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Environmental Effects On Drosophila Brain Development And Learning

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RESEARCH ARTICLE

Environmental effects on *Drosophila* brain development and learning

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ABSTRACT

Brain development and behavior are sensitive to a variety of environmental influences including social interactions and physicochemical stressors. Sensory input *in situ* is a mosaic of both enrichment and stress, yet little is known about how multiple environmental factors interact to affect brain anatomical structures, circuits and cognitive function. In this study, we addressed these issues by testing the individual and combined effects of sub-adult thermal stress, larval density and early-adult living spatial enrichment on brain anatomy and olfactory associative learning in adult *Drosophila melanogaster*. In response to heat stress, the mushroom bodies (MBs) were the most volumetrically impaired among all of the brain structures, an effect highly correlated with reduced odor learning performance. However, MBs were not sensitive to either larval culture density or early-adult living conditions. Extreme larval crowding reduced the volume of the antennal lobes, optic lobes and central complex. Neither larval crowding nor early-adult spatial enrichment affected olfactory learning. These results illustrate that various brain structures react differently to environmental inputs, and that MB development and learning are highly sensitive to certain stressors (pre-adult hyperthermia) and resistant to others (larval crowding).

KEY WORDS: Brain plasticity, Stress, Enrichment, Environmental influence

INTRODUCTION

Brain development is tightly regulated by genetic programs, yet environmental factors also play important roles in sculpting and refining neural circuitry and consequent behavior (Eisenberg, 1999; Rutter et al., 2006; Sale et al., 2009). Enrichment of the physical and social environment can have positive effects on brain development and function. For example, rodents raised in enriched environments show significant increases in neurogenesis, brain weight and size, and learning and memory relative to their sensory-impoverished siblings (Rosenzweig and Bennett, 1996; van Praag et al., 2000). Likewise, restricting memory-based tasks and experiences reduces hippocampal volume and neurogenesis in a passerine bird (LaDage et al., 2009, 2010). Alternatively, disruption of central nervous system (CNS) development by environmental stress exposure (nutritive, chemical, electromagnetic and thermal) has been shown in every model system studied to date, including humans (Ahmed, 2005; Rice and Barone, 2000; Roebuck et al., 1998; Weinstock, 2001). For example, neural tube defects, one of the most common birth defects of the CNS in humans, have been linked to hyperthermia during early pregnancy (Chambers, 2006; Chan et al., 2014; Dreier et al., 2014; Moretti et al., 2005).

Environmentally induced neuronal and behavioral plasticity is not limited to vertebrates, as both social and physicochemical cues affect insect brain development and function. The development of insect mushroom bodies (MBs), the conserved sensory integration, associative odor learning and memory center of insects, appears to be particularly sensitive to environmental influences. In *Drosophila melanogaster*, olfactory enrichment and social contact of adults increase the number of Kenyon cell fibers in the MB peduncle (Technau, 1984). Female flies reared in high-density larval cultures were shown to have more MB Kenyon cell fibers than females reared in low-density larval cultures (Heisenberg et al., 1995). Darkness and low-density rearing conditions during early adulthood reduce the volume of MB calyx (dendritic sensory input), although adult crowding in darkened rearing conditions increases MB calyx volume (Barth and Heisenberg, 1997). Similarly, olfactory or visual deprivation reduces MB neuronal proliferation in adult crickets (Cayre et al., 2007). We previously showed that daily episodes of physiologically relevant hyperthermia during larval and pupal development severely reduce MB calyx volume by decreasing Kenyon cell proliferation, with proportional reductions in Pavlovian odor learning abilities (Wang et al., 2007).

Given the demonstrated benefits of sensory enrichment and detrimental impacts of stress on central nervous system development, it is possible that these effects could offset each other when experienced concurrently. Indeed, enriched environments aid recovery from cortical and behavioral deficits associated with malnutrition and crowding in rats (Carughi et al., 1989). Environmental enrichment can delay cognitive impairment from brain disorders such as Huntington's disease, Alzheimer's disease and Parkinson's disease in rodent models (Hannan, 2014; Nithianantharajah and Hannan, 2006). In *Drosophila*, social interactions mitigate the negative effects of visual deprivation on MB growth (Barth and Heisenberg, 1997). In this study, we examined brain anatomy and learning in flies exposed to sub-adult heat stress, larval crowding, early-adult living condition enrichment and combinations thereof to further investigate the effects of multiple environmental factors and their interactions on brain development and function.

