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Quantification of Histamine and Carcinine in *Drosophila melanogaster* Tissues

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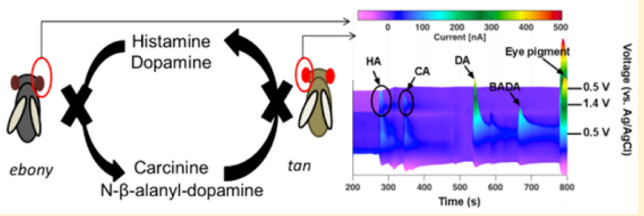
ABSTRACT: Histamine is a neurotransmitter crucial to the visual processing of *Drosophila melanogaster*. It is inactivated by metabolism to carcinine, a *β*-alanyl derivative, and the same enzyme that controls that process also converts dopamine to *N*-*β*-alanyl-dopamine. Direct detection of histamine and carcinine has not been reported in single *Drosophila* brains. Here, we quantify histamine, carcinine, dopamine, and *N*-*β*-alanyl-dopamine in *Drosophila* tissues by capillary electrophoresis coupled to fast-scan cyclic voltammetry (CE-FSCV). Limits of detection were low, 4 ± 1 pg for histamine, 10 ± 4 pg for carcinine, 2.8 ± 0.3 pg for dopamine, and 9 ± 3 pg for *N*-*β*-alanyl-dopamine. Tissue content was compared in the brain, eyes, and cuticle from wild-type (Canton S) and mutant (*tan*[3] and *ebony*[1]) strains. In *tan*[3] mutants, the enzyme that produces histamine from carcinine is nonfunctional, whereas in *ebony*[1] mutants, the enzyme that produces carcinine from histamine is nonfunctional. In all fly strains, the neurotransmitter content was highest in the eyes and there were no strain differences for tissue content in the cuticle. The main finding was that carcinine levels changed significantly in the mutant flies, whereas histamine levels did not. In particular, *tan*[3] flies had significantly higher carcinine levels in the eyes and brain than Canton S or *ebony*[1] flies. *N*-*β*-Alanyl-dopamine was detected in *tan*[3] mutants but not in other strains. These results show the utility of CE-FSCV for sensitive detection of histamine and carcinine, which allows a better understanding of their content and metabolism in different types of tissues to be obtained.



KEYWORDS: *D. melanogaster, histamine, carcinine, dopamine, capillary electrophoresis, fast-scan cyclic voltammetry*

Histamine is an important neurotransmitter in both *Drosophila* and humans. In mammals and flies, it functions to promote wakefulness and circadian rhythm regulation.[1,2] Histamine, and not glutamate, is the major photoreceptor transmitter in *Drosophila*.[3,4] Histamine is synthesized from histidine by histidine decarboxylase (hdc). When this enzyme is nonfunctional (*hdc*), flies are blind and behaviorally abnormal,[5] demonstrating the necessity of histamine for normal functioning of the visual system. In the brain and visual system of *Drosophila*, tight control of histamine content is necessary for the visual transduction process, and part of this control occurs through the metabolism of histamine to carcinine, which is "inactive".[6] Glial cells typically uptake histamine and then convert it to carcinine, which is *β*-alanyl histamine.[7,8] The enzyme for this conversion, *N*-*β*-alanyl-dopamine synthase (also called Ebony), also converts dopamine, and several other biogenic amines, into a *β*-alanyl derivative.[9] Conversion of dopamine to *N*-*β*-alanyl dopamine is important for cuticle scleritization and pigmentation; mutants deficient in *N*-*β*-alanyl-dopamine synthase appear dark (*ebony*) and also exhibit an altered visual processing phenotype.[10] Conversion of carcinine back to histamine is also important for regulating the amount of histamine available for neurotransmission, as the rate of histamine synthesis is slow. The enzyme *β*-alanyl-dopamine hydrolase (also called Tan) converts carcinine to histamine and *N*-*β*-alanyl dopamine to dopamine. Deficiency in *β*-alanyl-dopamine hydrolase also produces a phenotype that is visible in the cuticle (a light tan color) in addition to visual defects.[11] Furthermore, these mutations also influence the behavior of the flies,[12] causing abnormal locomotor activity rhythms. We hypothesize that *tan* and *ebony* mutants will have altered content of histamine, carcinine, and *N*-*β*-alanyl-dopamine, but levels of these neurotransmitters have not been explored in different tissues because of the lack of a good analytical technique.

Tissue content of histamine in *Drosophila* has typically been measured using high-performance liquid chromatography (HPLC). Borycz and co-workers reported tissue content determination from pooled whole heads using HPLC coupled to electrochemical detection (amperometry) in samples treated with *o*-phthaldialdehyde and mercaptoethanol to increase sensitivity; histamine is also natively electroactive and does not require treatment to be detected using electrochemical detectors.[13−15] However, they reported difficulty detecting carcinine using this method, and, later, carcinine quantification was performed by titrating [^{3}H]histamine.[16] These papers used samples of pooled whole heads,[11] likely due to the low concentrations of histamine present in the fly samples. Given the presence of histamine and carcinine in the eye and the

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brain, and its possible presence in cuticle (along with dopamine and *N*-β-alanyl-dopamine), quantitation in specific tissues is necessary to better understand the localization of these neurotransmitters and their metabolites in *Drosophila*. We have demonstrated the utility of capillary electrophoresis coupled to fast-scan cyclic voltammetry (CE-FSCV) for the separation and quantification of the monoamines dopamine, serotonin, octopamine, and tyramine from single ventral nerve cords from larvae[17] and brains from adult *Drosophila*.[18] Capillary electrophoresis with field-amplified sample stacking provides high sensitivity and uses small sample volumes, reducing the need for large pooled samples.

