Multiple Roles for Dopamine in Drosophila Development

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Manipulation of dopamine levels by inhibition of tyrosine hydroxylase activity was accomplished in Drosophila melanogaster larval instars by feeding enzyme inhibitors for a 24-hr period. Behavioral assays performed immediately after treatment demonstrated that larval phototaxis, salt aversion, and heptanol preference were unaffected by reduced levels of dopamine. Within a few hours of treatment, the larvae ceased exploratory behavior and were unresponsive to external stimuli; these larvae eventually died. This behavior is strikingly similar to that displayed by dopamine-deficient transgenic mice. Treated larvae placed immediately onto normal food (to replenish dopamine levels) showed significant developmental delays and decreased fertility as adults. The lethality, developmental retardation, and decrease in fertility were reversed by addition of L-DOPA to inhibitor-containing food, suggesting that these effects were due solely to inhibition of tyrosine hydroxylation. Depletion of dopamine in newly eclosed females resulted in abnormally developed ovaries. These results suggest that the enzymatic function of tyrosine hydroxylase is vital and that reduced levels of dopamine result in akinesia and lethality, developmental retardation, and decreased fertility.

INTRODUCTION

Catecholamines play a fundamental role as transmitters in nervous tissue; perturbation of catecholaminergic systems are known to be responsible for Parkinson’s disease and have been implicated in the etiology of schizophrenia, Alzheimer’s disease, and some depressive disorders (Hornykiewicz, 1966; Seeman et al., 1993). Although it is believed that catecholamines in the mammalian central nervous system modulate both motor skills and higher order cognitive function, these molecules also appear to act as neurotransmitters in evolutionarily diverse organisms, including cnidarians. Endogenous catecholamines are involved in planula metamorphosis (Kolberg and Martin, 1988), suggesting a role in growth regulatory signaling (Lauder, 1993).

Perturbation of catecholamine levels via manipulation of tyrosine hydroxylase, the first and rate-limiting biosynthetic enzyme, would enable one to assess the importance of catecholamines in normal physiological development and behavior. A full-length cDNA encoding Drosophila tyrosine hydroxylase (DTH, Neckameyer and Quinn, 1989) was isolated and characterized as a first approach toward generating the molecular tools to perturb catecholamine synthesis. The high degree of conservation of both sequence and biochemical mechanism between DTH and its mammalian counterparts lends credence to the hypothesis that at least some aspects of catecholamine metabolism regulation may be evolutionarily conserved. DTH is encoded by the pale (ple) locus (Neckameyer and White, 1993), originally recovered as a recessive embryonic lethal in a screen for mutant alleles on the third chromosome (Jurgesen et al., 1984). Reintroduction of an 8-kb genomic fragment from the DTH locus into the genome of ple flies rescued the ple phenotype from embryonic lethality to adult viability (Neckameyer and White, 1993).

Distribution of catecholamines in Drosophila has previously been considered to be limited to the hypoderm (dopamine is an intermediate in the cuticular sclerotization process, Wright, 1987) and to the central nervous system (CNS), where the stereotypic pattern of catecholaminergic neurons (as indicated by glyoxylic acid-induced histofluorescence) is confined to a small subset of neurons (Budnik and White, 1987). Analytical studies indicate that the predominant, if not exclusive, catecholamine present in these neurons is dopamine (see Restifo and White, 1990, for review).

To further elucidate the physiological requirements for dopamine in both development and behavior, we exposed the larval stage of Drosophila to competitive inhibitors of tyrosine hydroxylase. The results demonstrate that dopamine plays multiple and, in some cases novel, roles in differ-
ot organ systems and is absolutely required for normal development.

**MATERIALS AND METHODS**

**Fly Culture**

Several hundred wild-type Canton S adult flies were maintained for 1 week in a population cage kept at 25°C on a 12-hr light–dark cycle. The flies were allowed to lay eggs for 4 hr on apple juice–agar plates, and any larvae that had hatched were removed after 15 hr at 25°C. Staged larvae were collected after 4 hr by migration of the larvae onto yeast paste in the center of the agar plate. Larvae were maintained on yeast paste for an additional 24 hr. Equal gram amounts (25–50 mg) were placed in yeast paste (0.5 g baker’s yeast in 1 ml H2O) on a 2-in. square of Nitex mesh (25–16, Tetko, Inc.) containing different amounts of the tyrosine hydroxylase inhibitors α-methyltyrosine, methyl ester (AMT), or 3-iodotyrosine (3-I-Y), or the tyrosine hydroxylase inhibitors plus L-3,4-dihydroxyphenylalanine (L-DOPA). The Nitex mesh was screwed onto an upended 50-ml conical tube using a cap with an open top and placed in a 50-ml beaker containing 0.5 ml H2O. Twenty-four hours later the larvae were harvested and subjected to different assays.