MATERIALS AND METHODS

Flies, larval culture, thermal treatments and adult living condition enrichment

Wild-type *Drosophila melanogaster* Meigen 1830 adults from a large orchard population in southern Nevada were collected and

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used to establish populations in the laboratory in 2002. The lineage of these flies was used for all stress and enrichment studies in which we assessed anatomy and behavior. We cultured flies at 23°C (except for the heat stress treatment, below) on a 12 h:12 h light:dark cycle in plastic vials or bottles containing ∼8 cm^3 (vials) or ∼50 cm^3 (bottles) of standard *Drosophila* cornmeal medium (yeast, soy flour, cornmeal and corn syrup; recipe from the Bloomington Drosophila Stock Center at Indiana University).

In the larval density experiment, flies were allowed to oviposit overnight on Petri dishes containing 10% molasses and 1% agar. First-instar larvae (4–8 h after hatching) were counted and transferred into each vial (1–60 larvae cm^{-3}). The control group was reared at constant 23°C, while the heat-stressed (HS) group was exposed daily to a brief (35 min) 39.5°C pulse, by immersing culture vials in a circulating water bath, throughout larval and pupal development. Adult flies were collected every day after eclosion. As flies that emerged later in severely crowded densities varied considerably in size, only those flies emerging in the first 4 days were used.

In the adult living space experiment, both control and HS adult flies were reared from vials seeded with ∼20 first-instar larvae cm^{-3}. After eclosion, adult flies were either transferred into a bottle (∼150 cm^3) containing 50 cm^3 of standard medium at a density of 100 flies per bottle (spatially deprived) or transferred into a meshed cage [1.25×10^5 cm^3, i.e. $(50\ cm)^3$] containing 5 open food bottles (otherwise empty) at a density of 200 adult flies per cage (spatially enriched). Fly food was changed every 3–4 days.

In the adult deprived/enriched living condition experiment, adult flies (collected from control and HS vials seeded with ∼20 first instar larvae cm^{-3}) were held in isolation or in large groups. In the adult socio-spatial deprivation group, single pupae were transferred into each vial. After eclosion, individual adult flies were transferred to individual fresh vials (∼40 cm^3) with 8 cm^3 of standard medium every 3–4 days. In the adult socio-spatial odorant enrichment group, approximately 200 adult flies were released into each meshed cage (1.25×10^5 cm^3) with five open food bottles. Cages were decorated with visual stimuli, consisting of colorful plastic flowers and leaves. Ripe fruits (including apples, pears and bananas) were crushed and held in plastic cups with meshed covers to provide odors. Fly food and fruits were changed every 3–4 days. Cages were shaken twice a day to provide mechanical disturbance.

Histology and anatomy

We analyzed brain neuropil anatomy using a paraffin mass histology protocol as described previously (de Belle and Heisenberg, 1996; Wang et al., 2007) for 3–4 day old adults in the larval density experiments and 19–21 day old adults in the adult living condition experiment. Older flies were analyzed in the latter experiment to capture any brain reorganization occurring beyond early adulthood. Flies were cold-anesthetized (∼4°C), placed in fly collars, fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid; all chemicals are from Sigma-Aldrich, St Louis, MO, USA), dehydrated in ethanol (95% and 100%), embedded in paraffin (Leica Microsystems, Buffalo Grove, IL, USA) and cut into 7 µm serial frontal sections. Slides were photographed under a fluorescence microscope with an AxioCam digital camera (Zeiss, Jena, Thuringia, Germany). The volume of brain neuropil structures was derived from planimetric measurements of serial brain sections using AxioVision software (Zeiss).

Behavior assays

We analyzed associative odor learning with a Pavlovian conditioning T-maze paradigm as described previously (de Belle and Heisenberg, 1996; Wang et al., 2007) for 3–6 day old non-heat-shocked flies in the high larval density experiment and 19–21 day old flies in the adult living space experiment. Briefly, in an associative odor training procedure, groups of approximately 100 flies were transferred into a training tube embedded with internal double-wound electrifiable copper grids. Flies were exposed to an air current (750 ml min^{-1}) bubbled through one odor (2×10^{-3} dilution of 4-methyl cyclohexanol or 4×10^{-3} dilution of 3-octanol in heavy mineral oil; Sigma-Aldrich) paired with 1.25 s pulses of 90 V DC electric shock delivered every 5 s for 1 min. They were then exposed to fresh air for 1 min, followed by another air current bubbled through the other odor without shock for 1 min. To assay associative odor learning immediately after training, flies were exposed to both odors in converging air currents and allowed to choose between them for 2 min and then counted. Learning performance index was calculated as a normalized percentage of shock-paired odor avoidance. A second group of flies was trained in a reciprocal manner and tested similarly. Scores from both tests were averaged to account for possible odor preferences among different groups of flies.