In this study, we optimized parameters for CE-FSCV to separate and quantify histamine, carcinine, dopamine, and *N*-β-alanyl-dopamine in tissue from *Drosophila* melanogaster. With this technique, we can measure neurotransmitters separately from the brain, eyes, or cuticle of a single fly. The content of all three tissue types was analyzed in a wild-type fly, Canton S, and in two mutants, *tan*[3] and *ebony*[1], who are expected to have altered tissue content of histamine and carcinine. Eyes contained the highest levels of histamine and carcinine, followed by the cuticle and then the brain. Both of the mutants showed differences in tissue content of carcinine in the brain and eyes but not histamine. Ratios of histamine to carcinine were significantly altered in the brain and in the eyes in both the *ebony*[1] and *tan*[3] mutants. *N*-β-Alanyl-dopamine was detected only in the cuticle and eyes of *tan*[3]. Thus, Ebony and Tan are important for the metabolic production and inactivation of histamine, and mutating their function produces different ratios of histamine to carcinine. In addition, Tan is important for the metabolism of dopamine outside of the brain.

RESULTS AND DISCUSSION

Capillary Electrophoresis with Fast-Scan Cyclic Voltammetry for Histamine and Carcinine Detection. CE-FSCV can be used to analyze single brains from *Drosophila melanogaster*.[17,18] Many neurotransmitters, including histamine, and their metabolites are electroactive and are detected by FSCV using carbon-fiber microelectrodes.[14,15,19] In this study, we expanded the scope of CE-FSCV to include three new analytes: histamine (HA), carcinine (CA), and *N*-β-alanyl-dopamine (BADA). Histamine and carcinine were chosen because of their importance in the visual process of *Drosophila*,[3,6] and *N*-β-alanyl-dopamine was analyzed because it shares a synthesis pathway with carcinine.[11,20] These biogenic amines are found in a variety of tissues, so brains, eyes, and cuticle were studied. In order to detect both histamine and carcinine, the electrode was scanned from −0.4 to 1.4 V at a rate of 400 V/s (10 Hz repetition rate); this waveform provides high sensitivity for a wide variety of analytes,[18] and the higher switching potential is required for sensitive detection of both histamine and carcinine.[21,22] Using this waveform, limits of detection for the analytes in 10 μL of 0.5 mM perchloric acid were 4 ± 1 pg for histamine, 10 ± 4 pg for carcinine, 2.8 ± 0.3 pg for dopamine, and 9 ± 3 pg for *N*-β-alanyl-dopamine.

The separation buffer used for histamine, carcinine, dopamine, and *N*-β-alanyl-dopamine was modified from the buffer previously used for larval samples.[17] A pH 2, 200 mM phosphate buffer was used. Preliminary experiments showed that this lower pH was important, as migration times for histamine and carcinine were irreproducible at pH 4.0, likely due to wall interactions. At pH < 3.0, wall interactions are greatly reduced as silanol groups are fully protonated and do not react with protonated amines. Fewer silanol groups means that electroosmotic flow is largely suppressed, but our analytes of interest are all protonated and migrate toward the anode. Due to EOF suppression, wall coatings or added buffer modifiers are not needed, thus simplifying the separation.[23] All analytes appeared in 800 s (approximately 13.3 min) and were well-resolved from one another (Figure 1).

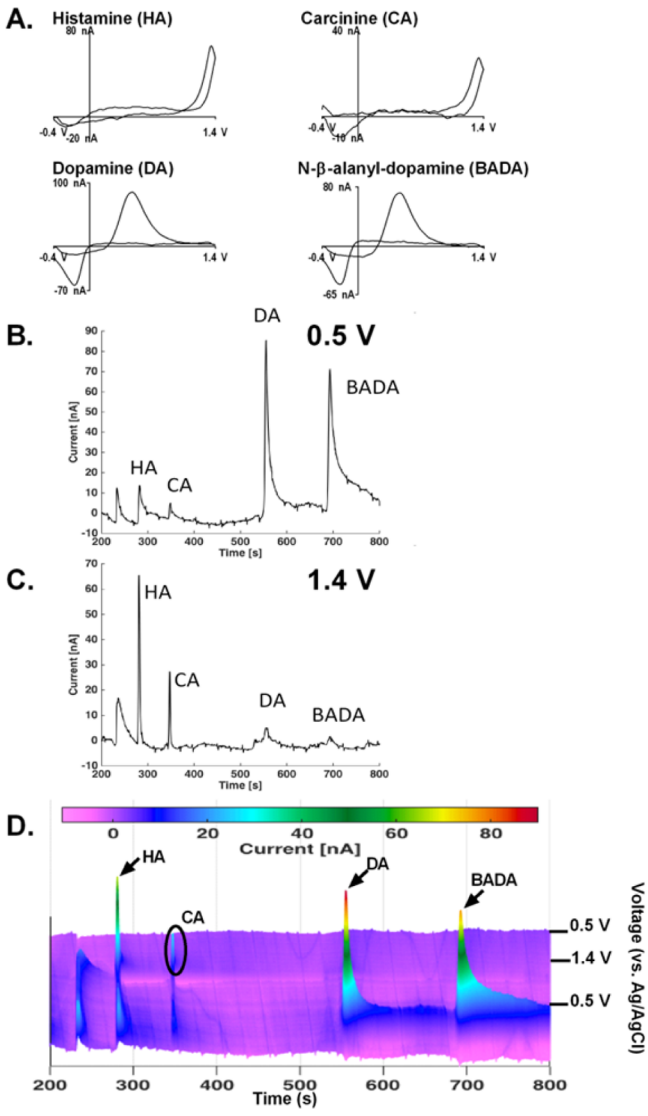

Figure 1. Separations of a standard mixture of neurotransmitters: 100 nM each of histamine (HA), carcinine (CA), dopamine (DA), and *N*-β-alanyl-dopamine (BADA) in 10 μL of 0.5 mM perchloric acid. (A) Cyclic voltammograms of the analytes. (B) Electropherogram at 0.5 V; DA and BADA are most visible at this potential. (C) Electropherogram at 1.4 V for the same separation as above; HA and CA are most visible at this potential. (D) A heat map showing the migrations of HA, CA (circled, in black), DA, and BADA.