**Assay of Dopamine Levels in Treated Larvae**

Cuticular depletion of dopamine was indicated by the reduction of melanized pigment in the mouthhooks. Treated larvae were placed in a solution of glycerol:acetic acid (1:4) for 24 hr at 65°C according to the procedure of van der Meer (1977), mounted in Hoyer’s medium on glass slides, and clarified by incubation for several days at 45°C.

Neuronal depletion of dopamine was demonstrated by the reduction in glyoxylic acid-induced histofluorescence of catecholamine-containing neurons in the central nervous system of treated larvae (Budnik and White, 1987).

Quantification of dopamine levels in treated animals was accomplished by HPLC analysis. Larvae or adults were quick frozen in liquid nitrogen and homogenized in 0.1 M perchloric acid (3 or 10 µl/mg of tissue, respectively). The chromatographic system consisted of an ESA pump (Model 580), a manual injector (Model 9125), and an HR-80 3-µm particle size column. Detection and quantitation were accomplished using a coulochem detector (ESA Model 5200A), an analytical cell (Model 5011, channel 1 set at ~50 mV and channel 2 set at 250 mV), and a guard cell (Model 5020, set at 400 mV). The mobile phase consisted of 75 mM NaH2PO4, 1.5 mM SDS, 100 µl triethylamine, 15% acetonitrile, 12.5% methanol. Under these conditions, dopamine eluted at 7 min. A standard concentration curve of dopamine was run in addition to the samples. Statistical comparisons between control and inhibitor-fed larvae for a given drug were done using an unpaired Student’s t test.

**Behavioral Analyses**

Larvae were assessed for behavioral abnormalities by subjecting them to five different assays. In all assays, staged larvae were allowed to feed on yeast paste or yeast paste plus 10 mg/ml 3-iodotyrosine for 24 hr before the behavioral tests. For the phototaxis, chemotaxis, and odor assays, animals were allowed to roam freely for 5 min, and a Response Index (R.I.) was calculated by dividing the number of animals found in the test quadrants over the total number of animals in all quadrants. Larvae remaining in the center of the dish were not included in the R.I. Statistical analysis (unpaired Student’s t test) of 10 independent assays of control larvae and larvae fed 10 mg/ml 3-iodotyrosine was done using the Macintosh Statworks program.

In the phototaxis assay, larvae were assayed for normal (negative) phototactic behavior by placing them in the center of an agar plate containing two opposing clear and two opposing dark quadrants illuminated from below by a light box in a dark room (Lilly and Carlson, 1990). Plates containing only clear agar were used for controls and run in parallel with the phototaxis assay. Larvae were tested for aversion to salt by placing them in the center of an agar plate containing two opposing quadrants of agar and two opposing quadrants of agar/1 M NaCl (Lilly and Carlson, 1990). Control plates were identical except that no salt was added. Larvae were tested for attraction to heptanol by placing them in the center of a 2% agar plate with 1.5-cm filter paper discs placed at opposite ends (Cobb, 1992). Seven microliters of odorant was placed on one disc, the top of the agar dish was replaced, and animals were allowed to roam freely for 5 min. Agar plates containing filter discs with no odorant were used for controls. The number of animals in each half was counted; however, animals found within a 2-in. radius of the center were discounted to ensure that only animals making a definite choice toward or away from the odor were included.

To assay locomotion, single animals were placed on a 2% agar surface, allowed to recover for 1 min 30 sec, and the number of contractile motions were counted for 1 min. Similarly, to assay feeding, single animals were placed on a petri dish containing 2% agar overlaid with a 2% solution of yeast with melanized pigment in the mouthhooks. Treated larvae were allowed to recover for 1 min 30 sec, and the number of mouthhook contractions in 1 min was counted (Sewell et al., 1975). 5 to 10 animals were assayed in four independent experiments.