Statistical analyses

All data were normally distributed ($P>0.05$, Shapiro–Wilk test) and all variances were homogeneous ($P>0.05$, Levene's test). Two-way MANOVA (reported as Pillai's trace) was used to test for thermal treatment, high larval density and interaction effects on all four brain structure volumes. Data passed Henze–Zirkler's test for multivariate normality ($P>0.05$) and Box's *M*-test for homogeneity of covariance matrices ($P>0.05$). Two-way ANOVA was used to test for thermal treatment, high larval density and interaction effects on volume for each brain structure. Two-way ANOVA was also used to test for thermal treatment, low larval density or adult environment and interaction effects on MB calyx volume and peduncle area. Tukey's multiple comparisons test was used for *post hoc* analysis to compare individual means. One-way ANOVA was used to test for effects of larval density on learning. Student's *t*-test (two-tailed) was used to compare learning between the bottle and cage adult holding groups. Pearson's product-moment correlation test was used to test for correlation between MB calyx volume and peduncle area. All data were analyzed using R statistics software (www.R-project.org/), in addition to 'biotools' and 'car' packages (da Silva et al., 2017; Fox and Weisberg, 2011).

RESULTS

Influence of high larval density and thermal stress on brain anatomy and learning

Increases in MB Kenyon cell fibers have been observed in adult female flies as a result of presumptive sensory enrichment derived from rearing in densely populated larval cultures (5–6 versus 20–25 larvae cm^{-3}; Heisenberg et al., 1995). To investigate a broad effect of larval culture density, as well as a possible interaction of larval density and heat stress on brain development, we measured the volume of adult brain structures (Fig. 1A) in flies reared at various larval densities ranging from ∼6 to ∼60 larvae cm^{-3}, either at constant temperature or in a thermal regime that imposed a daily heat stress.

We observed strong overall heat stress and larval density effects on brain development (MANOVA, Pillai's trace=0.55, $F_{4,69}=21.00$, $P<0.0001$ and Pillai's trace=0.61, $F_{12,213}=4.52$, $P<0.0001$, respectively). Follow-up ANOVA revealed variable levels of brain morphological plasticity in response to different rearing conditions (Fig. 1B–E). Pre-adult stage heat stress strongly decreased MB calyx volume (Fig. 1B; $F_{1,72}=49.41$, $P<0.0001$). All comparisons at each

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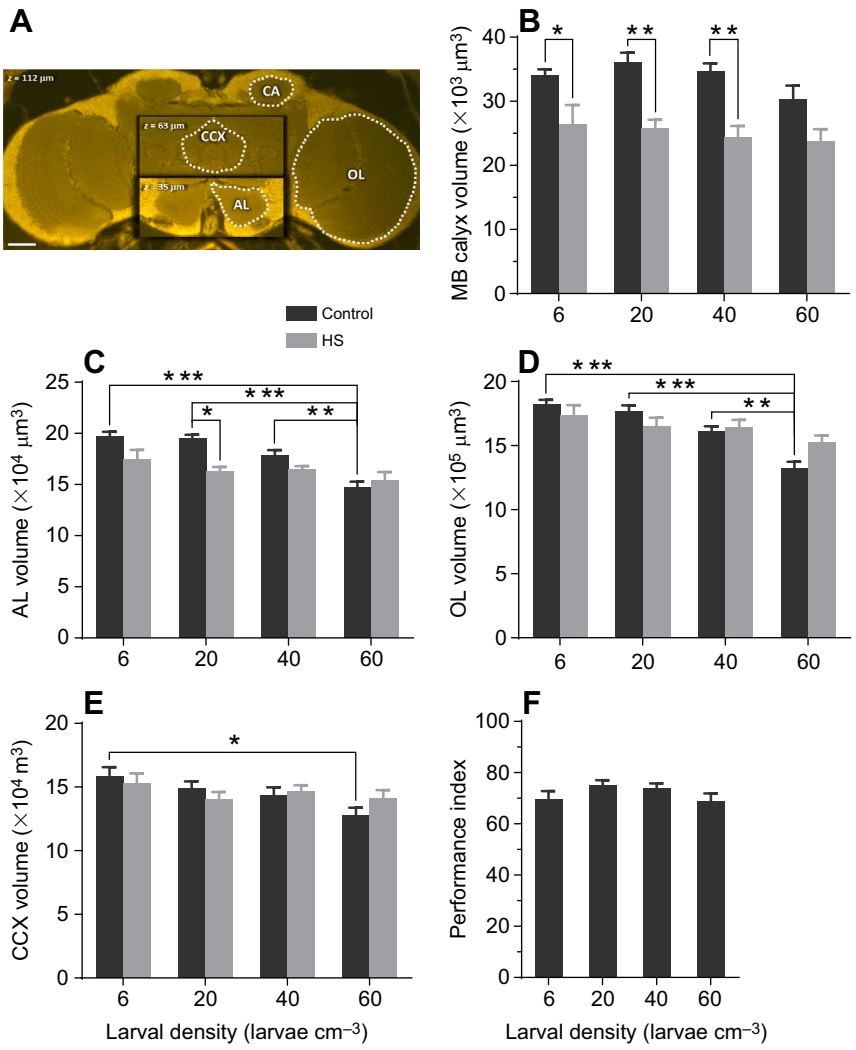

