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Influence of PCPA and MDMA (ecstasy) on physiology, development and behavior in *Drosophila melanogaster*

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Abstract

The effects of para-chlorophenylalanine (PCPA) and 3,4 methylenedioxy-methamphetamine (MDMA, 'ecstasy') were investigated in relation to development, behavior and physiology in larval *Drosophila*. PCPA blocks the synthesis of serotonin (5-HT) and MDMA is known to deplete 5-HT in mammalian neurons; thus these studies were conducted primarily to target the serotonergic system. Treatment with PCPA and MDMA delayed time to pupation and eclosion. The developmental rate was investigated with a survival analysis statistical approach that is unique for *Drosophila* studies. Locomotion and eating were reduced in animals exposed to MDMA or PCPA. Sensitivity to exogenously applied 5-HT on an evoked sensory–central nervous system (CNS)–motor circuit showed that the CNS is sensitive to 5-HT but that when depleted of 5-HT by PCPA a decreased sensitivity occurred. A diet with MDMA produced an enhanced response to exogenous 5-HT on the central circuit. Larvae eating MDMA from the first to third instar did not show a reduction in 5-HT within the CNS; however, eating PCPA reduced 5-HT as well as dopamine content as measured by high performance liquid chromatography from larval brains. As the heart serves as a good bioindex of 5-HT exposure, it was used in larvae fed PCPA and MDMA but no significant effects occurred with exogenous 5-HT. In summary, the action of these pharmacological compounds altered larval behaviors and development. PCPA treatment changed the sensitivity in the CNS to 5-HT, suggesting that 5-HT receptor regulation is modulated by neural activity of the serotonergic neurons. The actions of acute MDMA exposure suggest a 5-HT agonist action or possible dumping of 5-HT from neurons.

Introduction

Serotonin (5-HT), dopamine (DA) and octopamine are well known to act as neuromodulators in insects, particularly in *Drosophila melanogaster*, which produce behavioral and developmental alterations as well as organization in the central nervous system (CNS) circuits (Osborne, 1996; Monastirioti, 1999). 5-HT modulates voltage-dependent potassium channels and heart rate (HR) in *Drosophila* (Johnson *et al.*, 1997; Zornik *et al.*, 1999). DA is known to alter sexual behavior, sensory habituation (Neckameyer, 1998a,b) and increase activity in adult flies (Friggi-Grelin *et al.*, 2003) but depresses synaptic transmission at the neuromuscular junction in larval *Drosophila* (Cooper & Neckameyer, 1999). Octopamine expression is stress related in *Drosophila* (Hirashima *et al.*, 2000) and octopamine receptors are present in mushroom bodies in the *Drosophila* CNS, which is a region important for learning in adults (Han *et al.*, 1998). In fact, 5-HT, DA and octopamine all have some central effects in the *Drosophila* brain related to learning or behavior (Monastirioti, 1999; Blenau & Baumann, 2001). Recently, direct actions of these neuromodulators were shown to alter central neural activity (Dasari & Cooper, 2004).

These biogenic amines have broad differential effects on development and physiology in larvae as well as in adults. Here we attempted to focus on the serotonergic system and tissue sensitive to alterations in endogenous levels of 5-HT within the larvae and pupa by the use of two pharmacological compounds [i.e. para-chlorophenylalanine (PCPA) and 3,4 methylenedioxy-methamphetamine (MDMA, 'ecstasy')]. The development and distribution of 5-HT-immunoreactive neurons in the CNS have been established (Valles & White, 1988). 5-HT has a role in many physiological processes with regard to regulating locomotion and cardiac output (Kamyshev *et al.*, 1983; Johnson *et al.*, 1997, 2000; Nichols *et al.*, 1999; Zornik *et al.*, 1999; Dasari & Cooper, 2006). As 5-HT alters the activity of sensory-to-motor neuron central circuits in larval *Drosophila* (Dasari & Cooper, 2004) this opens the possibility that the serotonergic system could sculpt the formation of neural circuits by altering the neural activity in the developing CNS of *Drosophila*. The activity of developing neural circuits is well established to play a major role in the patterning of the adult CNS in mammals prior to critical periods (Hubel & Wiesel, 1963a,b, 1968, 1970).

MDMA (ecstasy), a drug of abuse, modulates the homeostasis of the serotonergic system in humans and animal models (Green *et al.*, 2003). Research is scant on the effects of MDMA in the developing CNS of mammals as well as in insects. Thus, we used the rapidly developing nervous systems of *Drosophila* larvae to provide an avenue to quickly screen the effects of MDMA on the larval CNS. The proposed mechanism of action of MDMA in mammals is an eventual depletion of 5-HT within neurons by the transporter working in reverse (Hilber *et al.*, 2005). Our findings suggest that the neurons are not depleted in the CNS within larval *Drosophila* over days but acute effects could be explained in part by dumping of 5-HT with possible

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up-regulation of 5-HT synthesis to replenish 5-HT. We tested other means, through pharmacological manipulation, throughout the early stages of larval development to deplete 5-HT for comparison with the effects induced by MDMA. Within this study we also provide a novel (for this field) approach based on statistical survival analysis to quantify developmental curves for *Drosophila* larva. Survival analysis is 'tailor-made' for time to event data.

Preliminary results of this study were presented in abstract form (Dasari & Cooper, 2005).

Materials and methods

Stock and staging of larvae

The common 'wild-type' laboratory strain of *D. melanogaster*, Canton S, was used in these studies. The methods used to stage fly larvae have been described previously (Campos-Ortega & Hartenstein, 1985; Li *et al.*, 2002). All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. All animals were kept on a 12/12 light/dark cycle. Introduction of pharmacological agents started with the first instar.

Behavioral assays

Early third instar larvae were used for behavioral assays. Feeding and locomotory behavior was assessed as described in Neckameyer (1996) and Li & Cooper (2001). In brief, single animals were placed on a 2% agar surface and the number of body wall contractions was counted for 1 min, after which an animal was placed in a 2% yeast solution overlaid on an agar plate (just covering the larva and allowing the spiracles to reach out of the solution). In this condition, *Drosophila* larvae immediately feed, initiating a pattern of repetitive mouth hook movements. The number of full mouth hook contractions in 1 min was counted (Sewell *et al.*, 1975). The results of these behaviors were plotted as body wall contractions or mouth hook movements per minute.

Dissection and electrophysiological recordings

Wandering third instar larvae were dissected as described previously (Cooper *et al.*, 1995). In brief, larvae were dissected dorsally removing the heart and viscera, which left a filleted larvae containing only a body wall, body wall muscles and the neural circuitry for the sensory, CNS and body wall (i.e. skeletal) motor units. The HL3 saline was prepared in the laboratory from component reagents (Sigma) and contained: 1.0 mM $CaCl_2.2H_2O$, 70 mM NaCl, 20 mM $MgCl.6H_2O$, 5 mM KCl, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose and 5 mM N,N-bis[2-hydoxyethyl]-2-aminoethanesulfonic acid (Stewart *et al.*, 1994).

The recording arrangement was essentially the same as previously described (Neckameyer & Cooper, 1998; Stewart *et al.*, 1994). Intracellular recordings in muscles were made with 30–60 MΩ resistance, 3 M KCl-filled microelectrodes. The amplitudes of the excitatory postsynaptic potentials elicited by the Is and Ib motor nerve terminals of muscle m6 were monitored. Primarily body segments 3 and 4 were used throughout these studies. Intracellular responses were recorded with a 1 × LU head stage and an Axoclamp 2A amplifier (Molecular Devices, Sunnyvale, CA, USA). To evoke a sensory–CNS–motor circuit, the tail segmental nerves were cut and stimulated using the suction electrode while using an intracellular electrode in an m6 muscle fiber (Dasari & Cooper, 2004). The stimulator (S-88, Grass) output was passed through a stimulus isolation unit in order to alter polarity and gain (SIU5, Grass). Electrical signals were recorded on-line via an A/D converter (powerlab 4s interface; ADInstruments). All events were measured and calibrated with SCOPE software version 3.5.4 (ADInstruments). All experiments were performed at room temperature (19–21 °C).

Developmental assays

Stored eggs were cleared from the females by a 15-min pulse, prior to a 2-h egg pulse, in which these eggs were then allowed to develop at 21 °C. Eggs were transferred to vials (15/vial) containing food with PCPA or MDMA so that first instars were in the food straight after hatching. The food was made with 0.5 g of corn meal with 0.5 mL of water mixed with the appropriate concentration of drug. We used corn meal instead of yeast-water for the food as we found a high rate of death with the yeast paste. The deaths were probably due to CO_2 build up with the growth of the yeast. When wandering third instars were seen, the vials were checked every 4 h and each individual pupa was marked on the side of the vials. The time to pupation and the time spent as a pupa were indexed for development.

Heart rate

The HR in third instars was examined and monitored in the same manner as detailed in Dasari & Cooper (2006).

Levels of serotonin and dopamine

The first instars were fed with pharmacological agents as described above. The wandering third instars were used for selective measures on the CNS. Larvae were dissected in HL3 as described above. Brains were placed in dry ice immediately after dissecting. 25 brains were pooled for each sample set and stored at −80 °C prior to high performance liquid chromatography (HPLC) analysis, which was performed at The Center for Sensor Technology (University of Kentucky, KY, USA).

The low level detections of 3,4-dihydroxyphenylacetic acid, 3-hydroxytyramine (DA), 5-hydroxytryptamine (5-HT), 5-hydroxyindole-3-acetic acid and 4-hydroxy-3-methoxy-phenylacetic acid (homovanillic acid) were performed as described previously by Hall *et al.* (1989). In brief, an isocratic HPLC system (Beckman, Inc., Fullerton, CA, USA), at a flow rate of 2 mL/min, was coupled to a dual-channel electrochemical array detector (model 5100A, ESA, Inc., Chelmsford, MA, USA) ($E_1 = +0.35$ mV and $E_2 = -0.25$ mV) using a dual analytical cell (model 5011, ESA, Inc.). The compounds of interest were separated with reverse-phase chromatography, using a C18 column (4.6 mm × 75 mm, 3 μm particle size, Shiseido CapCell Pak UG120, Shiseido Co., Ltd, Tokyo, Japan) with a pH 4.1 citrate-acetate mobile phase, containing 4.0% methanol and 0.34 mM 1-octane-sulfonic acid.