**Developmental Analyses**

Staged larvae were treated for 24 hr with yeast paste alone, or yeast paste containing 5, 10, or 15 mg/ml of 3-iodotyrosine, or 10 mg/ml 3-iodotyrosine plus 10 mg/ml L-DOPA. Larvae were also treated with 5 or 10 mg/ml of AMT or 10 mg/ml AMT plus 10 mg/ml L-DOPA. Immediately after harvesting, larvae from each group were placed in vials containing soft larval medium (5% Difco yeast extract, 0.8% bacto agar, 5% sucrose, and 2% baker’s yeast). Thirty larvae were placed in each vial and were maintained at 25°C until eclosion. Larvae were quick frozen in liquid nitrogen and homogenized in 0.1 M perchloric acid (3 or 10 µl/mg of tissue, respectively). The chromatographic system consisted of an ESA pump (Model 580), a manual injector (Model 9125), and an HR-80 3-µm particle size column. Detection and quantitation were accomplished using a coulochem detector (ESA Model 5200A), an analytical cell (Model 5011, channel 1 set at ~50 mV and channel 2 set at 250 mV), and a guard cell (Model 5020, set at 400 mV). The mobile phase consisted of 75 mM NaH2PO4, 1.5 mM SDS, 100 µl triethylamine, 15% acetonitrile, 12.5% methanol. Under these conditions, dopamine eluted at 7 min. A standard concentration curve of dopamine was run in addition to the samples. Statistical comparisons between control and inhibitor-fed larvae for a given drug were done using an unpaired Student’s t test.

**RNA in Situ Hybridizations to Whole Mount Embryos and Ovaries**

Sense and antisense digoxigenin-labeled RNA probes from the cloned DTH gene were made exactly according to the manufacturer’s protocols (Boeringer-Mannheim). Due to the extensive 3’ AT-rich untranslated region in the DTH transcript (Neckameyer and Quinn, 1996 by Academic Press, Inc. All rights of reproduction in any form reserved.
1989), DNA fragments containing only the coding region were generated using primers homologous to the translation start and stop sites in a polymerase chain reaction. The appropriately sized reaction products were subcloned into both the SK- and KS-plasmid vectors (Stratagene) and the coding sequence was confirmed by the dideoxy chain-terminating DNA sequencing method (Sanger et al., 1977). The digoxigenin-labeled probes were hybridized in situ to staged embryos (0–4, 4–8, 8–12, and 12–16 hr of embryogenesis) and to ovaries according to the method described by Lehmann and Tautz (1994). Hybridization temperature was 56°C.

Feulgen Staining of Ovaries

Newly emerged Canton S female flies were placed in standard plastic vials containing a 2.4-cm glass fiber filter paper disc (Baxter) saturated with 0.5 ml of a 2% baker's yeast/5% sucrose solution. Ten virgin females were placed in each vial. The yeast solution in the experimental vials contained 5 mg/ml of 3-iodotyrosine. The vials were maintained at 25°C for 24 or 48 hr, after which the ovaries were dissected and stained as described in Wayne et al. (1995). Alternatively, the flies were quick frozen in liquid nitrogen and dopamine content was determined by HPLC analysis.

RESULTS

Tyrosine Hydroxylase Inhibitors Severely Reduce Dopamine Levels in Treated Larvae

Treatment of second instar Drosophila larvae with 5 mg/ml AMT or 5 mg/ml 3-iodotyrosine for 24 hr resulted in a distinct reduction of dopamine in both cuticular structures and the central nervous system. Cuticle preparations of treated larvae showed no gross abnormalities except for decreased pigmentation of the heavily melanized mouthparts. The mouthparts of animals treated with AMT showed reduced pigmentation (from the original black color to brown); the mouthhooks of 3-I-Y-treated animals ranged from light brown to colorless (data not shown). There are approximately 80 larval tyrosine hydroxylase- and catecholamine-containing neurons in the nervous system of Drosophila which are distinctly distributed within the central nervous system (Budnick and White, 1988). Glyoxylic acid-induced histofluorescence of hand-dissected CNSs from treated larvae show little (AMT) or no (3-iodotyrosine) fluorescence in the catecholamine-containing neurons (Fig. 1). Four to five CNSs from each treated group were analyzed from four independent experiments, yielding consistently reproducible results.