Fig. 1. Effects of high larval density and pre-adult thermal stress on brain structure volume and learning in adult *Drosophila*. (A) Brain structures measured in this study. Overlaid images of three frontal paraffin sections of fly heads, viewed under a fluorescence photomicroscope. Perikarya and neuropil appear yellow and green, respectively. Structures are shown (outlined) in sections taken at various depths (*z*) from the anterior margin of the brain. Scale bar, 50 µm. (B–F) Brain structure volumes. Planimetric measurements of serial 7 µm paraffin sections of heads from adult flies. Bars represent means±s.e.m., n=10 per bar. Asterisks indicate a significant difference (ANOVA followed by Tukey's test, *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$). (B) Mushroom body (MB) calyx (left/right average). (C) Antennal lobe (AL; left/right average). (D) Optic lobe (OL; left/right average). (E) Central complex (CCX; fan-shaped body+ellipsoid body). (F) Associative olfactory learning performance index of flies reared at high larval density. Bars represent means±s.e.m., n=12 per bar. Differences were not significant (ANOVA).

density were significant (Tukey's test, $P<0.05$), except at very high density (60 larvae cm^{-3}). ANOVA did not reveal a significant influence of rearing density or an effect of thermal stress and density interaction on MB calyx volume. Heat stress had a relatively minor effect on antennal lobe (AL) volume (Fig. 1C; $F_{1,72}=13.74$, $P=0.0004$), which was only reduced in one rearing density (20 larvae cm^{-3}, Tukey's test, $P<0.05$). However, AL was reduced by 20–25% through larval crowding ($F_{4,72}=13.75$, $P<0.0001$), with significant differences observed at the highest rearing densities and most prominently in control flies (60 versus 6, 20 and 40 larvae cm^{-3}; Tukey's test, $P<0.05$). In addition, there was a significant effect of the interaction between rearing density and heat stress on AL volume ($F_{3,72}=4.13$, $P=0.009$). That is, the effect of heat stress on AL volume reduction depends on the rearing density (see Discussion). Heat stress had no significant impact on either optic lobe (OL) or central complex (CCX) volumes (Fig. 1D and E, respectively). However, both neuropils were sensitive to differences in rearing density ($F_{4,72}=15.50$, $P<0.0001$ and $F_{4,72}=3.81$, $P=0.014$, respectively). The OL was most strongly influenced by very high rearing density in control flies (60 versus 6, 20 and 40 larvae cm^{-3}; Tukey's test, $P<0.05$). The CCX volume was reduced by 15% only at the highest rearing density in control flies (60 versus 6 larvae cm^{-3}; Tukey's test, $P<0.05$).

We next examined the effect of crowded larval cultures on associative odor learning in adult flies developing at larval densities ranging from 6 to 60 larvae cm^{-3} (Fig. 1F). Differences were not significant, as we might expect with our observation of no impact of crowding on MB calyx volume. We did not measure odor learning in HS flies because our earlier work had demonstrated a strong impairment of both MB development and learning by heat stress (Wang et al., 2007).

