonstrating eGFP expression induced by the pHdc-5'UTR-eGFP transgene detected using an FITC filter (A,B; eGFP). Histamine immunoreactivity is shown in panels C,D (HA). E,F: merged images of A+C and B+D, demonstrating co-localization of eGFP and histamine (note yellow cells; eGFP+HA). Histamine staining and the eGFP flourescence appear to co-localize, indicating that the pHdc-5'UTR-eGFP transgene induces expression of eGFP in histaminergic cells. Specimens in these images are from the pHdc-5'UTR-eGFP transformant with insert on chromosome 2.

tion of living cells containing HDC activity remains elusive, and thus, the pHdc-5'-UTR-eGFP transgene may serve as such a marker for living histaminergic neurons. Thus, the co-localization of the transmitter histamine in the same cells expressing eGFP in the central nervous systems from pHdc-5'-UTR-eGFP transformants is shown in this paper, which should now allow the identification and study of living histaminergic neurons.

Identification of living cells containing Hdc activity, and consequently histamine, will allow the study of the differentiation and development of the histaminergic cells *in vivo*. It will also allow the identification of these cells for future physiological and other biochemical analysis, increasing our knowledge of the action of histamine in the central nervous system.

Methods and Materials

Histamine Immunocytochemistry: The central nervous systems from various developmental stages of pHdc-5'-UTR-eGFP transformant flies were dissected in a 2% EDAC fixative (Sigma Chemical Co., St. Louis, Mo) in a phosphate buffered saline, pH 7.2. The resultant tissue was then washed with *Drosophila* Ringer's solution twice for 10 minutes each, after which the tissue was incubated in 5% normal goat serum (NGS; Invitrogen, Inc.) for one hour. The tissue was then incubated overnight in a rabbit histamine polyclonal antibody (Immunostar Inc., Stillwater, MN) diluted 1:1000 in PBS containing 1% NGS, at 4°C. After the overnight incubation, the tissue was washed with TBS + 1% NGS + 0.3% Triton X-100 twice for 20 minutes each and then incubated in an Alexa-fluor 555 goat anti-rabbit antibody diluted 1:1000 (Invitrogen, Inc.) for 30-60 minutes. The tissue was then washed in TBS + 1% NGS + 0.3% Triton X-100 twice for 10 minutes each time and then washed in PBS and placed on microscope slides for microscopic analysis (protocol adapted from Pollack and Hofbauer, 1991).

Fluorescence Photomicrography: For bright-field microscopy, specimens were examined using an Olympus AX70 microscope using DIC optics, and images were captured using a high-resolution digital camera. To co-localize histamine and eGFP using epifluorescence microscopy, an Alexa-fluor 555 goat anti-rabbit antibody (detecting histamine)

was visualized using a rhodamine excitation filter set. eGFP was detected in whole mount tissue using epifluorescence microscopy using a FITC excitation filter set. Images were collected and digitally merged using Adobe Photoshop, adjusting only brightness and contrast.

Results

Specimens of *Drosophila melanogaster* from both the larval and adult stages that contain the pHdc-5'-UTR-eGFP transgene were dissected, fixed, and stained with a rabbit histamine antibody diluted 1:1000 and then incubated with a goat-anti-rabbit secondary antibody labeled with the Alexa-fluor 555 dye diluted 1:1000. After these incubations, each specimen was placed on a microscope slide, viewed and imaged using a fluorescent microscope equipped with a high resolution digital camera. Figures 1-3 show images with each column representing the image of the same specimen at the same focal plane using different excitation filters. The first row of images was generated using an FITC excitation filter, which allows excitation of eGFP, normally providing a green fluorescence and indicating the location of eGFP. The second row of images was generated using a rhodamine excitation filter, which in this case shows the location of histamine. The third row of images results from the digital merging of the first row of images with the second row of images.