HPLC analysis demonstrated that the dopamine content of treated larvae was clearly reduced (Fig. 2). In all three assays, 3-iodotyrosine appeared to have a more potent effect than AMT; this was not unexpected since the Kᵦ of the hydroxylation reaction using 3-iodotyrosine as a substrate is two orders of magnitude less than that for AMT (5 x 10⁻⁷ compared to 2.5 x 10⁻⁵, Udenfriend et al., 1965). These results confirmed that ingestion of added inhibitor in the food source was sufficient to drastically reduce, if not eliminate, catecholamine stores in both cuticular and nervous tissues.

Behavioral Analyses of Treated Larvae

Ingestion of 5 mg/ml 3-iodotyrosine for 24 hr was sufficient to eliminate catecholamine fluorescence in the larval CNS, suggesting that the level of dopamine was so low as
to be undetectable. Therefore, treated larvae were subjected to several behavioral paradigms to determine whether dopamine modulated these behaviors. However, in all assays, the larvae demonstrated the appropriate behavioral responses to the given stimuli (Table 1).

The phototaxis assay measures the normal tendency of second and early third instar larvae to avoid light. Generally, approximately 70% of the animals will seek out the dark quadrants (R.I. = 0.30); animals on the control plates will distribute themselves equally (R.I. = 0.50). Larvae fed 10 mg/ml 3-iodotyrosine (3-I-Y) for 24 hr were negatively phototactic on the assay plates relative to those on control plates (P < 0.001) and indistinguishable in their response from that of larvae fed yeast paste alone.

Drosophila larvae have a strong aversion to salt; approximately 80% will avoid the salt-containing quadrants. Similar to the animals fed yeast paste alone, inhibitor-fed larvae demonstrated a strong aversion to salt (R.I. = 0.19, P < 0.001).

Heptanol is an attractant substance for Drosophila larvae: greater than 70% of control animals, fed only yeast paste, were found in the region of the agar plate containing heptanol (R.I. = 0.769). Inhibitor-fed larvae responded in a similar manner (R.I. = 0.722).

Taken together, the behavioral results strongly suggest that dopamine does not modulate the multiple behaviors assayed. Although it cannot be excluded that a slight amount of residual dopamine in the CNS (although undetectable by glyoxylic acid-induced fluorescence) is sufficient to correctly modulate these behaviors, the extent and duration of treatment suggests this is unlikely. These experiments do not exclude the possibility that dopamine may modulate other larval behaviors, nor do they address dopamine modulation of adult behaviors.

Although the treated larvae behaved normally in the behavioral paradigms discussed above, it was noted that within a few hours after treatment, the larvae became akinesic. The numbers of larvae not responding to behavioral stimuli (those in the center of the plate) were similar in 3-I-Y treated and control animals; the behavioral experiments were completed well before the onset of akinesia. At first, they were still responsive if prodded gently with a needle, but later became unresponsive and eventually died. The akinesia was most likely independent of the larval molt, since several animals were observed in the process of shedding the old cuticle. In addition, many of the larvae had severely diminished pigmentation in their mouthparts, suggesting they had already shed their cuticles (pigmentation of the mouthparts occurs with each larval molt). These observations suggest the lethality was not due to any difficulty in achieving the next larval molt. Initial efforts to determine the extent of lethality indicated that the larvae did not burrow into the food when placed in vials and therefore did not eat. Under normal conditions larvae will feed continuously using their mouthhooks to aid in ingestion of the culture medium; the rate of feeding is stable during the first half of the third instar (Sewell et al., 1975). It was possible that lack of dopamine in the mouthpart structures might have weakened their integrity, although cuticle preparations displayed no gross abnormalities (data not shown). To determine whether weakened cuticular structures could be responsible for the ensuing lethality, feeding behavior was assayed immediately after treatment with 3-iodotyrosine (Table 2). The rate of locomotion, which remains constant over the first 72 hr of larval development (Sewell et al., 1975), was also assayed to determine whether cuticular abnormalities might affect the ability of the larva to burrow and seek food. Neither locomotive nor feeding abilities were

![Image](https://example.com/image.png)

**FIG. 2.** HPLC analysis of larval dopamine levels. After ingestion of 0 mg/ml inhibitor, 10 mg/ml α-methyl-p-tyrosine, methyl ester (AMT), or 10 mg/ml 3-iodotyrosine (3-I-Y) for 24 hr, larvae were homogenized in 3 μl/mg 0.1 M perchloric acid. Cross above bar indicates standard error. *P < .01 (n = 3); **P < .001 (n = 2) (Student’s unpaired t test).