With CE-FSCV, characteristic cyclic voltammograms are obtained for electroactive species present in the sample (Figure 1 A); these voltammograms are used to confirm the identity of analytes and for quantification of analytes.[17] Due to the high sampling frequency, a large number of cyclic voltammograms are collected during a separation, and these can be represented in a heat map. The heat map plots applied potential on the *y* axis, time on the *x* axis, and the current as a peak of color,

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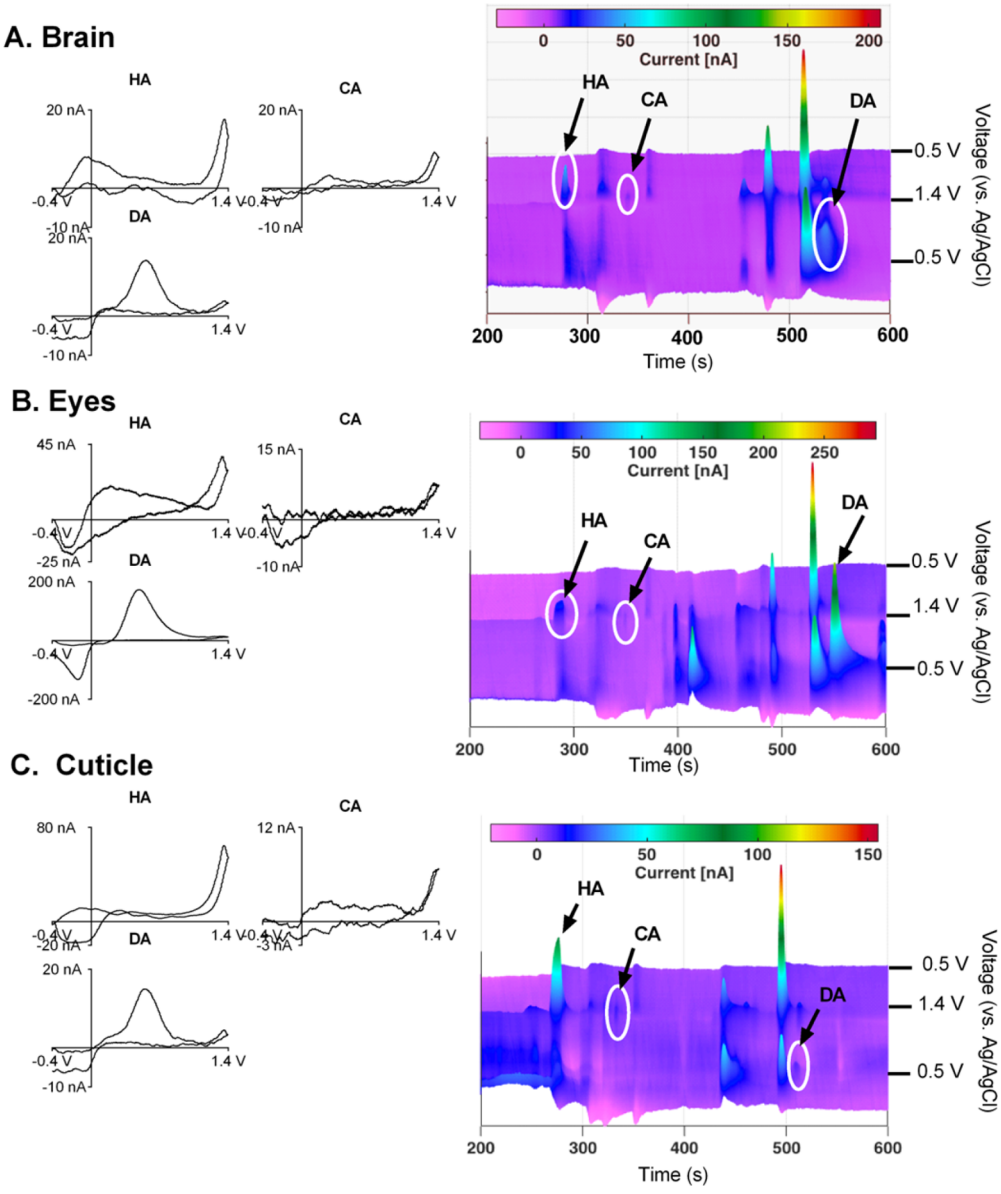

Figure 2. Separations of histamine (HA), carcinine (CA), and dopamine (DA) from (A) brain, (B) eyes, and (C) cuticle of a single Canton S fly. The cyclic voltammograms shown above confirm the identity of the analytes. The heat maps next to them show the separation of these analytes in different tissues. The peaks are circled and labeled to show their position.

which allows for easy visualization of all electroactive species in the separation (Figure 1D). The heat map displays only the oxidation peaks for the sake of clarity; however the reduction peaks are there and can be seen on the cyclic voltammograms (Figure 1A). From this heat map, one can visualize that histamine and carcinine migrate first and have higher oxidation potentials than dopamine and *N*-*β*-alanyl dopamine. In contrast, current vs time traces (Figure 1B,C) show only the current at a given potential, which means that not every analyte is visible on every trace. Histamine and carcinine are visible at higher potentials (1.4 V; Figure 1C), and dopamine and *N*-*β*-alanyl-dopamine are visible at lower potentials (0.5 V; Figure 1B).

Separations of biogenic amines were performed in the three different tissue types taken from a wild-type fly (Canton S). In each case, samples from a single adult fly were analyzed; for eye samples, both eyes from a single fly were combined. Histamine, carcinine, and dopamine were found in all of the tissue types tested (Figure 2). However, no *N*-*β*-alanyl-dopamine was detected in any of the Canton S samples; thus, the amount of *N*-*β*-alanyl-dopamine present in these tissues is below our limit of detection. The heat maps of the brain (Figure 2A), eyes (Figure 2B), and cuticle (Figure 2C) show that these tissues contain different amounts of histamine, carcinine, and dopamine. In the heat map for brain tissue (Figure 2A), histamine and carcinine are clearly visible. While dopamine is harder to see in the heat map because of an unknown electroactive peak, it is separated from the interferent and can be distinguished by looking at the cyclic voltammograms (Figure 2A,C). In the eye (Figure 2B), there is more carcinine than in the brain and more dopamine than in either the cuticle or brain. In the cuticle (Figure 2C), the heat map shows a high amount of histamine and a lesser amount of carcinine and dopamine. While there are other unidentified electroactive compounds in these tissue samples that appear on the heat maps, they do not interfere with detection of our analytes of

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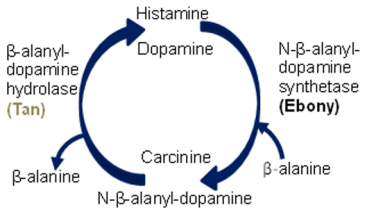
interest (Figure 2A,C). Migration times for these separations are consistent, even though they were obtained on different days.