Statistical analysis

When the basic assumption of the parametric Student's *t*-test was valid it was used; otherwise, the non-parametric Wilcoxon rank sum test was used.

Statistically, we wished to determine if the distribution of pupation and eclosion times differed across the treatment groups (controls vs. different doses of PCPA vs. different doses of MDMA). A common method of testing whether distributions are equal is a Kolmogorov-Smirnov (KS) test (Cesani *et al.*, 2006; Mentre & Escolano, 2006). The KS test is based on determining the largest deviation between the

observed distributions (usually called the 'D-statistic') and then comparing that difference with the known distribution of the difference given that the distributions are, in fact, equal. In our context, the KS test was inappropriate for two reasons. First, here we had 'interval-censored' data, meaning that we did not know the exact times of pupation, just the 4-h window in which pupation occurred. This rendered our data discrete (observations occur at a finite number of set times) and the KS test is not appropriate for discrete data (NIST/SEMATECH, 2006, section 7.2.1.2). Second, the KS test does not have an obvious generalization to multiple groups in the manner that a *t*-test generalizes to an ANOVA.

We thus chose to analyse our data with interval censored survival models, which are tailor-made for this type of experimental design. Survival models are implemented in all common statistical software packages (SAS, SPSS, S-plus, R, etc.) and are a standard technique in medical experiments (e.g. see Der & Everitt, 2006) where the event in question is typically death or the recurrence of a disease instead of pupation. Survival models also form the basis of many quality-control experiments (e.g. see Kalbfleisch & Prentice, 2002) where the event in question is the failure of a machine. A defining characteristic of survival analysis is the ability to handle censored data, where the exact time of the event is only known to lie in some region, here 4-h windows.

Survival models are flexible in that the underlying distribution of the event times may be assumed to be Weibull, lognormal, logistic or another common distribution (a semiparametric method, Cox regression, is also possible). The Weibull and lognormal are most common parametric distributions. We initially fit survival models with all of the common distributions to the observed data and found the lognormal to be the best fit (details provided in Results).

A lognormal distribution is described by two parameters, μ and σ, and is characterized by the observation that, if the pupation times have a lognormal (μ,σ) distribution, then the logarithms of the pupation times have a normal (gaussian) distribution with mean μ and SD σ. In fact, were the exact pupation times known, a straightforward analysis would be to take the log transform of the data and then perform an ANOVA. The purpose of the survival model is to perform much of the machinery of the ANOVA (testing for equality of distributions and pairwise comparisons) while accounting for the interval censoring inherent to the experimental design.

We used the statistical software R (a free version of the package S-plus, R Development Core Team, 2005). The built-in function survreg performs a survival analysis regression on the various treatments. Complicating this analysis is the observation that the σ parameters appear to vary across groups. Although ANOVA-like methods are typically robust to the assumption of equal variances, we wanted to allow for differing variances to further illustrate differences between groups.

Thus, for each of the seven groups we used survival analysis to estimate the μ and σ parameters and their SEs. We then performed pairwise comparisons between the groups to look for differences between the parameters. Due to the large number of pairwise comparisons (seven groups with two parameters each result in 42 different comparisons), we adjusted our α-level accordingly. We chose the conservative Bonferroni method, which in this case involves adjusting a standard $\alpha = 0.05$ to $0.05/42 = 0.0012$. The Bonferroni method is conservative (Neter *et al.*, 1990), meaning that we are guaranteed an overall error rate of $\alpha = 0.05$ at the cost of reduced power to detect differences. Fortunately the groups differed by such a degree that this caused no problems.

To summarize the results graphically, we also computed 95% confidence limits on the estimated distribution functions. These were not used to test for differences between groups but merely to demonstrate how the variation in the estimates of μ and σ translates into variation in the rate of pupation (or eclosion) over time. They also represent an exploratory method that looks for regions where the groups differ.

Results

Developmental curves

The first set of general analysis was to examine the time that it took for 50% of the population to pupate. The time for 50% of the population to reach eclosion from the start of pupation was also assessed. PCPA or MDMA was fed, in defined concentrations, to larvae throughout the first to third instar stages while monitoring time to pupation. The time taken by each larva from the laid egg (time = 0) to pupation was calculated. The highest concentration of PCPA (50 mM; 10 mg/mL) showed a drastic delay in the development such that 50% of larvae took a mean time of ~225 h for pupation, whereas 50% of controls pupated at ~125 h (Fig. 1A and B). There was a high death rate in this group. We conducted a study specifically of the death rate by monitoring the survival from first instar to pupa, by placing 10 larvae per vial and using five vials for each condition. These groups were control, PCPA (50 mM) and MDMA (1 mM). The percent survival for each group was: controls, 98% (± 2); MDMA, 60% (± 7) and PCPA, 36% (± 6). Both the MDMA and PCPA groups were significantly different from controls and each other ($P < 0.05$, ANOVA). Because of the significant death rate in flies fed high concentrations of PCPA and MDMA, as compared with controls, a relative cumulative sum graph of survivors better represented the delayed growth rates. Larvae fed PCPA at 50 mM were generally smaller in size on average as compared with controls (this was based on casual observation and not statistically analysed). PCPA at 0.5 mM showed no delay from controls in the time for 50% of animals to pupate and 5 mM showed a slight delay of 35 h from controls for pupation time (Fig. 1A and B). In calculating the amount of time taken for each pupa to eclose, the time point at which pupa were formed was readjusted to a zero time point (time = 0). The time taken for larvae that were fed 50 mM PCPA to eclose was longer than for controls (> 35 h, Fig. 1C and D). They seemed to catch up in eclosion time even though there was a delay in pupation time. A similar result was also seen with the other concentrations of PCPA (Fig. 1C and D).

Results similar to those found with PCPA were seen with MDMA-fed larvae. MDMA at the highest concentration (1 mM) showed a delay in time to pupation of approximately 36 h for 50% of larvae (Fig. 2A and B). The larvae that were fed the lower concentration of MDMA (10 μM) took less time (10 h) for 50% of the animals to pupate compared with controls. The 100 μM MDMA group took ~10 h more than controls for 50% of the animals to pupate (Fig. 2A and B). The eclosion time for larvae that were fed 1 mM was about 20 h more than the controls, whereas the other two concentrations did not show any significant difference from controls (Fig. 2C and D).

As discussed in Materials and methods, a survival analysis was performed on the developmental data sets. This analysis provided assessment over the entire developmental curve. Our first step was to examine how well different standard survival distributions fit the observed data. These analyses are summarized in Fig. 3. The dashed lines in Fig. 3 show the 1 mM MDMA group (the other groups produced similar results). We chose to plot time in hours vs. percent unpupated because the standard in survival analysis is to have the *y*-axis as the percent that have not had an event occur. If preferred, inverting the entire graph would show the percent of larvae pupated

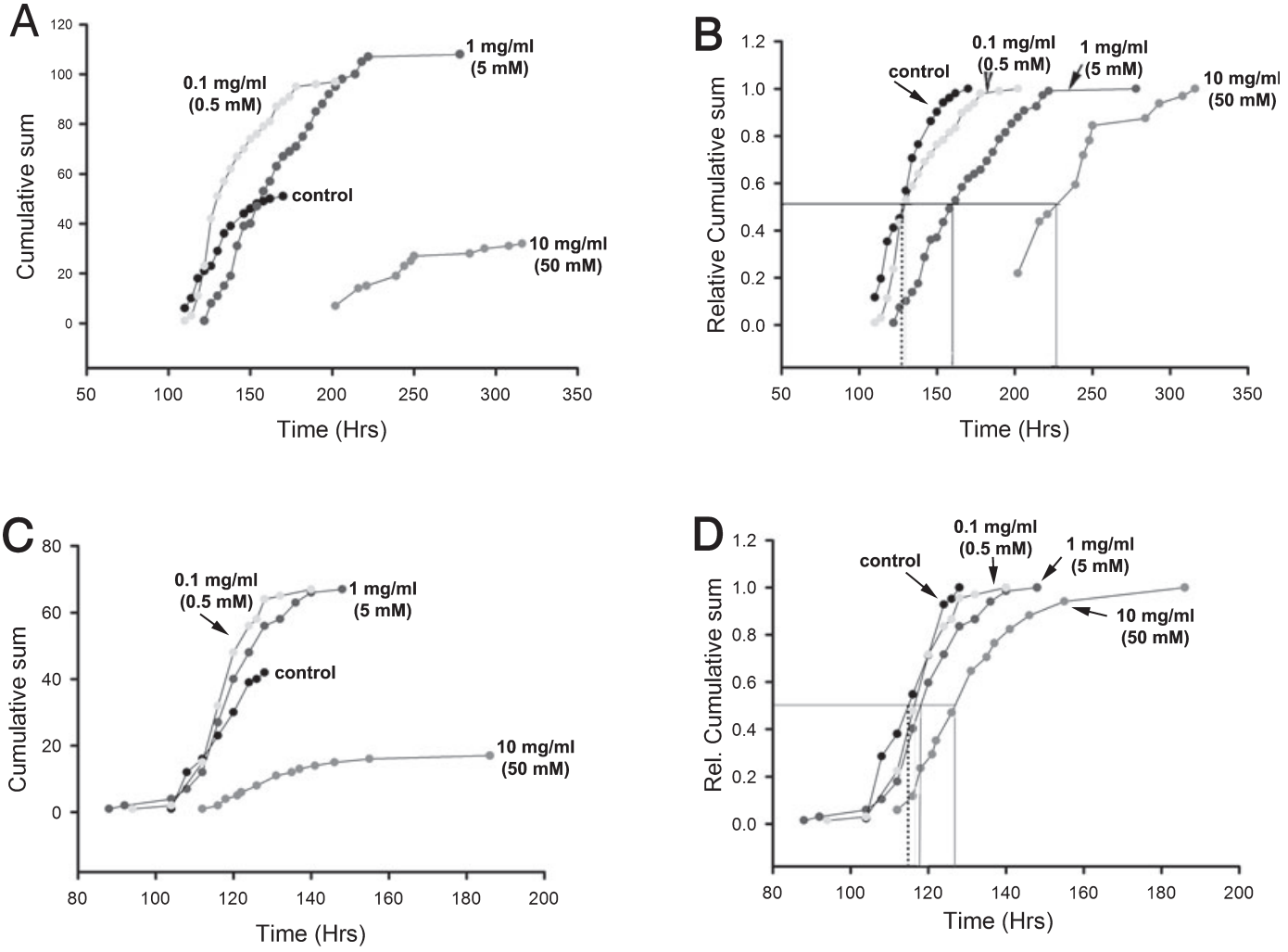

FIG. 1. PCPA growth curve. PCPA was fed from first to third instar stage. The time to pupation from eggs and time to eclosion from pupation were calculated for each larva. (A) Cumulative sum for time to pupation from eggs at different concentrations of PCPA. (B) Relative cumulative sum for A. Larvae fed the highest concentration of PCPA (50 mM) took the longest time to pupation. 50% of larvae took 100 h longer to pupate as compared with control and the lower concentration of PCPA. (C) Cumulative sum for time to eclosion from pupa formation for different concentrations of PCPA. (D) Relative cumulative sum for C. Time for each pupa was adjusted to 0 and calculated time taken from pupation to eclosion. The larvae fed 50 mM took the longest time to eclose but they seemed to catch up with the others in eclosion time.

and would not change the results. The thick curve is the best fitting Weibull distribution, the solid thin curve is the best fitting lognormal and the blue dashed curve is the best fitting logistic distribution. The Weibull distribution has some obvious lack of fit issues. Although the lognormal and logistic appear close, the lognormal is slightly better, particularly for the early observed times. This pattern is consistent across the groups. We also fit log-logistic and gaussian distributions that did not fit the data (note that the exponential distribution, also common in survival or reliability studies, is a special case of the Weibull distribution).