Influence of low larval density and thermal stress on MB anatomy

To address effects of low larval culture density, in addition to its possible interaction with heat stress on MB development, we examined MB calyx volume and peduncle cross-sectional area in adult control and HS flies reared at various larval densities ranging from 1 larva per vial (designated as <1 larva cm^{-3} in Fig. 2A,B) to ~6 larvae cm^{-3}. The results are similar to those in high larval density cultures. Pre-adult stage heat stress strongly decreased MB calyx volume (Fig. 2A; $F_{1,150}=152.60$, $P<0.0001$), as well as MB peduncle cross-sectional area (Fig. 2B; $F_{1,150}=125.88$, $P<0.0001$). All comparisons at each density were significant (Tukey's test, $P<0.05$). However, there was no significant influence of rearing density or an effect of thermal stress and density interaction on either MB calyx volume or peduncle area. Furthermore, Pearson's product-moment correlation test revealed a strong and significant correlation between MB calyx volume and peduncle cross-sectional

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area (Fig. 2C; r=0.81, P<0.0001), indicating that MB peduncle cross-sectional area and calyx volume changes were proportional. This association supports our use of MB calyx volume measurement as a reliable representation of the entire MB volume.

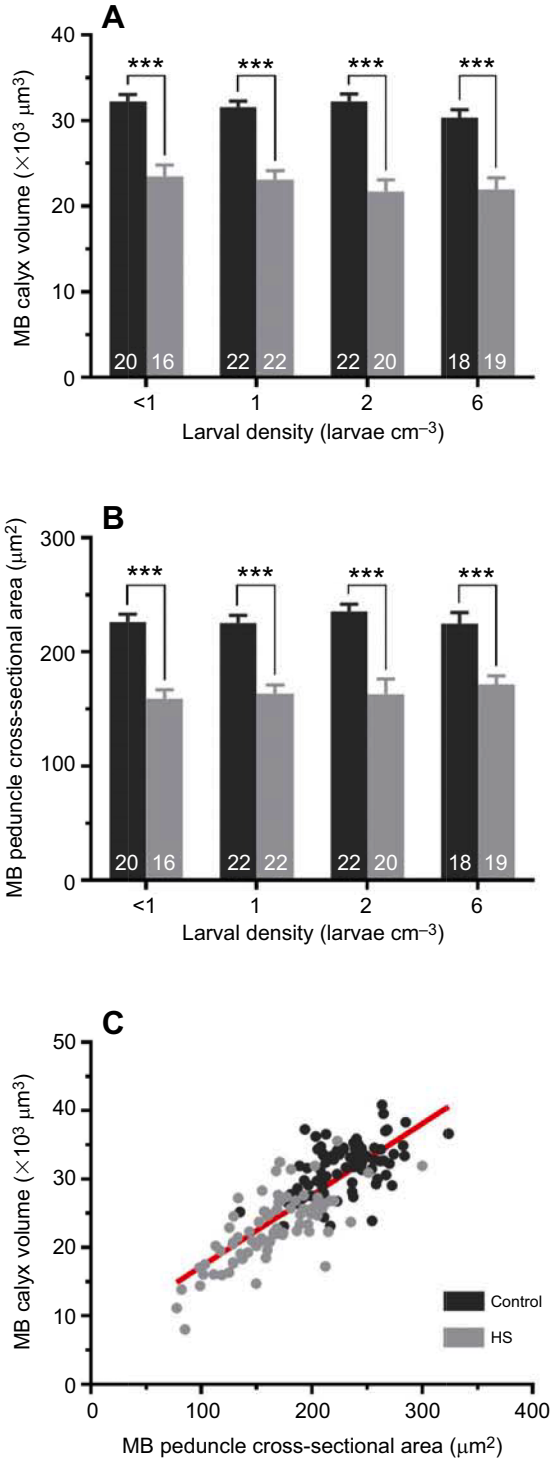

Fig. 2. Effects of low larval density and pre-adult thermal stress on MB calyx volume and peduncle cross-sectional area in adult *Drosophila*. (A,B) Bars represent means+s.e.m.; *n* values are shown on each bar. Asterisks indicate a significant difference (ANOVA followed by Tukey's test, ***P≤0.001). (A) MB calyx volume. (B) MB peduncle cross-sectional area. (C) Correlation between MB peduncle cross-sectional area and calyx volume (Pearson's product-moment correlation test, r=0.81, P<0.0001).