Quantification of Histamine, Carcinine, and Dopamine in the Brain, Eyes, and Cuticle of *Drosophila*. Using the CE-FSCV method, adult brains, eyes, and cuticle of three strains of *Drosophila* were compared to determine the content of histamine, carcinine, and dopamine. Canton S, a widely studied wild-type strain with normal metabolism of histamine, carcinine, and dopamine, was used as a control. Two strains of flies with nonfunctional enzymes for the synthesis of carcinine (*ebony*[1]) and the metabolism of carcinine (*tan*[3]) (Scheme 1) were selected to study the impact of mutations in enzymes on tissue content of histamine and carcinine. *Ebony*[1] mutants lack *N*-*β*-alanyl-dopamine synthetase (also called Ebony), which is responsible for the metabolism of histamine and dopamine to carcinine and *N*-*β*-alanyl-dopamine, respectively.[6,16] *tan*[3] mutants lack *β*-alanyl-dopamine hydrolase (also called Tan), which metabolizes carcinine and *N*-*β*-alanyl-dopamine to histamine and dopamine, respectively (Scheme 1).[11,16]

Scheme 1. Simplified Schematic of Histamine Metabolism[a]



[a]Histamine and dopamine are metabolized by *N*-*β*-alanyl-dopamine synthetase (Ebony) into their *β*-alanyl derivatives, carcinine and *N*-*β*-alanyl-dopamine. These metabolites can then be returned to histamine and carcinine by *β*-alanyl-dopamine hydrolase (Tan).

Tissue content was first compared in the different tissue types from single flies. Because different tissue samples had different masses, the amount of neurotransmitter was divided by the average weight of the tissue type to give picograms of neurotransmitter per milligram of tissue (Table 1). Overall, the tissue content of neurotransmitters was highest in the eyes, followed by the brain and then the cuticle. For histamine, there was a main effect of tissue type (two-way ANOVA, $p < 0.0001$) but no effect of genotype. Histamine content was significantly higher in the eyes than either the brain or cuticle (Bonferonni post test $p = 0.0009$ and 0.0003, respectively) in Canton S and higher in the eye than the cuticle ($p = 0.0372$) in *ebony*[1]. There were no differences in histamine content among tissue types for *tan*[3] ($p > 0.05$ for all tissues). Carcinine had a much different pattern than histamine, with large differences especially in the *tan*[3] flies. For carcinine, there was a main effect of genotype (two-way ANOVA, $p = 0.0004$) and tissue type ($p = 0.0012$), with a significant interaction between these factors ($p = 0.0006$). In Canton S or *ebony*[1] flies, tissue content of carcinine did not significantly differ in any of the tissue types. However, in *tan*[3] flies, carcinine content in the eyes was significantly higher than that in the brain or cuticle (Bonferoni post-test, $p < 0.0001$ for both). Dopamine also varied by tissue type and genotype (two-way ANOVA, $p = 0.0251$ and 0.0311, respectively), but there was no significant interaction between these variables. There were no significant differences among tissue content of dopamine in any of the Canton S or *ebony*[1] tissues. For *tan*[3], there was significantly more dopamine in the eye than in the cuticle or brain ($p = 0.0019$ and 0.0136, respectively).

Table 1. Tissue Content Per Milligram of Tissue of Histamine, Carcinine, and Dopamine in Brain, Eyes, and Cuticle[a]

Canton S			
tissue type	histamine	carcinine	dopamine
brain ($n = 7$)	1250 ± 400	1650 ± 400	1100 ± 200
eyes ($n = 7$)	12400 ± 3600	5460 ± 1600	4300 ± 2000
cuticle ($n = 7$)	390 ± 150	660 ± 300	83 ± 30
tan[3]			
tissue type	histamine	carcinine	dopamine
brain ($n = 5$)	750 ± 50	9350 ± 2700	4960 ± 1900
eyes ($n = 7$)	7700 ± 4000	58700 ± 9000	23600 ± 1000
cuticle ($n = 5$)	290 ± 100	920 ± 400	690 ± 300
ebony[1]			
tissue type	histamine	carcinine	dopamine
brain ($n = 5$)	1010 ± 200	1150 ± 400	1760 ± 1000
eyes ($n = 8$)	7720 ± 2000	1200 ± 300	1490 ± 400
cuticle ($n = 6$)	230 ± 90	190 ± 90	60 ± 20

[a]All tissue contents are pg neurotransmitter/mg tissue.

Differences in neurotransmitter distribution among tissue types are likely due to differing gene expression levels for the enzymes controlling the synthesis of these neurotransmitters and metabolites. For example, the expression of Ebony is highest in the eye, followed by the brain.[24] Similarly, the expression of Tan is highest in the eye, followed by the brain.[24] In both cases, there is also evidence of expression in the cuticle, due to the phenotypic effect of mutations to either gene. The eyes have the highest content of neurotransmitter for all three neurotransmitters, indicating large pools that are used for visual processing. In contrast, the cuticle has the lowest amounts, which is not surprising since this is not where neuronal communication is taking place.