With this established, we fit a lognormal survival regression, where the pupation times in each group are assumed to be lognormal with mean parameter μ and SD parameter σ varying across groups. Note that although it is standard to call μ the 'mean' parameter, it refers to the mean of the log-transformed values and not the pupation times themselves (Casella & Berger, 2002). Furthermore, $\exp(\mu)$ is not the mean of the lognormal distribution.

Table 1 shows the estimated parameters combined with their SEs. Note that for lognormal distributions it is better to work with the logarithm of σ rather than the SD σ itself (the asymptotic results based on the central limit theorem, underlying most commonly used methods, converge faster for $\log \sigma$, see McCullagh & Nelder, 1999). Again, these values are all obtained straightforwardly from the software R using the survreg function.

A hypothesis of equality of all groups with respect to either μ or σ is strongly rejected ($P < 10^{-8}$ for both hypotheses). Thus, we turned to pairwise comparisons to determine which groups differ. As mentioned in Materials and methods, we performed these tests conservatively using a Bonferroni adjusted $\alpha = 0.05/42 = 0.0012$. Table 2 summarizes the *P*-values for each pairwise comparison between means. Thus, each entry represents the *P*-value of comparing the estimated μ from one group with the estimate of μ from another group. A *P*-value of 0.0012 or less is viewed as significant after the Bonferroni adjustment. Values are only shown in the upper triangle of the table because the comparisons are symmetric (a significant difference between groups A and B is equivalent to a significant difference between groups B and A).

Considering the large number of significant differences between groups, it is easier to focus on the few instances where the groups are

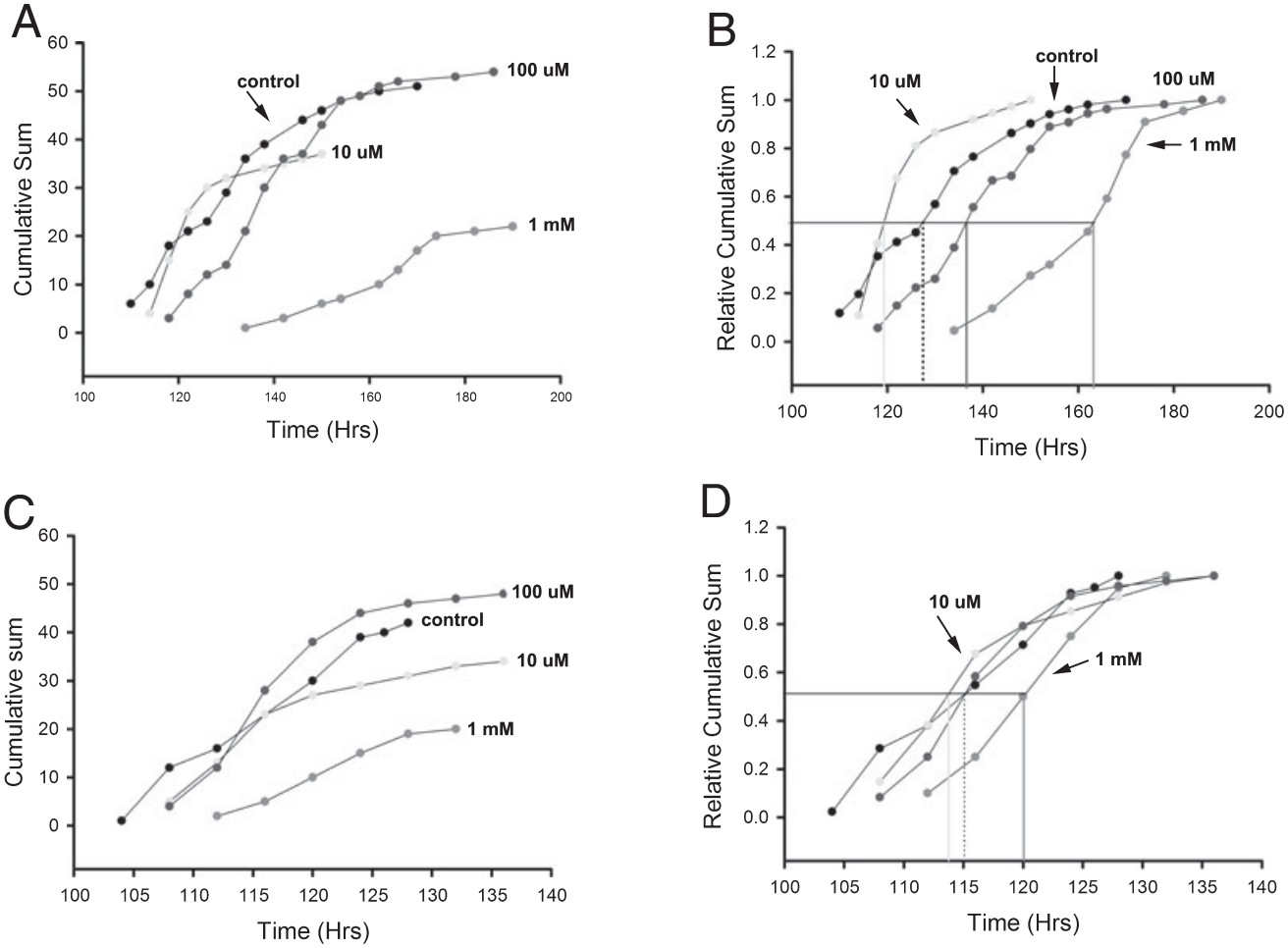

FIG. 2. MDMA growth curve. MDMA was fed from first to third instar stage. The time to pupation from eggs and time to eclosion from pupation were calculated for each larva. (A) Cumulative sum for time to pupation from eggs at different concentrations of MDMA. (B) Relative cumulative sum for A. 50% of larvae took ~165 h for pupation at the highest concentration of MDMA (1 mM). (C) Cumulative sum for time to eclosion from pupa formation for different concentrations of MDMA. (D) Relative cumulative sum for C. As described earlier, time for each pupa was adjusted to 0 and calculated time taken from pupation to eclosion. Pupae of larvae that were fed 1 mM MDMA took a little longer (~5 h more than controls) to eclose as compared with lower concentrations.

not significantly different. Although the *P*-values are relatively small, the low doses of both PCPA (0.1 mg) and MDMA (10 μM) do not significantly differ from the control group. Similarly, the low dose of PCPA (0.1 mg) does not significantly differ from the medium dose of MDMA (100 μM), whereas the medium dose of PCPA (1 mg) does not significantly differ from the high dose of MDMA (1 mM). Thus, both PCPA and MDMA appear to slow down pupation.

The variance parameters do not separate as well (the mean parameters account for most of the shifting between the distributions and we do not need both parameters to change for the groups to be different). What is interesting about the variance parameters (see Table 3) is that no significant differences occur between PCPA groups or MDMA groups after accounting for the multiple comparison issue. This is not statistical proof (not rejecting a hypothesis of equality is not the same as proving equality) but is nevertheless interesting. These results reflect the visual impression from Figs 3 and 4 (and the estimates in Table 1) that the MDMA groups may have less variation than the PCPA groups.

The overall fit of the model can be seen graphically in Figs 3 and 4. We plotted the fitted lognormal distributions and the observed data for each of the seven groups. The black circles and black lines indicate the observed control group data and the fitted control distribution. For the remaining groups, green indicates MDMA groups and red indicates PCPA groups. Solid lines and circles indicate the fitted lognormal distributions and data for the 'low-dose' groups (PCPA, 0.1 mg and MDMA, 10 μM). Dashed lines and triangles indicate the observed data and fitted lognormal distributions for the 'middle-dose' groups (PCPA, 1 mg and MDMA, 100 μM). Dotted lines and crosses indicate the fitted lognormal distributions and data for the 'high-dose' groups (PCPA, 10 mg and MDMA, 1 mM). In addition, 95% confidence limits for each percentile are plotted to illustrate the variation in the estimates. These limits are present for visualization and not used for testing.

The eclosion data (see Table 4) produce many fewer significant differences. Although the estimates of μ for all treatment (PCPA and MDMA) groups are higher than the estimate of μ for the control group, and the mean parameter increases with the dosage of both PCPA and MDMA, the differences between the groups do not reach the level of statistical significance except in a few cases. The corresponding *P*-values are shown in Table 5.