Influence of adult living space and pre-adult stage thermal stress on MB anatomy and learning

Adult rearing space has been suggested as an enrichment factor that increases MB size in flies (Technau, 1984). To examine the possible interaction effect of adult living space and pre-adult hyperthermic stress on MB development, we measured MB calyx volume in adult control and HS flies held as groups of 200 in cages and as groups of 100 in bottles. Among all comparisons, only pre-adult heat stress significantly reduced MB calyx volume (Fig. 3A; $F_{1,140}$=111.72, P<0.0001; Tukey's test, P<0.05). Increased adult rearing space [1.25×10^5 cm^3, i.e. (50 cm)^3, in cages versus 150 cm^3 in bottles] did not significantly affect MB calyx volume in either control or HS groups. Furthermore, to examine whether adult living space benefits learning ability, we measured associative odor learning in adult control flies held in cages and bottles. Enlarged adult living space did not significantly affect associative odor learning in flies either (Fig. 3B).

Influence of adult socio-spatial odorant experience and pre-adult-stage thermal stress on MB anatomy

Adult olfactory enrichment and social contacts have been observed as stimulations that can increase Keyon cell fiber numbers in the MB (Technau, 1984). To examine the possible interaction effect of adult socio-spatial odorant enrichment and pre-adult hyperthermic stress on MB development, we measured MB calyx volume and peduncle cross-sectional area in adult control and HS flies held singly in vials and as groups of 200 in cages with various odor sources. Pre-adult heat stress significantly reduced MB calyx volume and peduncle cross-sectional area (Fig. 4A; $F_{1,99}$=106.75, P<0.0001; and Fig. 4B; $F_{1,99}$=70.40, P<0.0001, respectively), in both singly isolated flies and socio-spatial odorant enrichment group flies (Tukey's test, P<0.05). We observed no significant influence of adult rearing condition or an interaction effect of pre-adult thermal stress and adult rearing condition on either MB calyx volume or peduncle area. There was a strong and significant correlation between MB calyx volume and peduncle cross-sectional area (Fig. 4C; r=0.86, P<0.0001).

DISCUSSION

Influence of larval crowding on MB, AL, OL and CCX volume

Larval crowding in cultured *Drosophila* increases larval mortality, prolongs sub-adult development and decreases adult body size and fecundity, increases developmental time, increases variability of adult body mass, size and developmental time, and increases larval mortality (Lints and Lints, 1969; Miller and Thomas, 1958; Zwaan et al., 1991). Nonetheless, adult flies derived from high larval culture density have increased longevity and thermal stress resistance (Miller and Thomas, 1958; Sørensen and Loeschcke, 2001; Zwaan et al., 1991). Modest increases in brain structure size, especially the MBs, have been observed in adults eclosing from densely populated larval culture (Heisenberg et al., 1995). Female flies grown in crowded larval cultures (20–25 larvae cm^{-3}) had 20% more MB Keyon cell fibers than flies from low density cultures (5–6 larvae cm^{-3}). Crowded larval cultures also showed increased variability in developmental time (13–15 days) compared with low density larval cultures (13 days). However, the significant effect on MB calyx volume was only observed in female flies with the most extended sub-adult developmental time (15 days), but not in female flies with a regular sub-adult developmental time (13 and 14 days), or in any male flies (Heisenberg et al., 1995). In this study, we were interested in the broad effect of larval culture density. Therefore, we examined MB calyx volume of flies reared at seven larval densities

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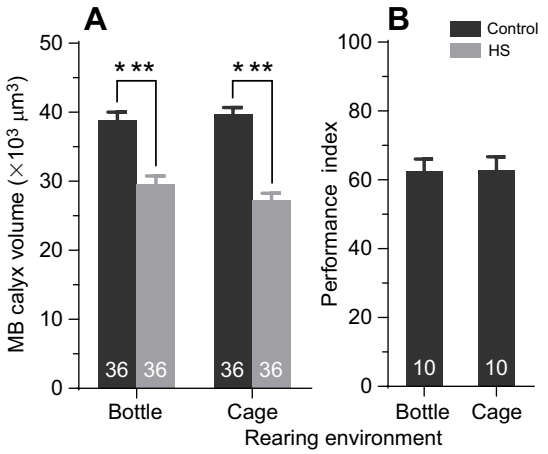

Fig. 3. Effects of adult living space and pre-adult thermal stress on MB calyx volume and learning in adult *Drosophila*. Bars represent means±s.e.m.; *n* values are shown on each bar. (A) MB calyx volume. Asterisks indicate a significant difference (ANOVA followed by Tukey's test, ***$P \leq 0.001$). (B) Associative olfactory learning performance index. Difference was not significant (*t*-test).