Another way to compare the data is to look at each tissue type and compare the effects of genotype and type of neurotransmitter. Because the mass would be the same for each sample, we compared picograms of neurotransmitter instead of picograms per milligram (Figure 3). Overall, the main finding is that histamine does not vary much with the different genotypes but that carcinine varies dramatically. In the brain (Figure 3A), there was a significant main effect of genotype (two-way ANOVA, $p = 0.0001$) and neurotransmitter ($p = 0.0048$) and a significant interaction between these variables ($p = 0.0025$). While there was no difference for histamine between any of the genotypes in the brain, carcinine was significantly higher in the *tan*[3] brain than in the Canton S or *ebony*[1] brains (Bonferroni post test, $p < 0.0001$ for both). *tan*[3] also had increased dopamine compared to that in Canton S brains ($p = 0.0333$). In the eyes (Figure 3B), the greatest effect of genotype was again on carcinine content and not histamine content; *tan*[3] flies again showed the largest differences in carcinine content. Overall, there was a significant effect of genotype in the eyes (two-way ANOVA, $p < 0.0001$) but no significant effect of neurotransmitter ($p = 0.0603$). There was also a significant interaction between genotype and neurotransmitter in the eyes (two-way ANOVA, $p = 0.0009$). There were no significant differences in histamine or dopamine content in eyes between the strains of flies, but carcinine

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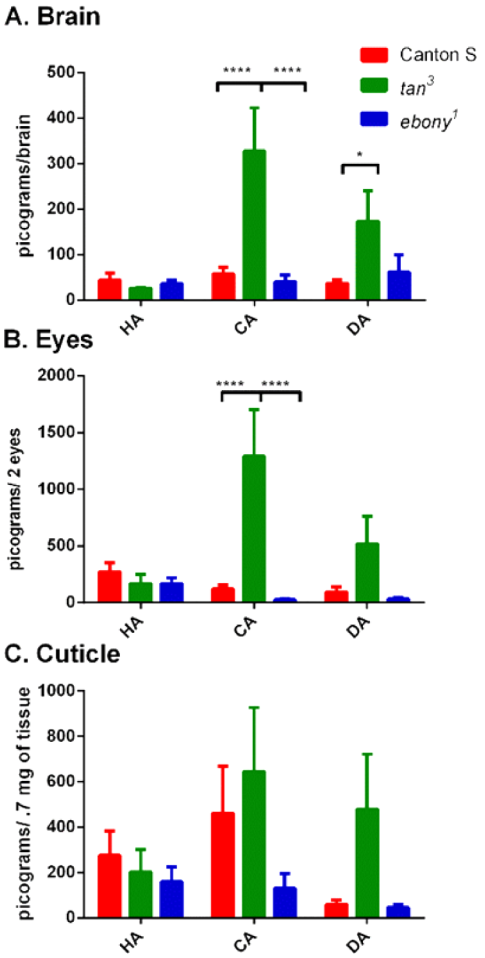

Figure 3. Comparisons of histamine (HA), carcinine (CA), and dopamine (DA) tissue content for all strains: (A) brain (n = 5−7), (B) eyes (n = 7−8), and (C) cuticle (n = 5−7). Two-way ANOVA and Bonferroni post tests were used to compare the tissue content of each neurotransmitter. (A) In brain, there was a significant effect of genotype (p = 0.0025) and neurotransmitter (p = 0.0048) and a significant interaction (two-way ANOVA, p = 0.0025). (B) In eyes, there was a significant effect of genotype (p < 0.0001) and a significant interaction between neurotransmitter and genotype (p = 0.0009). (C) In cuticle, there is a significant effect of genotype (two-way ANOVA, p = 0.0347) but not neurotransmitter. *, p < 0.05; ****, p < 0.0001.

content was significantly higher in *tan*^{3} eyes than in either Canton S or *ebony*^{1} eyes (p < 0.0001 for both). In the cuticle (Figure 3C), there was a significant effect of genotype (two-way ANOVA, p = 0.0347). However, Bonferroni post tests showed that there were no significant differences in any neurotransmitters in any of the tested strains.

Another way of analyzing the differences between the strains of flies is to compare the ratio of histamine to carcinine (HA/CA; Figure 4). This ratio provides information on the balance between histamine and carcinine and how this is altered by mutations in the metabolic cycle such as *tan* and *ebony*. In the brain (Figure 4A), there was a main effect of genotype on the HA/CA ratio (one-way ANOVA, p = 0.0012), and *ebony*^{1} brains had a significantly higher ratio than the *tan*^{3} brains (Bonferroni post test, p = 0.0021). Additionally, the HA/CA ratio in Canton S brains was higher than in *tan*^{3} brains (p = 0.0044). Even though the amount of histamine in the brain was not significantly different, the difference in ratios indicates that there are alterations in the metabolism of histamine relative to

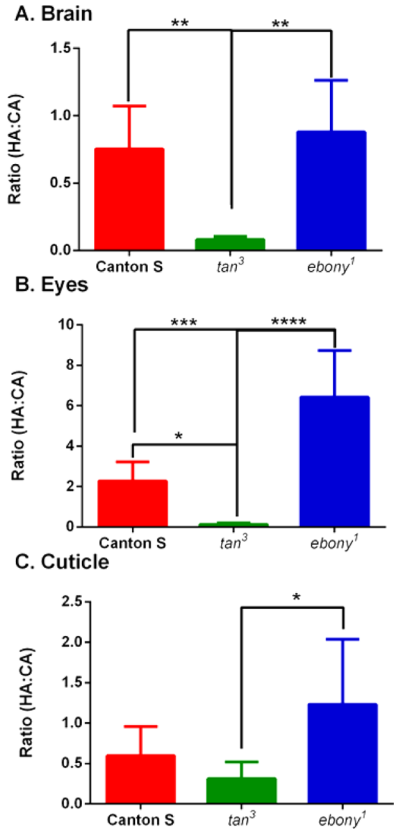

Figure 4. Comparisons of ratios of histamine to carcinine ratios (HA/CA) for (A) brain (n = 5−7), (B) eyes (n = 7−8), and (C) cuticle (n = 5−7). One-way ANOVA with Bonferroni post test was used to compare these ratios. (A) In the brain, there was a significant effect of genotype (p = 0.0012). (B) In the eyes, there was a significant effect of genotype (p < 0.0001). (C) In the cuticle, there was a significant effect of genotype. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

that in the wild-type fly. In the eyes (Figure 4B), there was a main effect of genotype on the HA/CA ratio (one-way ANOVA, p < 0.0001) and the HA/CA ratio was significantly higher in *ebony*^{1} than Canton S or *tan*^{3} eyes (Bonferroni post test, p = 0.0001 and p < 0.0001, respectively) and in Canton S than *tan*^{3} eyes (p = 0.0451). In the cuticle, there was a main effect of genotype on the HA/CA ratio (one-way ANOVA, p = 0.0297), and the HA/CA ratio was only significantly different between *ebony*^{1} and *tan*^{3}, with *ebony*^{1} flies having a higher ratio than *tan*^{3} flies (p = 0.0350). The consistent trend here is that the ratio of HA to CA is lowest in *tan*^{3}, due to deficits of the metabolism of histamine to carcinine, and highest in *ebony*^{1} flies, due to deficits in the synthesis of carcinine from histamine.