### TABLE 1

<table>
<thead>
<tr>
<th>Assay</th>
<th>n</th>
<th>Control (±SE)</th>
<th>Assay (±SE)</th>
</tr>
</thead>
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<tr>
<td>Phototaxis</td>
<td></td>
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<td></td>
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<tr>
<td>Control</td>
<td>12</td>
<td>0.519 ± 0.051</td>
<td>0.361 ± 0.066*</td>
</tr>
<tr>
<td>Depleted</td>
<td>10</td>
<td>0.491 ± 0.034</td>
<td>0.342 ± 0.061*</td>
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<tr>
<td>Chemotaxis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0.538 ± 0.077</td>
<td>0.170 ± 0.023*</td>
</tr>
<tr>
<td>Depleted</td>
<td>5</td>
<td>0.490 ± 0.037</td>
<td>0.190 ± 0.012*</td>
</tr>
<tr>
<td>Heptanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>0.502 ± 0.074</td>
<td>0.769 ± 0.068*</td>
</tr>
<tr>
<td>Depleted</td>
<td>12</td>
<td>0.475 ± 0.070</td>
<td>0.722 ± 0.136*</td>
</tr>
</tbody>
</table>

Note. Control animals were fed yeast paste (0.5 g/ml) for 24 hr; experimental animals were fed yeast paste containing 10 mg/ml 3-iodotyrosine (depleted). n, number of independent behavioral assays. SE, standard error of the mean. Control and assay plates for each behavior were run in parallel.

* Significant at P ≤ 0.001 (Student’s t test)
diminished after treatment with 3-iodotyrosine. Overall, the data from these experiments suggest that locomotive and feeding abilities were unaffected by any weakening of cuticular structures and that the ensuing lethality must arise as a consequence of some other aspect of dopamine depletion.

### Reduction of Larval Dopamine Levels Results in a Lower Eclosion Rate

The extent of lethality after 24 hr treatment with tyrosine hydroxylase inhibitors was determined by immediately placing the larvae onto soft larval medium (30 larvae per vial) and scoring the number that eclosed as adults following pupariation. The data in Table 3 clearly demonstrate an increasing lethality (fewer flies eclosing) with increasing amounts of either 3-iodotyrosine or AMT. In addition, flies treated with greater amounts of tyrosine hydroxylase (TH) inhibitors eclosed significantly later than flies with normal levels of dopamine. However, the flies that did eclose displayed no obvious defects in cuticular structure or pigmentation and were indistinguishable from controls. No melanization around the leg joints (indicating weakening of the cuticle) was observed. Unlike the published ple transgenic flies (Neckameyer and White, 1993), these animals appeared to have normal cuticles and show no differences in fragmentation. Therefore, 24 hr of exposure to TH inhibitors during a larval instar did not appear to affect adult cuticle formation or pigmentation. The observed lethality and developmental retardation of inhibitor-fed animals were reversed by the addition of L-DOPA, strongly suggesting that dopamine is solely responsible for both the increase in lethality and the delay in development.

### Expression of Tyrosine Hydroxylase mRNA in Situ

Given that reduction of dopamine by inhibition of tyrosine hydroxylase resulted in delayed development, expression of DTH mRNA was examined in the developing embryo to help elucidate the role of dopamine in normal development. No tyrosine hydroxylase expression was detected in the early embryo (0–8 hr after egg laying, data not shown). At later stages, strong expression of DTH was detected in certain tissues of ectodermal origin (Fig. 3). The observed staining is specific for DTH, since detection of expression in these tissues was seen only with the antisense and not the sense probe (Fig. 3A). Tyrosine hydroxylase mRNA is found in the foregut, hindgut, anal pads, and in part of the proventriculus (Fig. 3B, 12–16 hr post-egg laying). Figure 3C (12–16 hr post-egg laying) displays a pattern of expression highly reminiscent of the larval denticle belts; this pattern arises prior to any detectable cuticle deposition. Expression was also detected in certain midline neural cells (Fig. 3D, 8–12 hr post-egg laying).