As can be seen, the only group that is significantly different from all other groups is the high-dose PCPA (10 mg) group. The only other significant difference is the MDMA (1 mM) group compared with the control group. Thus, it still seems that PCPA and MDMA slow down

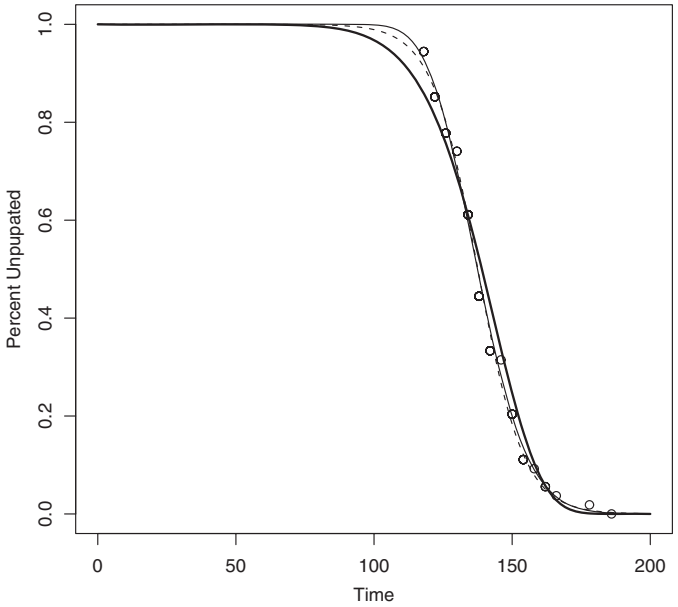

FIG. 3. Illustration of goodness of fit for Weibull (thick line), lognormal (solid, thin line) and logistic (dashed line) distributions to the MDMA (1 mM) group. The Weibull distribution has clear lack of fit problems, whereas the lognormal and logistic curves are both reasonable. The lognormal curve has a slight advantage for earlier survival times, so the lognormal distribution was used in the subsequent analyses.

TABLE 1. The estimated parameters for pupation combined with their standard errors (SEM)

Group	Estimate for μ	Estimate for σ	Log(σ)	SEM (μ)	SEM (log(σ))
Control	4.839	0.123	−2.096	0.017	0.109
PCPA 0.1 mg	4.906	0.136	−1.992	0.014	0.073
PCPA 1 mg	5.087	0.171	−1.764	0.017	0.069
PCPA 10 mg	5.423	0.144	−1.943	0.026	0.131
MDMA 10 μM	4.796	0.070	−2.654	0.012	0.128
MDMA 100 μM	4.923	0.104	−2.263	0.014	0.101
MDMA 1 mM	5.070	0.089	−2.417	0.019	0.159

eclosion in high doses but the effect is much less for eclosion than for time to pupation (see Table 6). The information on σ, apart from allowing the data to be fit well, does not seem to provide much evidence concerning differentiating groups. Figures 5 and 6 are analogs to Figs 3 and 4 and provide 95% confidence limits for the percentiles of the fitted distributions (Fig. 7).

Behavior (mouth hook and body wall contractions)

Simple feeding (mouth hook movements) and locomotor (body wall movements) behaviors were tested for larvae that were fed PCPA (50 mM) and MDMA (10 μM). Larvae ate these compounds from the first to third instar stage and the mid-third instars were used for behavioral analysis. Each larva was first used to count body wall contractions and then for mouth hook contractions. Body wall and mouth hook contractions were counted for 1 min and averaged. Compared with controls, PCPA- and MDMA-fed larvae showed significant lower body wall and mouth hook contractions ($N = 20$, Fig. 8, ANOVA < 0.0001). Some of the PCPA larvae were seen to be crawling with difficulty and some just contracted their body once or twice.

Spontaneous activity (serotonin and MDMA)

The intrinsic activity of the CNS was assessed by monitoring motor commands to muscles m6 or m7. Both of these muscles receive the same innervation by two motor axons (Kurdyak *et al.*, 1994). We noted substantial variation among preparations in the extent of the spontaneous activity and bursting frequency but the spontaneous activity can be ensured to occur by pulling the edges of the cuticle taut in the experimental chamber. Measurements prior to and during exposure to various compounds give an approach to determine if the CNS circuit that initiates the motor commands is sensitive to the particular agent of interest. We utilized this experimental approach for examining the sensitivity of the CNS to 5-HT and MDMA at various doses. However, the approach has some difficulties as an enhanced response can be observed with an initial exposure but rapid desensitization can occur after only a few minutes depending on the dose and compound. For example, application of MDMA resulted in a marked prolonged busting behavior followed by short high-frequency bursts (Fig. 9A for saline and Fig. 9B for MDMA). This was subsequently followed by decreased basal activity and inactivity to evoke a sensory–CNS–motor response by electrical stimulation of an afferent nerve root. The bursts over time became less frequent and shorter in duration (Fig. 9C).

In quantifying the bursting behavior all of the peaks within each burst and the occurrences of the burst were recorded. The frequency of bursts per second followed by average frequency per second was used for comparisons. A percent difference before and during the drug exposure was calculated for each preparation and compared among treatment groups (Fig. 10).

The spontaneous activity measured in sham saline trials showed a small increase of 20% (Fig. 10A). This was probably due to the disturbances in the bath of sensory hairs, which results in a motor unit response. Control larvae showed a dose-dependent increase in activity in the presence of 5-HT (Fig. 10A), whereas lower concentrations of

TABLE 2. *P*-values for each pairwise comparison between means of μ for pupation

	Control	PCPA 0.1 mg	PCPA 1 mg	PCPA 10 mg	MDMA 10 μM	MDMA 100 μM	MDMA 1 mM
Control		0.0027	< 0.0001*	< 0.0001*	0.0392	0.0002*	< 0.0001*
PCPA 0.1 mg			< 0.0001*	< 0.0001*	< 0.0001*	0.4038	< 0.0001*
PCPA 1 mg				< 0.0001*	< 0.0001*	< 0.0001*	0.4989
PCPA 10 mg					< 0.0001*	< 0.0001*	< 0.0001*
MDMA 10 μM						< 0.0001*	< 0.0001*
MDMA 100 μM							< 0.0001*
MDMA 1 mM							

**P*-value of 0.0012 or less is viewed as significant after the Bonferroni adjustment

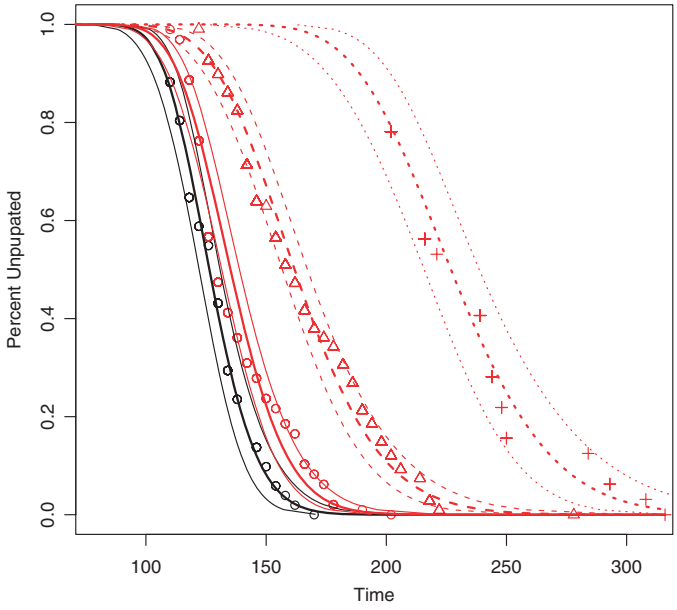

FIG. 4. Fitted lognormal distributions to each para-chlorophenylalanine (PCPA) group for the pupation data. The black circles and black line indicate the observed control group data and the fitted control distribution. Solid lines and circles indicate the fitted lognormal distribution and data for the low-dose group (PCPA, 0.1 mg). Dashed lines and triangles indicate the observed data and fitted lognormal distributions for the middle-dose group (PCPA, 1 mg). Dotted lines and crosses indicate the fitted lognormal distributions and data for the high-dose group (PCPA, 10 mg). 95% confidence limits for each percentile are also shown around the fitted distributions.

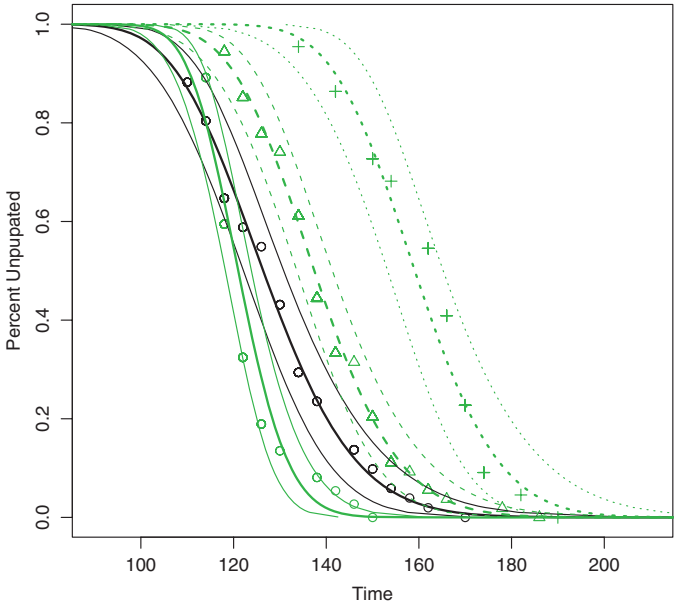

FIG. 5. Fitted lognormal distributions to each MDMA group for the pupation data. The black circles and black line indicate the observed control group data and the fitted control distribution. Solid lines and circles indicate the fitted lognormal distribution and data for the low-dose group (MDMA, 10 μM). Dashed lines and triangles indicate the observed data and fitted lognormal distributions for the middle-dose group (MDMA, 100 μM). Dotted lines and crosses indicate the fitted lognormal distributions and data for the high-dose group (MDMA, 1 mM). 95% confidence limits for each percentile are also shown around the fitted distributions.

MDMA (100 nM and 10 μM) showed a small increase and 100 μM MDMA showed a decrease in activity (Fig. 10A). There was a substantial amount of variation from preparation to preparation.

Larvae that were fed 50 mM PCPA showed an increase in activity on application of 10 and 100 nM 5-HT, whereas at 10 μM 5-HT a decrease in responsiveness was observed (Fig. 10B). The receptors probably undergo desensitization at this high concentration of 5-HT. It is known that some 5-HT receptor subtypes can desensitize rapidly (i.e. in seconds; Hu *et al.*, 2006). In addition, at this high concentration there may be activation of different receptor subtypes with a lower affinity, which might inhibit the neural circuit. Larvae that were fed 100 μM MDMA showed an increase in activity at all concentrations of 5-HT, although it was a very small increase (Fig. 10C). As for the acute studies, the CNS activity for the long-term effects of p-PCPA or MDMA also showed a large variation among preparations. No statistical significance was seen among these groups (ANOVA, $P > 0.05$; Fig. 10A–C).