While mutations to Ebony and Tan were anticipated to alter the histamine content, our main finding is that the greatest effects these mutations are on the carcinine content and not on histamine content. Previous studies utilized whole heads for histamine and carcinine determination, whereas we analyzed more specific tissue types.[11,13] For example, the whole head histamine content reported in Oregon R (another wild-type strain of *Drosophila*) was 1980 ± 150 pg.[13] As the contribution of the eyes is larger than that of the brain (270 ± 80 pg in the eyes of Canton S vs 44 ± 15 pg in the brain), previous whole head reports likely reflect eye and cuticle values more than they do brain values. The whole head contents of histamine in *tan*^{3} and *ebony*^{1} were 200 and 900 pg/head, respectively.[16] Previous

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studies could not detect carcinine directly but used feedings of [^{3}H]histamine to produce [^{3}H]carcinine, which could be measured. However, carcinine was detected only in *tan*^{3} flies, which is consistent with our findings of elevated carcinine in *tan*^{3} tissue types. Our method allows for direct comparison of histamine and carcinine and reveals that changes in metabolism affect carcinine more than histamine in the eyes, where visual processing is expected to take place.

The HA/CA ratios illustrate that there are differences in the relative amounts of these neurotransmitters that are dependent on genotype; *ebony*^{1} flies have a greater amount of histamine relative to carcinine than *tan*^{3} flies in both their brains and eyes. While there are phenotypical changes associated with deficits in either of *N*-*β*-alanyl-dopamine synthetase (*ebony*, which results in substantially darker cuticle than wild type) or *β*-alanyl-dopamine hydrolase (*tan*, which results in lighter cuticle than wild type), these changes do not appear to significantly change the neurotransmitter content of cuticle. The largest effects of Tan and Ebony on neurotransmitter content are in the brain and eye, which agrees with immunohistochemical staining showing histamine reactivity in the eye and optic lobes.[5] As such, future work on histamine and carcinine in *Drosophila* should focus on the analysis of eyes and brains from other mutants.

Quantification of *N*-*β*-Alanyl-dopamine in *tan*^{3} Flies. *N*-*β*-Alanyl-dopamine (BADA) is a metabolite of dopamine and is synthesized by *N*-*β*-alanyl-dopamine synthetase (Ebony). This metabolite is typically found in the cuticle and eyes of insects, where it is involved in the pigmentation and sclerotization of these tissues.[20,25,26] The primary role of *N*-*β*-alanyl-dopamine in *Drosophila* appears to be in cuticle sclerotization; however, there is evidence that Ebony is expressed in the head and brain of *Drosohila*.[9] To the best of our knowledge, there is no evidence of *N*-*β*-alanyl-dopamine in mammals, and *N*-*β*-alanyl-dopamine is primarily of importance in insects.

N-*β*-Alanyl-dopamine was not detected in most tissue types or strains of *Drosophila* tested; however, it was detected consistently in *tan*^{3} cuticle and eye samples (Figure 5). *N*-*β*-Alanyl-dopamine appears later in the separation than dopamine (Figure 5A,B), around 700 s (or ∼11.5 min), and has a similar cyclic voltammogram to dopamine (Figure 1A), due to similar electrochemistry. *N*-*β*-Alanyl-dopamine is well-separated from dopamine and other interferents in eye samples (Figure 5).

The tissue content (in pg/mg tissue) of *N*-*β*-alanyl-dopamine of the cuticle and eyes for *tan*^{3} was significantly different (Figure 6A) (unpaired *t*-test, $p = 0.0148$). The average content of *N*-*β*-alanyl-dopamine in *tan*^{3} eyes was 56 000 ± 16 000 pg/mg tissue and cuticle was 440 ± 200 pg/mg tissue. The ratio of the precursor to metabolite (DA/BADA) was calculated, and no significant difference was found between the cuticle and eyes (Figure 6B) (unpaired *t*-test, $p = 0.2193$). *N*-*β*-Alanyl-dopamine was not detected in any other strain of fly, indicating that levels are below our limit of detection (9 ± 3 pg). Higher levels of *N*-*β*-alanyl-dopamine are present in *tan*^{3} flies due to the absence of *β*-alanyl-dopamine hydrolase, which allows it to accumulate in tissue. While there is evidence of *N*-*β*-alanyl-dopamine synthetase activity in the head of *Drosophila*,[9] and we observed carcinine in the brain, *N*-*β*-alanyl-dopamine levels may be lower because dopamine may not be available as a substrate in the brain or *N*-*β*-alanyl-dopamine synthetase may be compartmentalized in different cell types than dopamine. The presence of *N*-*β*-alanyl-dopamine in both the cuticle and eye of *tan*^{3} mutants agree well with our histamine and carcinine results, where the amount of metabolite in a tissue increases as *N*-*β*-alanyl-dopamine hydrolase activity is abolished. Overall, *N*-*β*-alanyl-dopamine content of wild-type tissues appears to be extremely low and, as such, may not be an important metabolite of dopamine, other than its role in normal pigmentation of cuticular tissues. Additionally, the extremely low level of *N*-*β*-alanyl-dopamine in the brain of *tan*^{3} flies also indicates that *N*-*β*-alanyl-dopamine is unlikely to be an important product of dopamine metabolism in brain tissue.