The images in Figure 1 contain the results from different 2nd instar stage larval brains, clearly demonstrating that all cells that contain eGFP (Fig. 1A, B) were also positive for histamine (Fig. 1C, D); see also merged images in Fig. 1E, F. While there were differences in the intensity of the eGFP staining between preparations, co-localization of eGFP and histamine was consistently demonstrated. Next, a 3rd instar larval brain was examined for both eGFP and histamine, demonstrating results from different focal planes, one more ventral (Fig. 2 A, C, E) and the other more dorsal (Fig. 2B, D, F). Both sets of images support the previous observation in 2nd instar larval brains that eGFP expressing cells also contain histamine (note the merged images which indicate only "yellow" colored cells, indicating the presence of both red and green fluorescence). The histamine staining observed appears to be identical to previously published reports of histamine staining in the brain of the 3rd larval instar brain (Python and Stocker, 2002).

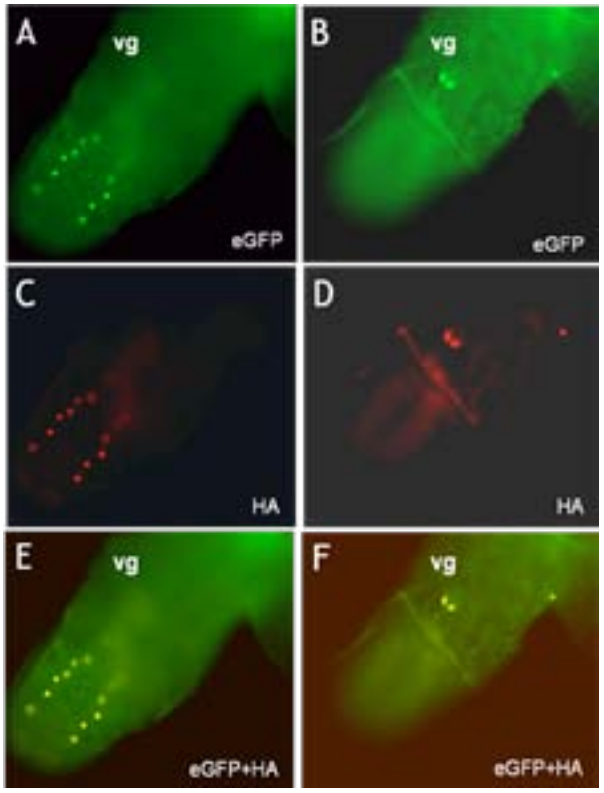

Figure 2: Images from whole-mount 3rd instar larval brains demonstrating eGFP expression induced by the pHdc-5'UTR-eGFP transgene detected using an FITC filter (A,B; eGFP). Histamine immunoreactivity is shown in panels C and D (HA). E: merged image of A and C; F: merged image of B and D. Both E and F demonstrate co-localization of eGFP and histamine (note yellow cells; eGFP+HA). A, C, E are the same image using either the FITC (A), rhodamine-cutoff filter set (B), or combined image (E) from a more ventral focal plane as compared to the image in B, D, F, which is a more dorsally oriented focal plane. vg, ventral ganglia; anterior toward top of image.

While larval brains examined previously for histamine appeared as expected (Python and Stocker, 2002), intact adult nervous tissue was also examined for histamine localization, which has not been as well characterized. Figure 3 demonstrates the results from co-localization experiments carried out showing 2 different focal planes taken of the adult thoracic ganglia from pHdc-5'-UTR-eGFP trasnformant flies. While eGFP expression appears to be stronger than in the larvae, the eGFP appears to still co-localize to cells that contain histamine (Figs 3A, C, E as well as 3B, D, F). Thus, results demonstrate that, in all tissues thus far examined, eGFP and histamine co-localize in cells of the central nervous systems from pHdc-5'-UTR-eGFP transformant flies.

Discussion

Histamine, synthesized by Hdc, likely acts as a neurotransmitter in *Drosophila melanogaster*. Currently, the only effective way to view histamine in the central nervous system of *Drosophila melanogaster* requires fixation of the tissue. Results from this study demonstrate that, in the pHdc-5'-UTR-eGFP tran-

formant flies, eGFP can be localized in neurons that also contain histamine, and thus, can serve as a marker for living histaminergic neurons.

The co-localization of eGFP and histamine shows that the 5' UTR region of Hdc is sufficient to drive expression of histamine in most histamine-containing neurons of the central nervous system. This also means that histaminergic neurons can be viewed in live tissue of transformant flies with the eGFP insert, opening many avenues of histamine-related research and potentially enabling the discovery of more pertinent information regarding the function of histamine in the central nervous system.