Whole mount ovaries from wild-type adult flies were also hybridized in situ to digoxigenin-labeled sense and antisense DTH probes. No staining is seen using the sense probe (Fig. 3E). However, and somewhat unexpectedly, expression of DTH mRNA was found in the nurse cell cytoplasm and in follicle cells through at least the first 12 stages of egg chamber development (Fig. 3F). No expression was detected in the mature eggs; although this may be due to the chorionic membrane pre-
FIG. 3. DTH in situ mRNA expression. Digoxigenin-labeled sense and antisense DTH probes were hybridized in situ to staged embryos and ovaries. (A) Sense probe. (B, C) 12- to 16-hr embryos. (D) 8- to 12-hr embryos. (E) Sense probe, ovaries. (F) Antisense probe, ovaries. ap, anal pads; pr, proventriculus; nb, neural bodies; fg, foregut; hg, hindgut; db, denticile belts; fc, follicle cells; nc, nurse cells.

To determine what effect developmental depletion of dopamine may have had on fertility, three male and three female flies emerging from each of the vials set up as described above were allowed to mate for 1 week, and the number of flies emerging from the crosses were determined. In several instances, crosses between flies treated during the larval stage with TH inhibitors were not productive (no eggs laid or eggs laid did not hatch). Of those vials that were productive, increasing amounts of tyrosine hydroxylase inhibitor fed to the parents as larvae correlated with fewer progeny (Table 4).
FIG. 5. Feulgen staining of ovaries. Newly eclosed females were maintained at 25°C for 48 hr and fed either 2% yeast/5% sucrose (A, control) or yeast/sucrose plus 10 mg/ml 3-iodotyrosine (B, experimental). The ovaries were then hand-dissected and subjected to Feulgen staining. Samples were photographed and enlarged at the same magnification.

in Table 4 thus reflect a decrease in fertility from productive matings only. The number of progeny from flies fed L-DOPA as larvae in addition to the tyrosine hydroxylase inhibitors was indistinguishable from that of controls. These experiments strongly suggest that reduced levels of dopamine during a larval instar affects adult fertility.

Reduction of Dopamine Levels in Newly Eclosed Females Inhibits Ovarian Development

Due to the unexpected expression of tyrosine hydroxylase in ovarian tissue, and the decreased numbers of progeny resulting from crosses established with flies that had been fed TH inhibitors as larvae, it seemed likely that dopamine was required for normal fertility. Therefore, the effect of dopamine depletion on adult ovaries was examined. Newly eclosed virgin females were maintained in vials for 24 or 48 hr with or without 10 mg/ml 3-iodothyrosine. Newly eclosed females require more than 24 hr to produce mature eggs, indicating that ovarian development is not complete at this stage and may be vulnerable to agents during this time period. HPLC analysis of whole flies treated with the inhibitor indicated a severe and significant reduction in dopamine levels.
TABLE 4
Reduction in Fertility of Animals Fed 3-Iodotyrosine (3-I-Y) as Larvae

<table>
<thead>
<tr>
<th>3-I-Y (mg/ml)</th>
<th>L-DOPA (mg/ml)</th>
<th>Males eclosing (mean ± SE)</th>
<th>Females eclosing (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>32.8 ± 3.5</td>
<td>33.3 ± 3.2</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>12.6 ± 1.6*</td>
<td>15.4 ± 2.0*</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>15.7 ± 2.5*</td>
<td>16.8 ± 2.8*</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>31.0 ± 3.2</td>
<td>37.4 ± 3.0</td>
</tr>
</tbody>
</table>

Note. Larvae were fed the indicated concentrations of 3-I-Y and L-3,4-dihydroxyphenylalanine (L-DOPA) for 24 hr. After treatment, 30 larvae from each experimental point were placed in soft larval medium. After eclosion, 3 males and 3 females from each vial were allowed to mate for 1 week. Numbers of male and female flies emerging from these crosses were counted over a 10-day period. SE, standard error of the mean. There was no significant difference between the numbers of males and females emerging for each drug concentration. *P < .01; **P < .001 (one-way ANOVA; Bonferroni posttest) comparing inhibitor-fed larvae with control. Differences in values for inhibitor-fed larvae and larvae fed inhibitor plus L-DOPA were also significant (P < .001, females eclosing from 5 mg/ml and 10 mg/ml parental crosses; P < .01, males eclosing from 5 mg/ml crosses; P < .05, males eclosing from 10 mg/ml crosses).