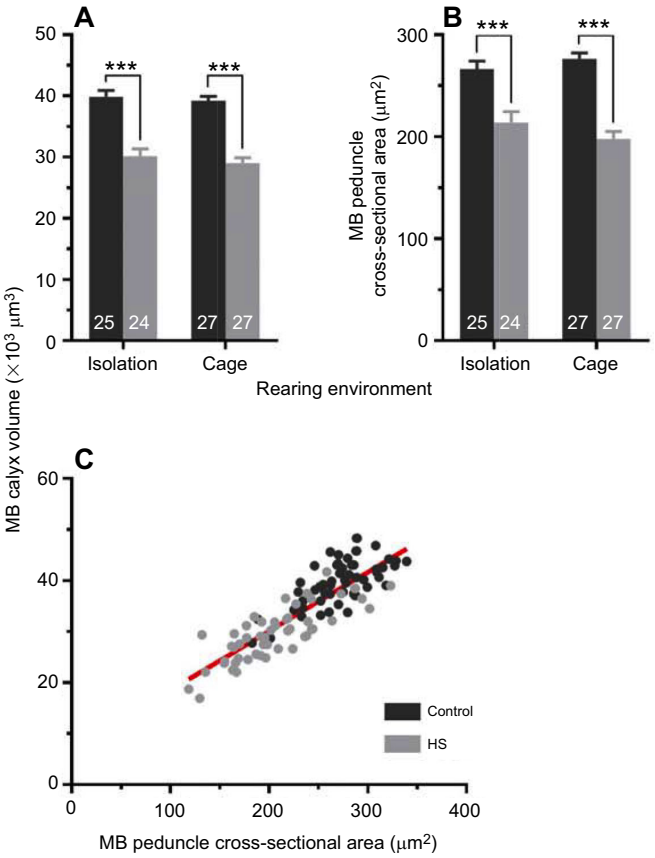

Fig. 4. Effects of adult socio-spatial odorant experience and pre-adult thermal stress on MB calyx volume and peduncle cross-sectional area. (A,B) Bars represent means±s.e.m.; *n* values are shown on each bar. Asterisks indicate a significant difference (ANOVA followed by Tukey's test, ***$P \leq 0.001$). (A) MB calyx volume. (B) MB peduncle cross-sectional area. (C) Correlation between MB peduncle cross-sectional area and calyx volume (Pearson's product-moment correlation test, $r=0.86$, $P<0.0001$).

(1 larva per vial, and 1, 2, 6, 20, 40 and 60 larvae cm^{-3}), without separating flies from different developmental times. We analyzed 3–4 day old flies (instead of 1 h old flies analyzed in Heisenberg et al., 1995), to allow flies to fully mature so they could also be tested in a learning paradigm. We did not find any significant increase in MB calyx volume in crowded culture densities (≥20 larvae cm^{-3}) relative to low culture densities (≤6 larvae cm^{-3}), nor any significant differences between female and male flies. Furthermore, elevated rearing density did not increase any brain structure volume; instead, extremely crowded larval cultures (60 larvae cm^{-3}) impaired volumetric development of the AL, OL and CCX. High larval density may be potentially stressful or even harmful because of the excessive utilization and interference competition of food and space (Beebee and Wong, 1992; Roberts, 1998; Rodriguez-Munoz et al., 2003; Walls, 1998). Many studies have reported that larval crowding in *Drosophila* during development has negative effects on growth, especially in body size (Imasheva and Bubliy, 2003; Lefranc and Bundgaard, 2000; Miller and Thomas, 1958). Interestingly, Lin et al. (2013) reported that during development, MB neuroblasts can continue to proliferate under starvation conditions, whereas AL lineage cell cycles are slowed down by nutrient deprivation. These results support our observations that MB calyx volume was not sensitive to larval density, even in extremely crowded cultures (60 larvae cm^{-3}) in which access to food was likely limited, while AL volume decreased under these conditions.