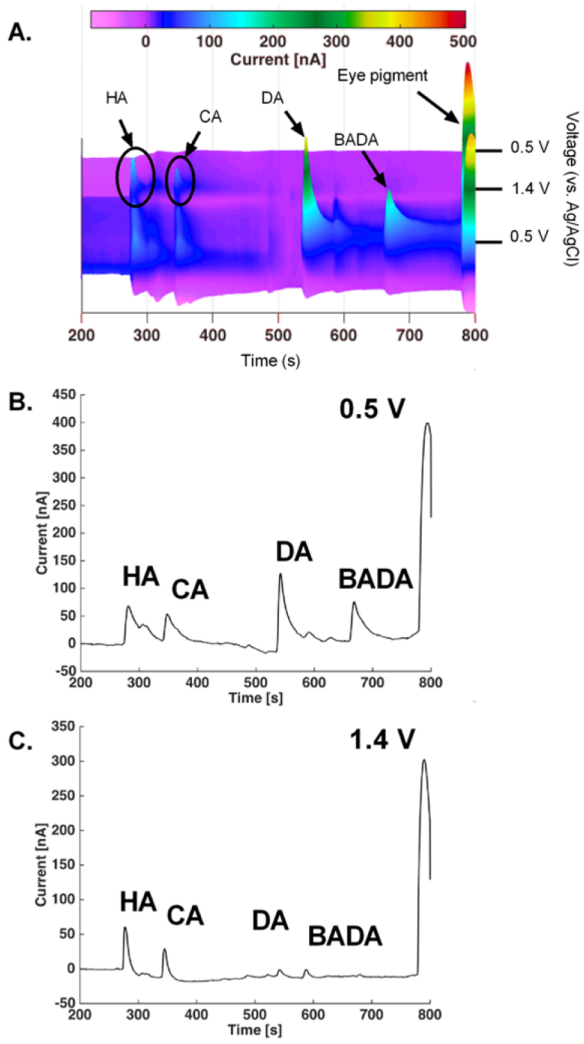

Figure 5. Separations of neurotransmitters in the eyes of a *tan*^{3} fly. (A) Heat map of the separation, with histamine (HA), carcinine (CA), dopamine (DA), and *N*-*β*-alanyl-dopamine (BADA) circled. The last peak is an eye pigment observed only in eye samples. The bottom traces are electropherograms at different potentials. (B) At 0.5 V, DA and BADA are more visible. (C) At 1.4 V, HA and CA are more visible.

CONCLUSIONS

Here, we report the first amount of histamine and carcinine in single brains as well as in eyes and cuticle from several strains of *D. melanogaster*. CE-FSCV allows for highly sensitive detection of these analytes and resolves previous issues with carcinine detection without the need for sample pretreatment or derivitization. Furthermore, peak identities were confirmed by the use of cyclic voltammatery in addition to comparisons of

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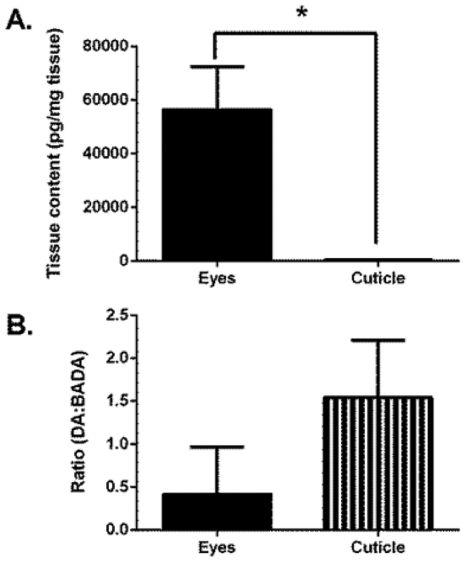

Figure 6. (A) Content of *N*-β-alanyl-dopamine (BADA) in *tan*^{3} tissues: cuticle (n = 5) and eyes (n = 7). No *N*-β-alanyl-dopamine was detected in the brain of *tan*^{3} flies. The eyes contained significantly more *N*-β-alanyl-dopamine than the cuticle (unpaired t-test, p = 0.0148). (B) Comparisons of ratios of dopamine to β-alanyl-dopamine content (DA/BADA) in *tan*^{3} flies. There was no significant difference in the ratios from these tissues (t-test, p = 0.0940). *, $p < 0.05$.

migration times. Further application of CE-FSCV to study histamine and carcinine in a wider variety of *Drosophila* strains in the brain and eyes may help elucidate the impact of a variety of mutations on the visual system of *Drosophila*. Our method could also be used to determine histamine and carcinine in a variety of other invertebrates where histamine is suspected to be important for vision.

METHODS

Chemicals. Histamine and dopamine were purchased from Sigma-Aldrich (St. Louis, MO). Carcinine, perchloric acid, and sodium phosphate monobasic monohydrate were purchased from Fisher Scientific (Pittsburgh, PA). *N*-β-Alanyl-dopamine was obtained from NIMH Chemical Synthesis and Drug Supply Library (compound A-902). Perchloric acid was diluted to 5 mM for fly tissue samples and was diluted to 0.1 M for the preparation of neurotransmitter stock solutions for calibrations.

Capillary Electrophoresis. A small diameter fused silica capillary (10 μm i.d., 151 μm o.d., Polymicro Technologies, Phoenix, AZ) cut to 40−42 cm long was used. The detection end of the capillary had the polyimide coating removed from the first ∼2 cm by burning and was polished at a right angle on a polishing wheel (Sutter Instruments, Novoto, CA). The separation capillary was filled with separation buffer prior to use and allowed to equilibrate for 15 min. Sample injection was electrokinetic: + 5 kV was applied for 15 s at the injection end of the capillary through a platinum wire in the sample vial using a dc power supply (Spellman, Plainview, NY). The +15 kV separation voltage was applied at the injection end of the capillary through a platinum wire placed in a buffer reservoir. The detection end was grounded through stainless steel tubing attached to the detection cell, a Lucite block.[17] The block was placed on a Stereomaster microscope (Fisher, Fair Lawn, NJ) to align the capillary and disk electrode, as previously reported.[17] A cross-flow buffer (detection buffer) flowed slowly (0.5 mL/min) between the other arms to flush the area around the electrode.