levels, similar to that seen in larvae (Fig. 4). Examination of the ovaries from flies treated for 24 hr showed that they were clearly smaller and less well-developed; the oocytes appeared shrunken and withered. The difference was even more pronounced after treatment for 48 hr (Fig. 5). Immediately after treatment, the adult flies appeared to have normal locomotor and feeding skills; therefore, it is unlikely that cessation of feeding (as in inhibitor-fed larvae) was responsible for this effect. Partial rescue was obtained if 10 mg/ml L-DOPA was added to vials treated with inhibitor; the ovaries from females so treated were larger than those of flies treated with 3-I-Y alone, but smaller than those of flies fed no inhibitor. HPLC analysis demonstrated that dopamine concentrations of adult flies fed L-DOPA were at least an order of magnitude greater than that of control flies, suggesting that deviations in normal dopamine levels (higher and lower) adversely affect ovarian maturation. Titration of both inhibitor and L-DOPA is thus required to assess how much dopamine is necessary for normal ovarian development.

DISCUSSION

Dopamine synthesis from its amino acid precursor, tyrosine, requires the activities of several enzymes. Hydroxylation of tyrosine to L-DOPA occurs primarily, if not exclusively, via the enzyme tyrosine hydroxylase. Decarboxylation of L-DOPA to dopamine is accomplished by the action of a phenol oxidase, usually dopa decarboxylase. In addition, the activity of tyrosine hydroxylase is dependent upon the tetrahydrobiopterin cofactor, and thus the regulation of dopamine synthesis is also governed by the activities of the enzymes in the pteridine biosynthetic pathway.

Mutant alleles arising from the loci encoding these enzymes have been characterized in Drosophila. The known alleles of ple, the locus encoding tyrosine hydroxylase, are embryonic lethals with unpigmented denticles and mouthparts (Jurgens et al., 1984; Neckameyer and White, 1993). Dopa decarboxylase (Ddc) is one of several phenol oxidase genes clustered on the second chromosome (Wright, 1987); generally, the lethal Ddc phenotype is similar to that of ple. The Punch (Pu) locus encodes GTP cyclohydrolase I, the first and rate-limiting enzyme in the synthesis of all pterins, including the cofactor required for tyrosine hydroxylase activity; most mutant Pu alleles have a phenotype indistinguishable from that of ple.

Although there have been extensive molecular and genetic characterizations of these loci, the specific functions of dopamine have not been directly addressed. Confirmation of isolation of hypomorphic or conditional alleles of ple has not yet been accomplished; it is not yet known whether currently described ple embryonic lethal alleles are true nulls. This study was undertaken to induce a hypomorphic state by inactivating wild-type tyrosine hydroxylase with exogenous inhibitor. The results from this work suggest novel functional roles for tyrosine hydroxylase and dopamine unlikely to have otherwise been uncovered.

Behavioral Analyses

Several lines of evidence suggest that the biogenic amines are involved in the physiological regulation of several be-
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Behavior in insects; primarily, these studies have focused on feeding behavior and conditioned responses to olfactory stimuli. Long and Murdock (1983) have demonstrated that injection of dopamine 45 min before presentation of sucrose to blowflies significantly decreased food consumption. In addition, Mercer and Menzel (1982) have shown that response levels of the honeybee Apis mellifera to a conditioned olfactory stimulus were significantly reduced by dopamine, which apparently inhibited retrieval of the conditioned response. These studies only addressed the effects of increased levels of dopamine. Few studies have addressed dopamine-modulated behaviors in Drosophila and other insects, and none have specifically targeted behaviors occurring during a juvenile life stage.

The data presented here suggest that larval phototaxis, salt aversion, and heptanol preference behaviors are unaffected by reduced levels of dopamine; the animals were tested immediately after harvesting, a few hours before the onset of akinesia. The observations that the larvae exposed to TH inhibitors are capable of normal locomotive and feeding behaviors immediately after treatment suggests that the structural integrity of the cuticle and mouthparts is not significantly compromised despite the reduction in pigmentation.