Evoked sensory–central nervous system–motor circuit (serotonin and MDMA)

The sensitivity of central circuits to 5-HT and MDMA at various doses was examined by stimulating sensory nerves and monitoring motor units before and during exposure to the compounds. The experiments above addressed intrinsic spontaneous activity, whereas in this set of experiments the circuit was driven by electrical stimulation. There was a clear dose-dependent effect of both 5-HT and MDMA, but in opposing actions, in altering the evoked CNS activity (Fig. 11A). Sham controls were performed as the disturbance of changing solutions could result in sensory activity and thus drive a motor response. A slight increase occurred in a few preparations but overall this sham was non-significant. The preparations were used once for each condition. The mean percent increase in response increased as well as the variability from exposure to 10 nM, 100 nM and 10 μM 5-HT (ANOVA, $P < 0.0001$, $n = 7$). Tukey posthoc test showed only exposure to 10 μM 5-HT as significantly different from shams ($P < 0.05$). To our surprise, MDMA caused a decrease in evoked responsiveness that was dose-dependent except that the variation among preparations was very consistent unlike that for 5-HT at higher doses (Fig. 11A). The effects of MDMA were significantly different from shams (ANOVA, $P < 0.0001$).

PCPA-fed larvae showed a decreased response when exposed to 5-HT. The decrease in activity was dose-dependent. 5-HT at 10 μM showed an approximately 50% decrease compared with saline whereas 100 nM showed a 30% decrease in activity (Fig. 11B). This was unexpected. Possibly preparations were desensitized very fast on application of 5-HT. However, MDMA-fed larvae showed an increase in activity when exposed to 5-HT. Exposure to 10 nM 5-HT showed a larger increase as compared with 100 nM or 10 μM 5-HT (Fig. 11B). Both PCPA- and MDMA-fed larvae showed a significant difference as compared with shams (ANOVA, $P < 0.0001$). Tukey test showed that MDMA-fed larvae exposed to 10 nM 5-HT showed a significant difference ($P < 0.05$).

Heart rate

To examine if larvae altered their 5-HT sensitivity in cardiac function to long-term depletion of 5-HT, the larvae were fed PCPA from the first to third instar. In mid-third instar the larvae were dissected and the HR monitored for alteration to exogenous application of 5-HT. The

TABLE 3. *P*-values for each pairwise comparison between means of σ for pupation

	Control	PCPA 0.1 mg	PCPA 1 mg	PCPA 10 mg	MDMA 10 μM	MDMA 100 μM	MDMA 1 mM
Control		0.0102	0.4258	0.3683	0.0971	0.0009*	0.2630
PCPA 0.1 mg			0.0233	0.2297	0.0002*	< 0.0001*	< 0.0001*
PCPA 1 mg				0.7432	0.0153	< 0.0001*	0.0297
PCPA 10 mg					0.0217	0.0001*	0.0535
MDMA 10 μM						0.2470	0.4159
MDMA 100 μM							0.0168
MDMA 1 mM							

**P*-value of 0.0012 or less is viewed as significant after the Bonferroni adjustment

TABLE 4. The estimated parameters for eclosion combined with their standard errors (SEM)

Group	Estimate for μ	Estimate for σ	Log(σ)	SEM (μ)	SEM (log(σ))
Control	4.746	0.062	−2.782	0.009	0.107
PCPA 0.1 mg	4.769	0.069	−2.674	0.008	0.082
PCPA 1 mg	4.776	0.098	−2.324	0.011	0.082
PCPA 10 mg	4.868	0.086	−2.452	0.016	0.131
MDMA 10 μM	4.760	0.068	−2.693	0.010	0.111
MDMA 100 μM	4.763	0.058	−2.852	0.008	0.096
MDMA 1 mM	4.792	0.043	−3.154	0.008	0.144

larval HR increased upon exposure to 5-HT (100 nM); however, there was no significant increase above controls for larvae depleted of 5-HT (Fig. 12). Controls increased their HR by 31% and PCPA-fed larvae by 28% (not significantly different from controls). The rationale for examining the HR is that it can serve as an additional assay for responsiveness to pharmacological agents that work through 5-HT receptors as the heart is sensitive to 5-HT.

Levels of serotonin and dopamine

It is important to determine the amount of depletion of 5-HT in the CNS as well as the whole body to correlate with the delayed development in PCPA- and MDMA-treated animals. The brains from mid-third instar larvae were individually dissected out for HPLC analysis. At least five samples, with each sample containing 25 pooled brains, were analysed. The HPLC results showed that control brains ($n = 6$) contained about 12–14 pg/brain of 5-HT and DA (Fig. 13A). Treatment with PCPA (50 mM) caused a significant decrease in 5-HT levels in larval brains, by approximately 90% ($n = 5$, ANOVA, $P < 0.05$, Fig. 1A). Larvae that ate PCPA also showed a significant decrease in DA levels ($n = 5$, ANOVA, $P < 0.05$, Fig. 13A). MDMA treatments did not produce a difference in the levels of DA or 5-HT ($n = 5$, Fig. 13A). Treatment with 5,7-DHT, a compound that kills serotonergic neurons in vertebrates, showed a small decrease in the mean 5-HT levels but it was not significant (Fig. 13A). 5,7-DHT appears not to function in killing 5-HT neurons in *Drosophila* as established in vertebrates, as otherwise there would be little, if any, 5-HT left in the CNS samples that were exposed to 5,7-DHT for 4 days.

TABLE 5. *P*-values for each pairwise comparison between means of μ for eclosion

	Control	PCPA 0.1 mg	PCPA 1 mg	PCPA 10 mg	MDMA 10 μM	MDMA 100 μM	MDMA 1 mM
Control		0.0483	0.0362	< 0.0001*	0.2889	0.1360	0.0002*
PCPA 0.1 mg			0.6406	< 0.0001*	0.4870	0.5889	0.0506
PCPA 1 mg				< 0.0001*	0.3116	0.3633	0.2473
PCPA 10 mg					< 0.0001*	< 0.0001*	< 0.0001*
MDMA 10 μM						0.8097	0.0185
MDMA 100 μM							0.0127
MDMA 1 mM							

**P*-value of 0.0012 or less is viewed as significant after the Bonferroni adjustment

TABLE 6. *P*-values for each pairwise comparison between means of σ for eclosion

	Control	PCPA 0.1 mg	PCPA 1 mg	PCPA 10 mg	MDMA 10 μM	MDMA 100 μM	MDMA 1 mM
Control		0.4254	0.0007*	0.0524	0.5642	0.6256	0.0382
PCPA 0.1 mg			0.0026	0.1530	0.8913	0.1593	0.0038
PCPA 1 mg				0.4051	0.0075	< 0.0001*	< 0.0001*
PCPA 10 mg					0.1621	0.0142	0.0003*
MDMA 10 μM						0.2777	0.0111
MDMA 100 μM							0.0814
MDMA 1 mM							

**P*-value of 0.0012 or less is viewed as significant after the Bonferroni adjustment

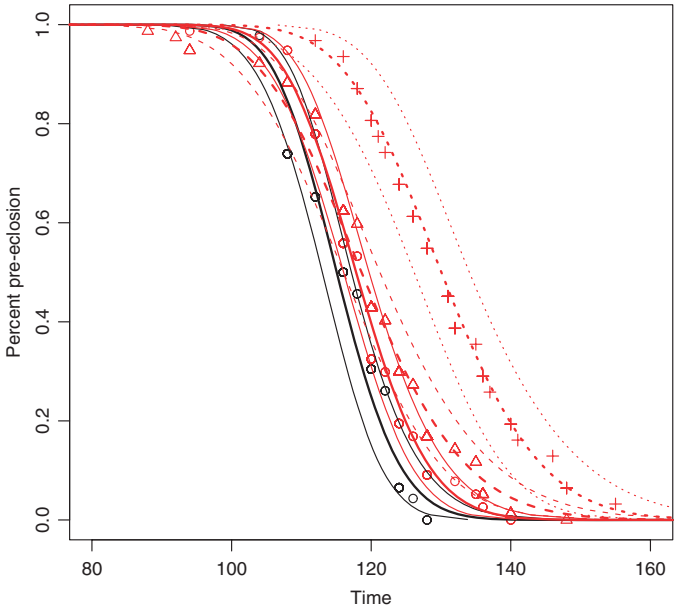

FIG. 6. Fitted lognormal distributions to each para-chlorophenylalanine (PCPA) group for the pupation data. The black circles and black line indicate the observed control group data and the fitted control distribution. Solid lines and circles indicate the fitted lognormal distribution and data for the low-dose group (PCPA, 0.1 mg). Dashed lines and triangles indicate the observed data and fitted lognormal distributions for the middle-dose group (PCPA, 1 mg). Dotted lines and crosses indicate the fitted lognormal distributions and data for the high-dose group (PCPA, 10 mg). 95% confidence limits for each percentile are also shown around the fitted distributions.

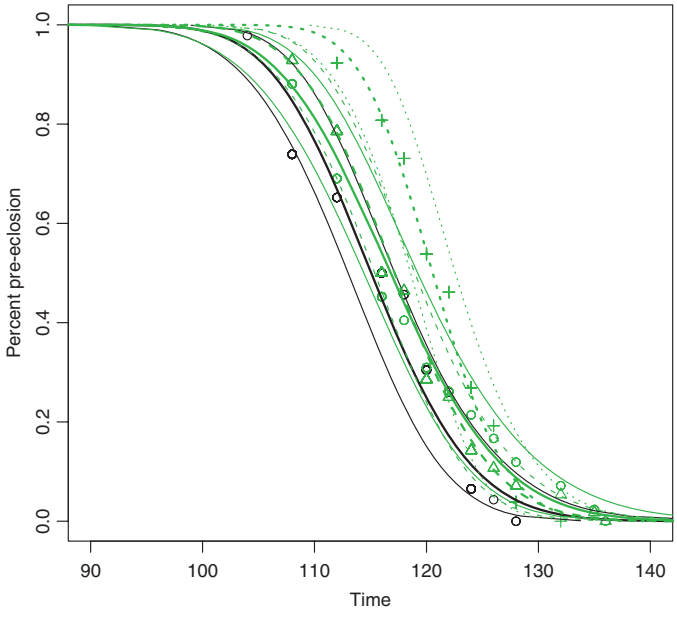

FIG. 7. Fitted lognormal distributions to each MDMA group for the eclosion data. The black circles and black line indicate the observed control group data and the fitted control distribution. Solid lines and circles indicate the fitted lognormal distribution and data for the low-dose group (MDMA, 10 μM). Dashed lines and triangles indicate the observed data and fitted lognormal distributions for the middle-dose group (MDMA, 100 μM). Dotted lines and crosses indicate the fitted lognormal distributions and data for the high-dose group (MDMA, 1 mM). 95% confidence limits for each percentile are also shown around the fitted distributions.