Buffer Composition. Dissection buffer for *Drosophila* (modified PBS, pH 7.4) was made as follows: 131.25 mM NaCl, 3.0 mM KCl, 10 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.0 mM Na_2SO_4, 1.2 mM $CalCl_2$. After buffer was made and brought to the proper pH, 50 mL aliquots were made; 0.1 g of both trehalose and glucose were added to these aliquots to maintain tissue viability. Buffer with added sugars was discarded after use to reduce the possibility of bacterial contamination.

Separation buffer was 200 mM phosphate, pH 2.0, with pH adjusted using HCl. The cross-flow buffer was 100 mM phosphate buffer, pH 7.0, with pH adjusted using NaOH.

Fast-Scan Cyclic Voltammetry and Data Collection. Detection was performed with fast-scan cyclic voltammetry in a two electrode configuration using a Dagan ChemClamp potentiostat (Dagan, Minneapolis, MN, with a custom-modified headstage). Data acquisition software and hardware were as previous described.[17] The electrode was scanned from −0.4 to 1.4 V and then back at 400 V/s every 100 ms. The CE-FSCV set up was kept inside of a Faraday cage in order to minimize the effects of external noise sources. Concentrations of neurotransmitters in fly tissue samples were determined by standard samples run before or after the fly sample. The oxidation peak current of the standard was used to determine the concentration per unit current (nM/nA). This ratio was then used to convert the peak oxidation current (nA) of the same analyte in the fly sample by multiplying by this ratio (nM/nA).

Heat Map Generation. Heat maps were generated using a MATLAB program, written in-house. The program takes color plot data generated by TarHeel CV or HDCV during the entire separation. These files are then concatenated, and background subtraction is performed within the program to generate the surface plots. Surface plots allow easier visualization of peak heights and intensities in a way that combines the advantages of standard current vs time traces (i.e., electropherograms) traditionally used to show migration order with the advantages of false color plots traditionally used to display FSCV data.

***D. melanogaster* Sample Preparation.** *D. melanogaster* strains Canton S, *ebony*^{1}, and *tan*^{3} were maintained on a standard molasses yeast medium at 17 °C. Stocks of Canton S and *ebony*^{1} flies were obtained from Bloomington Drosophila Stock Center at Indiana University. *tan*^{3} flies were generously provided by the Hirsh lab (University of Virginia). In *ebony*^{1} flies, there is a large-scale deletion of the gene *N*-β-alanyl-dopamine synthetase. In *tan*^{3} flies, a spontaneous mutation in *N*-β-alanyl-dopamine hydrolase makes the gene nonfunctional. The mutants are commonly used for investigations into histamine and carcinine in the visual system. Canton S flies are a wild-type strain of fly and are used here to compare normal function of the visual system and normal histamine and carcinine cycling.

Fly Homogenate Preparation. Adult female flies were selected (3 days post eclosion) and dissected, removing the brain, both eyes, and the cuticle from the scutellum of the fly. These tissues were then placed in dissection buffer to maintain tissue viability and stored on ice in the dark. Sample vials were made as previously described,[17] and a new vial was used for each sample. Each sample vial was filled with 10 μL of 0.5 mM perchloric acid prior to adding tissue. Tissue samples (brain, eye, or cuticle) were removed from dissection buffer by pipetting, using a 10 μL micropipette, and excess buffer was ejected into a waste container. The pipet tip was then stacked inside the sample vial. Each sample vial was loaded with a single brain, piece of cuticle, or the eyes of a single fly; no pooled samples with tissue from multiple flies were used. This allows for determination of neurotransmitter tissue content in a given tissue type obtained from individual flies. Both were then placed inside of a 2 mL centrifuge tube and were then centrifuged (Eppendorf, Brinkman Instruments, Westbury, NY) for 2 min at 9800 rpm for 1 min at room temperature. After this, the pipet tip was checked to ensure that the tissue had moved into the sample vial. The tissue in the sample vial was homogenized using a thin, silver wire (28 gauge, o.d. 0.325 mm). The sample vials were then transferred to capped 2 mL Eppendorf tubes and were sonicated in a bath sonicator for 15 min. After sonication, the sample vials are inverted on top of Ultrafree centrifugal filter tubes (Millipore, Billerica, MA, USA) and were centrifuged for 4 min at 11 000 rpm. The filter was then removed, and the filtrate was transferred to labeled 500 μL Eppendorf tubes. Injections were performed from these tubes.

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Fly tissue weights were obtained by weighing tissue from several flies and obtaining an average tissue weight for the brain (0.035 mg/brain), eyes (0.022 mg/two eyes), and cuticle (0.7 mg/cuticle piece). Pooled weights were necessary as the balance was not sensitive enough to weigh single tissue samples. These weights were then used to determine the tissue content per milligram of each tissue tested (picograms per milligram of tissue).

Statistics. Error bars are standard error of the mean (SEM). Statistics were performed in GraphPad Prism 6 (La Jolla, CA). Outlier testing (Q-test, conservative) was performed on all data to remove definitive outliers. For comparisons of two groups, *t*-tests were used. For comparisons of three groups, one-way ANOVA with Bonferonni post test was performed, and for comparisons with two variables, two-way ANOVA with Bonferonni post tests was performed.

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Author Contributions

M.E.D. wrote the manuscript, prepared figures, and conducted the experiments described in this work. E.P. provided *D. melanogaster* tissue samples for this work and advice on *Drosophila* biology. R.B. designed the MATLAB program to generate the heat plots shown in this work and provided technical support for the program. D.W. provided *D. melanogaster* tissue samples for this work. B.J.V. assisted in the development and design of experiments, helped with data interpretation, and assisted in writing the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HA, histamine; CA, carcinine; DA, dopamine; BADA, *N*-*β*-alanyl-dopamine; CE-FSCV, capillary electrophoresis coupled to fast-scan cyclic voltammetry

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