The akinesia displayed by inhibitor-fed larvae most likely results from behavioral and not physiological consequences of reduction in dopamine levels, since the assays demonstrate the larvae are physiologically capable of normal feeding and locomotive behaviors. Additionally, it appeared that the akinesia seen in TH inhibitor-fed larvae is strikingly similar to that displayed by dopamine-deficient transgenic mice (Zhou and Palmiter, 1995). These mice have TH activity only in noradrenergic and not in dopaminergic cells, and although apparently normal at birth, within a few weeks these mice become hypoactive and discontinue feeding. Similarly, although apparently behaviorally normal immediately after treatment, TH inhibitor-fed larvae became unresponsive and ceased exploratory and feeding behaviors within a few hours. Our data suggest that, as in the transgenic mice, dopamine is essential for exploratory behavior and thus for normal feeding and locomotion.

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The Role of Dopamine in Fertility

DTH mRNA is present in situ in ovarian tissue in the developing egg chambers. DTH mRNA is apparently expressed in some, but not all, tissues of ectodermal origin during embryogenesis; the ovaries arise from pole cells and mesodermal cells, suggesting a very distinct regulation of DTH in gonadal tissue. It is unlikely that DTH mRNA is transported into the oocyte from the nurse cells, since no expression is detected in early embryos (0–4 hr post-egg laying). This finding is somewhat different from that found for Ddc, the second enzyme in the dopamine biosynthetic pathway: Ddc mRNA expression has been detected in ovaries from virgin females, but the transcript has also been found in 2-hr embryos (Gietz and Hodgetts, 1985). It is possible that Ddc expression persists because of the stability of the message relative to that of DTH and/or because it is required for serotonin synthesis in early embryogenesis; previous studies have detected tryptophan hydroxylase (the rate-limiting enzyme in serotonin synthesis) mRNA and protein in 0- to 40-min embryos (Neckameyer and White, 1992; O'Donnell et al., 1993). Pu transcripts and protein are found in the nurse cells during oogenesis, and the protein, but not the mRNA, is transported into the oocyte cytoplasm (Chen et al., 1994). The DTH pattern of expression may be similar to Pu in that only the protein is transported into the embryo.

The co-expression of Ddc, DTH, and Pu in the developing egg chambers of the ovaries is a likely indicator of dopamine production. That the presence of dopamine in the ovaries is pivotal for normal ovarian development is clearly shown by the vastly decreased size of the ovaries when dopamine levels are reduced during the first 2 days of adult life (Fig. 5). Interestingly, the family of related genes forming the diphenol oxidase cluster on the second chromosome (including Ddc) are involved in female fertility as well as in catecholamine metabolism, and at least four of these genes are required for male fertility (Wright, 1987). DTH mRNA is also expressed in adult testicular tissue (Neckameyer, unpublished observation) and is likely to affect male fertility as well. The additional observation of decreased fertility of those flies that lack Th inhibitor over a 24-hr period during the larval stage suggests that normal function of adult gonadal tissue is dependent on dopamine levels in a juvenile stage. At this point, however, one cannot exclude a behavioral component to the sterility.

ple is thus a vital gene which is also necessary for oogenesis. ple mutations affecting only oogenesis may not have been isolated due to its requirement during embryogenesis. DTH mRNA is expressed in cells of both somatic and germinal origin; further studies are needed to determine whether the requirement for dopamine is in the germ line or soma and whether there is a neural component for normal fertility. These questions will be addressed by generating germ-line clones (see Ruohola et al., 1991).

Several vital genes are also expressed in ovarian tissue (including the neurogenic loci Notch, Delta, mastermind, neuralized, and Enhancer of split). These genes are required for specifying a neural fate in ectodermal tissue (Ruohola et al., 1991). It is possible that if tyrosine hydroxylase is required for terminal differentiation of specific tissues, dopamine would thus act as a signal to aid these undifferentiated cells in selecting a specific fate. The studies presented here support the hypothesis that dopamine may have been used as a signaling molecule previous to the development of the nervous system and continues to perform this role in both neural and nonneural tissues.

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