Intact larvae were also collected from the same culture vials as above for each group and washed in water to remove food residues. 20 larvae were pooled together for each set and subjected to HPLC. Control larvae had approximately 500 pg/larva of DA and 300 pg/larva of 5-HT. No significant differences in levels of 5-HT and DA occurred with either PCPA- or MDMA-treated groups as compared with controls (Fig. 13B).

Larvae were fed different concentrations of PCPA (0.5, 5 and 50 mM) and MDMA (10 μM, 100 μM and 1 mM) and allowed to form pupae. Vials were checked every 4 h for newly eclosed adults. The adults were collected and frozen immediately. The heads were chopped off these adult flies and five heads were pooled for each set. The analysis of HPLC data revealed no significant difference in DA or 5-HT levels in either of the groups as compared with controls, except for the group treated with 1 mM MDMA (Fig. 13C and D). The larvae fed 1 mM MDMA showed a significant increase above controls in levels of both 5-HT and DA ($n = 5$, ANOVA, $P < 0.05$, Fig. 13C and D).

Discussion

In this study we demonstrated that feeding larvae PCPA or MDMA retards development and decreases activity associated with crawling and eating behaviors. HPLC analysis of 5-HT and DA for the whole larvae did not show any effects of the drug treatments, whereas selectively measuring the brains of larvae showed significant effects. The levels of PCPA that slowed development also reduced the concentrations of 5-HT and DA in third instar larval brains. This effect of PCPA is dose-dependent. However, treatment with MDMA did not produce any alterations in the 5-HT and DA in the CNS of larvae but produced a dose-dependent increase in both 5-HT and DA in newly eclosed adults that ate MDMA as larvae. Initial exposure of the larval brain to MDMA caused an increase in spontaneous activity, which was short lived (~3 min), and in fact decreased evoked sensory–CNS–motor activity afterwards. In contrast, 5-HT increased evoked activity in a dose-dependent manner but did not significantly alter spontaneous activity. However, when the larvae were depleted of 5-HT throughout development they did not increase their sensitivity to the excitatory response of 5-HT but showed a more pronounced inhibitory action of 5-HT on the central circuit. The opposite effect occurred for larvae fed MDMA, in that treatment with MDMA throughout larval development resulted in the central circuit being more responsive to 5-HT.

As there was a dose-dependent effect in larvae fed PCPA or MDMA in slowing down the rate of development as well as increasing the mortality rate, it would appear that the involvement of the targets for PCPA and MDMA is vital to the health of larvae. Direct and indirect effects are both likely to contribute to the offset development. As larvae fed PCPA or MDMA showed reduced mouth hook movements one would logically ask whether a reduced diet was a limiting factor. We could assay the total amount of food within the digestive system of larvae with a dye technique but such approaches do not show if the nutrients are absorbed from the digestive system. Possibly animals absorbed even more nutrients if retention within the digestive system was enhanced due to a decrease in gut motility from treatments with PCPA or MDMA. Whole animal HPLC assays did not show a significant decrease in 5-HT with PCPA treatments and, given that 5-HT can modulate gut motility in many invertebrates (Katz & Harris-Warrick, 1989; Ayali & Harris-Warrick, 1999), it is possible that there was no effect on digestive processes in *Drosophila* larvae. It is just as likely that there may well be neural regulation of gut motility in larval

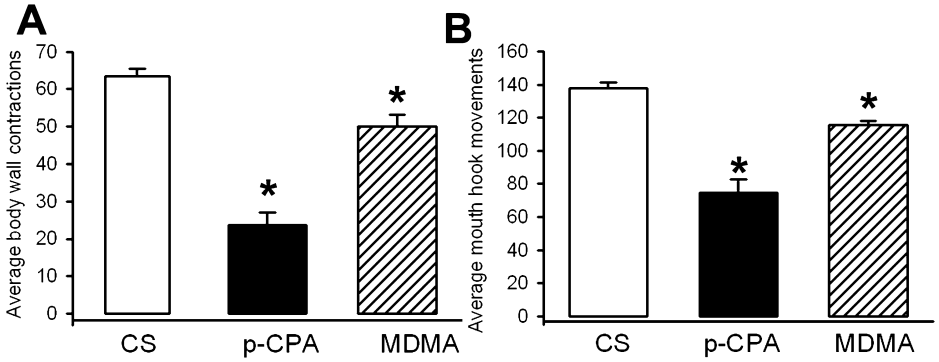

FIG. 8. Body wall and mouth hook contractions. (A) Body wall contractions were counted for 1 min and averaged per minute. PCPA- or MDMA-fed larvae showed a decrease in body wall movements as compared with controls ($N = 20$, ANOVA, $P < 0.05$). (B) Mouth hook contractions were counted in yeast solution for 1 min and averaged per minute. Both PCPA- and MDMA-fed larvae showed a decrease in mouth hook movements as compared with controls ($N = 20$, ANOVA, $P < 0.05$). *$P < 0.05$.

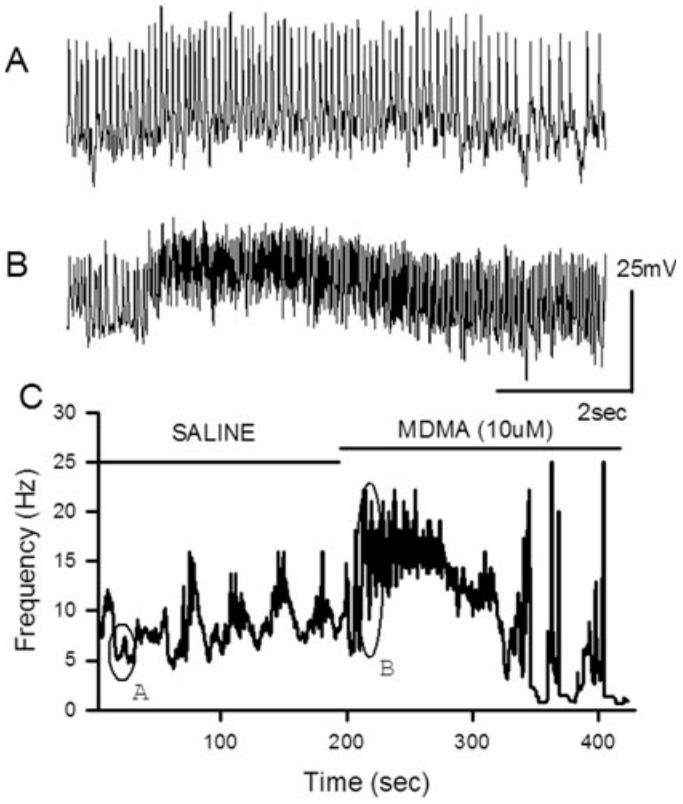

FIG. 9. The intrinsic activity of motor neurons in saline (A) and during the initial exposure to MDMA (B). The initial enhancement by MDMA is short-lived followed by short high-frequency bursts and then an overall reduced basal activity (C). The circled regions in C represent the traces shown in A and B.

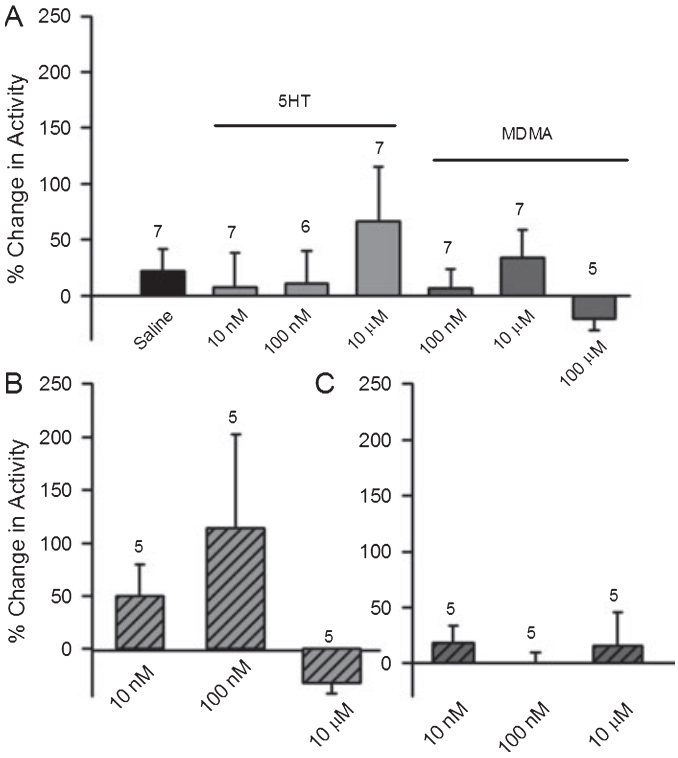

FIG. 10. Spontaneous activity. Percent change in average frequency per second (activity) for acute drug exposures (A), PCPA-fed larvae (B) and MDMA-fed larvae (C). Three different concentrations of serotonin (5-HT) and MDMA were used on the larval central nervous system. The number above the bar shows the sample size.

Drosophila that can be altered by the decrease in 5-HT within the CNS. Autonomic neural function of digestive properties in insects (Copenhaver & Taghert, 1989, 1991; Zavarzin, 1941; Penzlin, 1985) and crustaceans (Shuranova *et al.*, 2006) is established but how the autonomic digestive function may be regulated by serotonergic or DA circuits effected by PCPA treatments within the CNS has not been addressed. As for the mechanism of MDMA slowing down development at high doses, it is clearly not a lack of 5-HT in the CNS as the HPLC results showed that 5-HT is not substantially reduced as it is in mammals with MDMA treatment.