Future studies will need to be conducted to determine if all histaminergic cells in the central nervous system co-localize with eGFP in the pHdc-5'-UTR-eGFP transformant fly. It is unclear whether the 3' UTR region of the Hdc gene is necessary for increasing the levels of Hdc gene expression. Construction of a transgene containing both the 5'-UTR and the 3'-UTR controlling expression of eGFP, and comparison of eGFP expression to the pHdc-5'-UTR-eGFP transgene, may demonstrate what function the 3'-UTR has with respect to cell-specific expression or level of expression.

Whether copy number of the pHdc-5'-UTR-eGFP transgene can improve eGFP detection has yet to be clearly determined. Transposition of the pHdc-5'-UTR-eGFP transgene to other locations in the genome is necessary before this type of analysis can continue. Currently, attempts to transpose the pHdc-5'-UTR-eGFP transgene to another chromosome are being initiated.

There were also differences in the signal intensity for eGFP when directly compared to the levels of signal observed in histamine detection. To improve the signal of eGFP in fixed tissue, a monoclonal antibody against eGFP will be used, which is planned to be done in future work. Once carried out, this enhanced detection for eGFP should clearly demonstrate whether all histamine-containing neurons express eGFP in the pHdc-5'-UTR-eGFP transformants at various developmental stages.

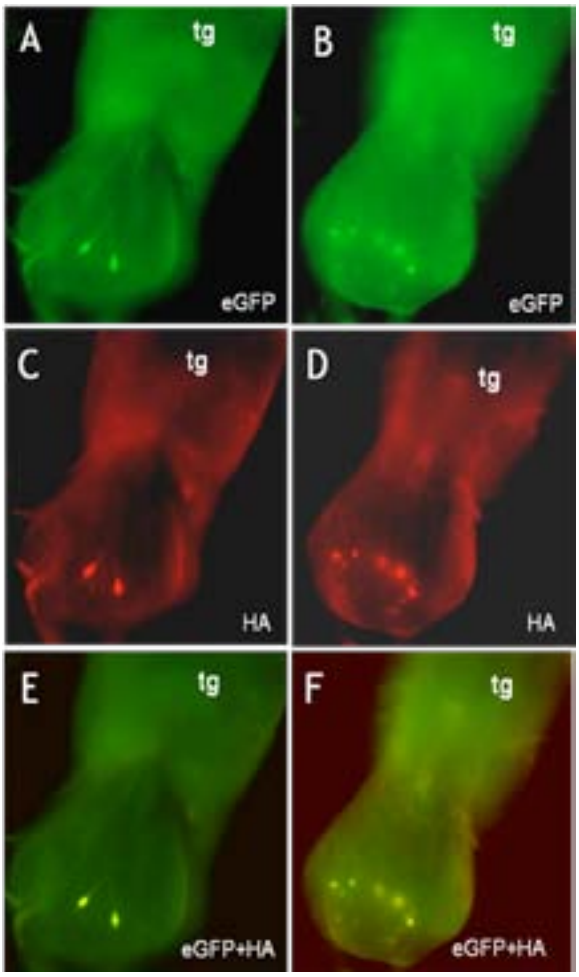

Figure 3: Images from whole-mount adult thoracic and abdominal CNS demonstrating eGFP expression induced by the pHdc-5'UTR-eGFP transgene in adult tissue, detected using an FITC filter (A, B). Histamine immunoreactivity is shown in C and D. E: merged image of A and C; F: merged image of B and D. A, C, E are the same image using either the FITC (A), rhodamine-cutoff filter set (B), or combined image (E) from a more ventral focal plane as compared to the image in B, D, and F. Histamine staining and the eGFP fluorescence appear to co-localize, indicating that the pHdc-5'UTR transgene induces expression of eGFP in histaminergic cells. While all histaminergic cells in the thoracic and abdominal nervous system appear to be expressing eGFP, the neurons in the central brain do not appear to be as easily detected (data not shown). This suggests that while the majority of the pHdc-driven expression is present, it may not be completely expressed in all histamine-containing cells. tg, fused thoracic ganglia; anterior toward top of image.

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