The development of some circuits within the CNS of arthropods is known to be dependent on 5-HT. The olfactory neurons in *Manduca sexta* (moth) are 5-HT sensitive (Kloppenburg & Hildebrand, 1995) and the correct development of neuronal processes within the olfactory center is dependent on 5-HT (Hill *et al.*, 2003). The central development of the olfaction circuits in crustaceans (Sandeman *et al.*, 1995) and *Caenorhabditis elegans* (Nuttley *et al.*, 2002) is also dependent on 5-HT. 5-HT alters the visual sensitivity in the eyes of flies (Chen *et al.*, 1999) as well as crustaceans (Aréchiga & Huberman, 1980). This, in turn, could have a substantial effect on development of the neural circuit as it is well established from invertebrates (Payne, 1911; Roach & Wiersma, 1974; Cooper *et al.*, 2001; Scott *et al.*, 2003) to mammals (Hubel & Wiesel, 1970) that activity in the visual system sculpts the peripheral and central circuits. As the neuroendocrine axis

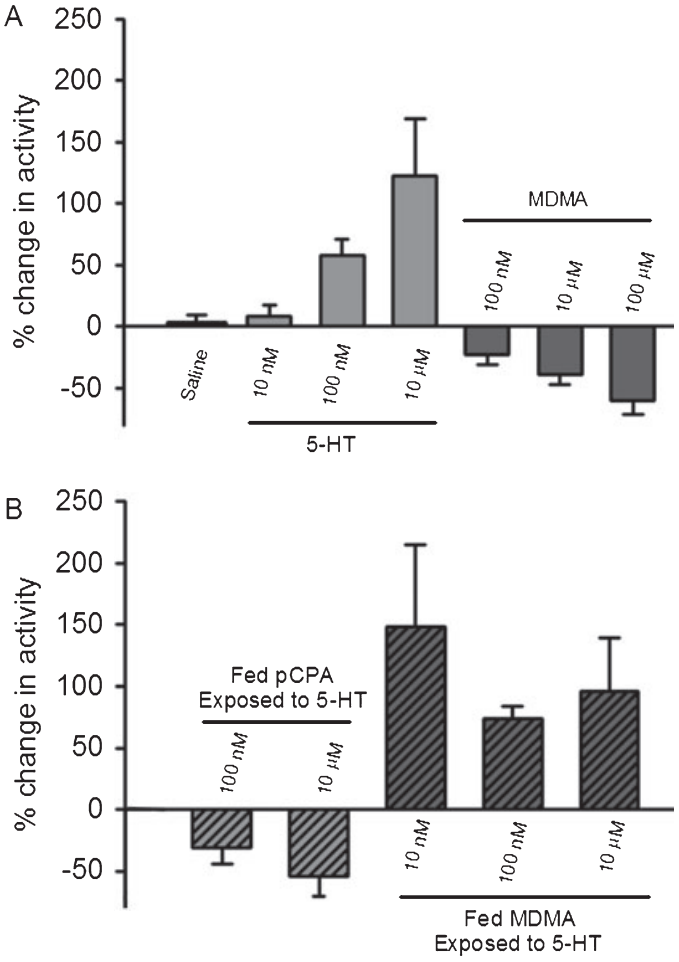

FIG. 11. Evoked sensory–central nervous system–motor circuit. (A) A percent difference in the activity of motor units to muscle 6 before and during application of serotonin (5-HT) or MDMA. Saline shams showed a small increase. 5-HT produced a dose-dependent significant increase, whereas MDMA showed a dose-dependent significant decrease in activity from shams (ANOVA, $P < 0.0001$). (B) Larvae fed 50 mM PCPA and 100 μM MDMA were exposed to 5-HT at different concentrations. The percent difference in activity is recorded before and after 5-HT exposure. PCPA-fed larvae showed a significant decrease in sensitivity to 5-HT, whereas MDMA-fed larvae showed a significant increase in activity in the presence of 5-HT (ANOVA, $P < 0.0001$).

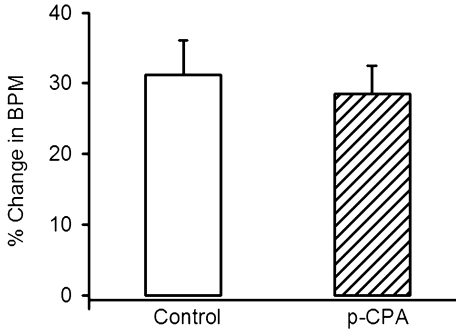

FIG. 12. The percent change in heart rate [beats per minute (BPM)] upon exposure to serotonin (100 nM). Both controls and larvae fed PCPA showed a similar and significant ($n = 7$; $P < 0.05$, Student's *t*-test) increase in heart rate without a significant difference between the groups.

in invertebrates is sensitive to 5-HT (Lee *et al.*, 2000), this could alter the development of the whole animal as well as specific neuronal processes and neurohormones with very broad actions (Nässel, 2002). Likewise, if the circadian patterns are altered due to decreased neuronal 5-HT or direct action of MDMA on 5-HT receptors then one would expect endocrine-related developmental abnormalities. Given that PCPA did not reduce just 5-HT but also DA in the larval brain there are many likely effects of having two very important chemical substances reduced within the larval CNS. Until there is more information on the role of DA and 5-HT in the CNS of flies it may be too early to speculate on mechanisms of action to account for the phenomenon that we report in this study.

Genes that regulate tryptophan hydroxylase (TPH) for the biochemical synthesis of 5-HT are known in *Drosophila* and there are two different genes encoding for two different forms of the enzyme. One type is expressed in the periphery and another within the CNS (Coleman & Neckameyer, 2005). A disease state of phenylketonuria can occur when there are defects in phenylalanine hydroxylase in mammals (Lenke & Levy, 1980). In this pathological state it is known that tyrosine hydroxylase and TPH are inhibited, which leads to reduced 5-HT and associated neuronal damage (Roux *et al.*, 1995; Colas *et al.*, 1999) that impinge on developmental rates, thus a similar situation may occur in *Drosophila*. The associated higher death rate with larvae fed MDMA and PCPA in high doses points to some significance of the serotonergic and possibly the dopaminergic (for PCPA) system in overall health as these systems are probably the primary site of drug action.

The slowed developmental time from egg to pupation and pupation to eclosin with PCPA and MDMA treatments is surprising as MDMA did not result in reduced 5-HT in the larval brain. Thus, the retardation in development cannot be solely due to reduced 5-HT but may be due to altered central neural activity as both changed spontaneous as well as evoked patterns of central circuits. An easy approach to index the rate of development was a time measure for 50% of the larvae to pupate or pupae to eclose but this does not provide a full spectrum of the developmental dynamics; thus, we took the laborious task of checking fly cultures every 4 h throughout the day and night for weeks to obtain the developmental curves. Likewise, the survival analysis undertaken is tailored specifically to the analysis of 'time-to-event' data observed at intervals. Although a KS test also analyses distributions over the entire time span, the KS test is not designed for data observed at intervals and nor is there an obvious extension to multiple curves, which the survival model handles naturally. The interval-censored survival models that were designed for this type of experimental design for time to pupation or eclosion allowed 95% confidence intervals to be placed along the whole developmental time for comparisons on the groups. The ability to fit a parametric survival model also allows, through the parameters, an investigation separating the effects of PCPA (or MDMA) on the mean response as opposed to the variation in the response. This is seen in Tables 1–3. Finally, when a parametric model is appropriate, the ability of the experiment to detect differences between groups (statistical power) is increased.

The behavioral similarities, as with overall development, associated with both PCPA and MDMA indicate that the overall health of the animal might be comprised by treatments in a dose-dependent manner. Despite 5-HT levels not declining with MDMA treatments, it is possible that just the dis-synchronization of appropriate neural activity did not allow the animal to perform coordinated motor commands, whereas the depletion of 5-HT in the CNS by PCPA may have produced altered neural activity differently from that induced by MDMA. Both compounds may have involved the serotonergic system. 5-HT is associated with the modulation of eating/digestion in

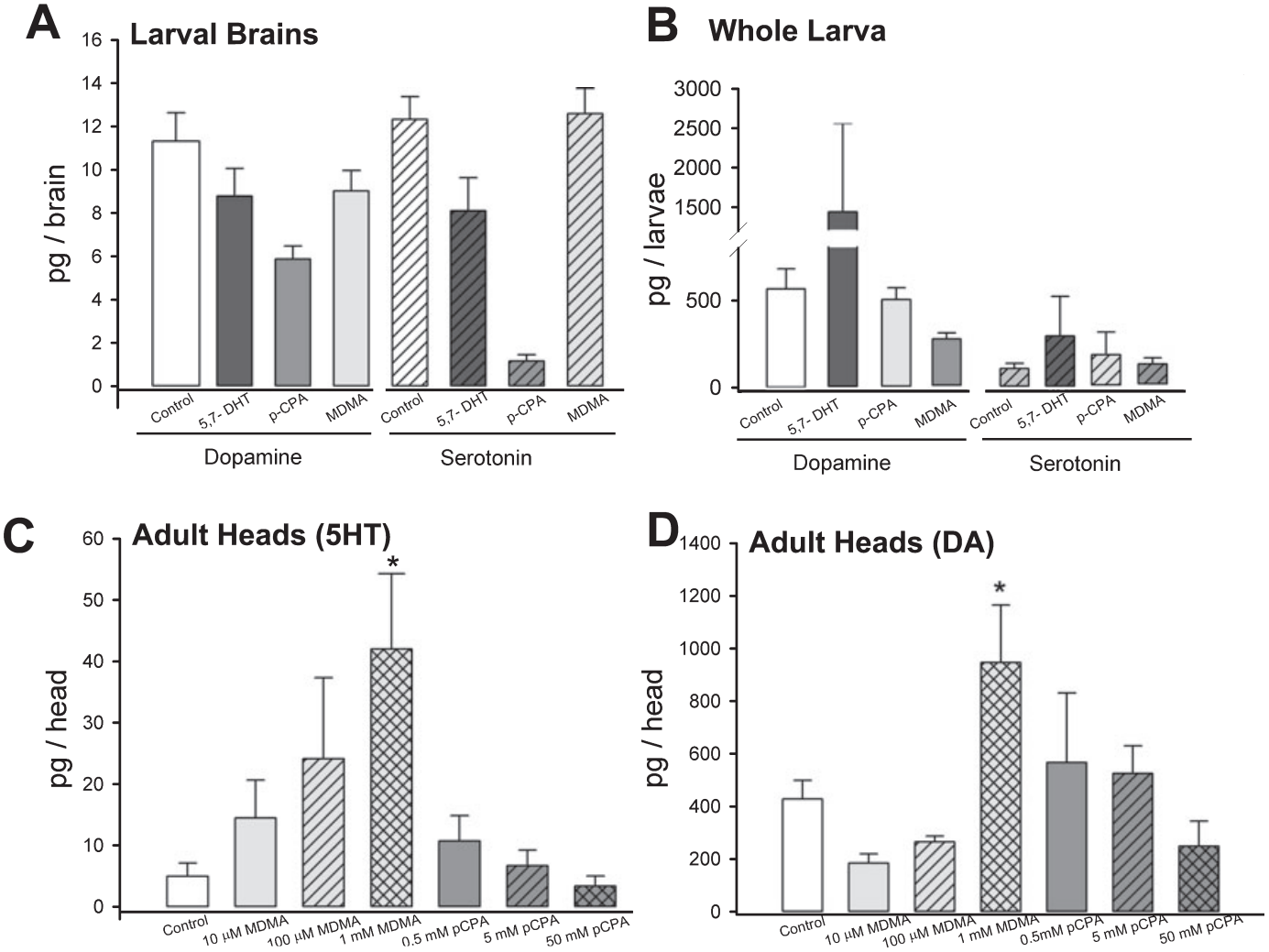

FIG. 13. High performance liquid chromatography (HPLC) analysis of third instar larvae. (A) HPLC analysis on third instar larval brains. PCPA-treated larval brains ($n = 5$) showed a decrease in dopamine (DA) (open bars, ANOVA, $P < 0.05$) and serotonin (5-HT) (hashed bars, ANOVA, $P < 0.05$) levels. MDMA and 5,7-DHT did not show a difference ($n = 5$ and 3, respectively). (B) DA and 5-HT levels in whole larvae. None of the treated groups showed any significant difference from the control group ($n = 5$). (C) DA levels in adult heads collected after they had been treated with drugs from first to third instar stage. 1 mM MDMA showed a significant increase in DA levels ($n = 5$, ANOVA, $P < 0.05$). (D) 5-HT levels in adult heads. Except for 1 mM MDMA other groups did not show any difference in levels compared with controls ($n = 5$). *$P < 0.05$.

crustaceans (Shuranova *et al.*, 2006) and humans (Aubert *et al.*, 2000) as well as with motor unit coordination (Weiger, 1997; Strawn *et al.*, 2000; Dasari & Cooper, 2004; LeBeau *et al.*, 2005) and behavior (Bicker, 1999; Barnes & Sharp, 1999; Toth *et al.*, 2005) in a wide variety of animals. Likewise, MDMA in humans promotes mastication and heightened activity for motor function. The few minutes of increased central motor commands in the larvae on exposure to MDMA prior to the decrease-evoked responses is contrary to what was expected as we predicted that a further increase in 5-HT release would mimic the exogenous application of 5-HT. However, the proposed synaptic model of MDMA action in mammals (Green *et al.*, 2003; Simantov, 2004; Sprague & Nichols, 2005) may not hold for *Drosophila*. In addition, the surprising results of PCPA reducing not only 5-HT but also DA indicate careful assessment of extrapolating mechanisms of drug action noted in mammals to invertebrates. Such oversights have also been noted to occur for pharmacological serotonergic agents used in crustacean behavioral research (Sparks *et al.*, 2003), which is due to various cellular cascades and receptor subtypes (Tierney, 2001; Clark *et al.*, 2004).

The activity of the CNS in the absence of electrical evoking of sensory neurons but by having the larvae dissected open, stretched and pinned down probably results in some sensory activity that elicits the spontaneous bursting of motor neurons that is often seen. The action of 5-HT and MDMA shown in this study demonstrates that this induced state of the CNS is sensitive to 5-HT as well as MDMA but the exact mechanisms have not been elucidated in this report. It would be of interest to try selective 5-HT receptor blockers and to repeat these descriptive studies. As the electrically evoked sensory–CNS–motor circuit shows that defined stimulatory conditions can induce a motor command, the location of action within the circuit can now be dissected out with more refined experimental paradigms. The actions of MDMA or 5-HT in general in enhancing sensory responsiveness in humans, such as tactile mechanoreception, being mimicked in an insect as well as in a crustacean (Strawn *et al.*, 2000), suggests a commonality in serotonergic modulation of sensory input in animals.

We were fortunate to have carried out the HPLC analysis as we would not otherwise have realized the broad action of PCPA in reducing DA in parallel with 5-HT. It appears that PCPA probably

works on TPH as well as the biochemical pathway for synthesis of DA in *Drosophila*. The probable enzyme targeted by PCPA in DA production is tryptophan-phenylalanine hydroxylase, as both 5-HT and DA synthesis share this similar enzyme and both compounds are reduced by PCPA. This enzyme is also used in part for 5-HT production for *Drosophila* (Coleman & Neckameyer, 2005). The long-term treatment of larvae that were followed throughout pupation and eclosion offered even more fodder for speculation, as the actions were dose-dependent in increasing 5-HT as well as DA in adults when treated with MDMA. The PCPA decreased both 5-HT and DA in new adults, as it did in larvae. The speculation at present is that MDMA promotes the production of 5-HT and DA by either turning off the inhibitory feedback balance or directly stimulating biosyntheses in the CNS. We also noted that the levels of 5-HT or DA in the CNS do not parallel whole larval body analysis and PCPA treatments had no discernible effect on the whole body 5-HT levels. Tissue-specific analysis for 5-HT and DA is required in this model organism. It is possible that PCPA may only work on the tryptophan-hydroxylase enzyme produced centrally, as it is coded by a different gene that that expressed peripherally (Coleman & Neckameyer, 2005). As in vertebrates, there are differentially expressed TPH genes centrally and peripherally that produce different enzyme isoforms (Walther *et al.*, 2003). It is possible that, even if the gene sequence is the same, they may undergo alternative splicing differentially making one form more sensitive to drug treatments. Enzyme splicing variants that show various drug affinities are due to various 5-HT receptor subtypes. Such a phenomenon is now commonly observed in multiple species (Krobert & Levy, 2002; Kishore & Stamm, 2006). With the dual effects of PCPA on 5-HT and DA concentrations in the CNS, one now has to determine if the observed behavioral changes are related to alterations in 5-HT or DA as well as the delayed development. As in vertebrates, DA in *Drosophila* has an effect on behavior and locomotion (Kume *et al.*, 2005; Neckameyer, 1996, 1998a; Cooper & Neckameyer, 1999).

The larval heart did not show any altered responsiveness to exogenously applied 5-HT (10 μM) in the animals fed PCPA. This was probably due to the peripheral 5-HT levels not being altered by PCPA treatment as assayed by whole larva HPLC. As peripheral 5-HT levels were not altered we did not expect a change in HR sensitivity. The heart does serve as an independent assay for alteration in the CNS responses to 5-HT as the heart is also responsive to 5-HT (Dasari & Cooper, 2006) but the aorta of the larval heart does appear to be innervated (Johnstone & Cooper, 2006) and neuromodulatory action on this innervation is not known. This is one of the reasons why we removed the CNS when conducting the heart assay so as to only examine direct effects of 5-HT on the heart.

These studies have produced some unexpected findings on the actions of MDMA, particularly that 5-HT was not depleted in the brains of larvae and that the 5-HT levels were raised in pupae/adults of larvae fed MDMA. Possible enzymatic assays would resolve if MDMA could stimulate synthesis. The pronounced inhibitory effects of 5-HT after PCPA treatment were also surprising as acute application produced excitation of the central circuit. In addition, the reduction of DA by PCPA treatment in larvae was serendipitously found, which could be accounted for by PCPA blocking not only TPH but also tryptophan phenylalanine hydroxylase and tryosine hydroxylase that is used to produce DA. An enzymatic analysis is needed to resolve this issue. The excitatory and depressing effects of 5-HT could be accounted for by alternative expression of the four known 5-HT receptor subtypes or even alternative splicing of the D5-HT_2 subtype as is known to occur in mammals (Pauwels, 2000; Kishore & Stamm, 2006). The four (5-HT_{7dro}, 5-HT_{1Adro}, 5-HT_{1Bdro} and 5-HT_{2dro}) receptor subtypes are analogous to mammalian systems in classification. A possible up-regulation of the 5-HT_1 subtype could cause a depression of cellular excitability (Barnes & Sharp, 1999; Tierney, 2001; Nichols *et al.*, 2002). The location of particular receptor subtypes in a neuronal circuit could still result in excitation on one set that ultimately inhibits motor neurons through a GABA-ergic path.

MDMA can act as a weak 5-HT receptor agonist, which might account for some of the acute as well as the long-term effects in this study. There are various scenarios to explain the actions of MDMA first increasing neural activity followed by an overall decrease over several minutes: (i) a fraction of the 5-HT is dumped by the reversal of a transporter over a short term; (ii) the transporter reversal may be negatively regulated; (iii) 5-HT synthesis is up-regulated; and/or (iv) the transporter desensitizes to MDMA. A 5-HT_{1A} agonist can lead to desensitized 5-HT_{1A} receptors in the vertebrate CNS within just 3 days of treatment (Assie *et al.*, 2006). Even a single exposure of an agonist can produce a long-lasting inhibition of 5-HT_{2A} receptor-mediated neuroendocrine responses (Carrasco *et al.*, 2007). In a human embryonic kidney 293 cell line, made to express 5-HT_{3A} receptors, the average desensitization time constants of currents activated by 3, 30 and 1000 μM 5-HT are related within 6–1 s to exposure concentration (Hu *et al.*, 2006). Therefore, it is feasible that even an acute high concentration exposure of the fly CNS may result in a rapid desensitization. In addition, our long-term treatments of MDMA could have consequences on 5-HT receptor expression levels, as MDMA can act as a 5-HT receptor agonist.

Obviously, more work is needed to investigate receptor localizations, regulation in expression and cellular responses to make sense of the sensory–CNS–motor circuits described in this study as well as in studies on vertebrate neuronal circuits (McMahon *et al.*, 2001; Vitalis & Parnavelas, 2003; Sodhi & Sanders-Bush, 2004). By accomplishing such a task we may then understand the actions of various pharmacological agents and the potential role of endogenous neuromodulators on the serotonergic circuits as well as developmental influences on the larval brain and the brain during the transformation from pupa to adult.

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Abbreviations

CNS, central nervous system; DA, dopamine; HPLC, high performance liquid chromatography; HR, heart rate; 5-HT, serotonin; KS, Kolmogorov-Smirnov; MDMA, 3,4 methylenedioxy-methamphetamine, 'ecstasy'; PCPA, para-chlorophenylalanine; TPH, tryptophan hydroxylase.

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