Adult living condition influences on MB volume

MB fiber number and MB calyx volume of flies kept in flight cages enriched with odor sources (enrichment) were larger than those of flies kept singly in vials (deprivation) (Heisenberg et al., 1995; Technau, 1984). The hypothesis that living space by itself can act as an enrichment parameter for brain structure volume has also been suggested for *Drosophila* (Heisenberg et al., 1995). However, experimental data in support of this are inconsistent. For example, Balling et al. (1987) reported that the difference of MB fiber number in one of their enrichment/deprivation experiments was very small and non-significant. Following the lead of Heisenberg et al. (1995), we wanted to further investigate the notion that living space is a critical enrichment parameter. In the current study, we did not find any significant effect on MB anatomy as a result of an enriched environment during adulthood. Such inconsistent results in brain structure volumes have been noted as 'problematic' (Heisenberg et al., 1995). For example, MB calyx volume of flies kept singly in vials has been observed to be equal to and, at other times, larger than that of flies kept in group in standard food bottles (Heisenberg et al., 1995). Moreover, both an increase and a decrease in MB fiber number during the first week of fly adulthood have been reported (Balling et al., 1987). These observations might reflect the sensitivity of the *Drosophila* brain to subtle differences between rearing environments of separate experiments. Indeed, Heisenberg et al. (1995) have cautioned against comparing flies from different treatment groups in different experiments. In this study, all flies were descended from the same fly population. In each experiment, flies were reared with the same batches of food and living conditions, except for the experimental factors of thermal stress treatment, density or adult living condition treatments. We showed that MB calyx volume in flies kept in space-enriched cages (with and without odor sources) is not significantly different from that of flies kept in groups in bottles or singly in space-deprived vials during early adulthood. However, our data are consistent in demonstrating that pre-adult stage heat stress strongly decreased MB calyx volume in HS flies relative to control flies. We also observed a robust heat stress

effect on MB peduncle cross-sectional area, and a strong correlation between MB calyx (pack of Keyon cell dendrites) volume and MB peduncle (bundle of Keyon cell axons) cross-sectional area.

Influence of larval crowding and adult living condition on olfactory associative learning

Genetic and environmentally induced reductions in MB calyx volume impair olfactory associative learning (de Belle and Heisenberg, 1994, 1996; Wang et al., 2007). In this study, we did not find any significant effect on odor learning of larval crowding or space-enriched adult living conditions. The consistency in learning ability across groups reared at different larval densities and in different adult living space conditions is not surprising given the similarity in MB calyx volume among these treatment groups. We did not measure learning in flies raised at low density (including 1 larva per vial) or in individually kept flies because of the large number of flies needed for the associative odor learning assay. This assay requires flies to be tested in groups of about 100. A comprehensive comparison would include tests of sensory acuity and learning, and would require many thousands of individually reared flies. Nonetheless, it would be interesting to investigate the effects of low larval density and adulthood isolation on learning and other behavior, using methods that require single flies (Claridge-Chang et al., 2009).

Possible environmental effects on brain development and behavior

Environmental enrichment has been shown to enhance neuroblast proliferation, neuronal survival and morphological changes such as synaptogenesis and dendrite branching (Kempermann et al., 1997; Sandeman and Sandeman, 2000; van Praag et al., 1999b; Volkmar and Greenough, 1972). Crowded larval cultures and enriched adult living conditions might induce neuronal re-growth or re-sculpture, but those fine changes may have eluded discovery by our volumetric measurements with light microscopy. Application of confocal microscopy and electron microscopy might be required to locate subcellular changes in the fly brain. Although rodents reared in enriched laboratory environments were found to have improved learning and problem-solving abilities (Renner and Rosenzweig, 1987; van Praag et al., 1999a; Wainwright et al., 1993), the results were often short lived and depended on multiple factors, such as the age at which enrichment was experienced, and the tasks that were learned and measured (Rosenzweig, 2003). Rosenzweig (2003) has cautioned against over-interpretation of enrichment experiments on learning ability, as early enrichment may improve learning of one task but have no effect on others. In the olfactory aversive Pavlovian conditioning paradigm, we found no significant learning differences among flies reared at variable larval densities or between flies with different early-adult living space. In line with Rosenzweig's (2003) suggestion, alterations in behavior might be stimulated in flies that experienced crowded larval cultures and space-enriched cages, but be more prominent in certain neural circuits outside the MBs. Additional behavior assays (Pitman et al., 2009), such as courtship conditioning (Siegel and Hall, 1979), olfactory appetitive conditioning (Tempel et al., 1983), visual learning (Dill et al., 1993), heat box spatial memory (Putz and Heisenberg, 2002) and aversive phototaxic suppression (Le Bourg and Buecher, 2002), would be helpful to uncover possible differences induced by those environmental enrichment factors.

It is possible that neither larval crowding nor increased space in a flight cage constitute enriched environments for flies. Enrichment can be defined as 'a combination of complex inanimate and social stimulation' (Rosenzweig et al., 1978), although the so-called experimentally enriched environment should also be defined relative to the regular laboratory impoverished settings, rather than enrichment over natural living conditions. Studies in rats revealed that the effects of an enriched environment were mostly associated with an increase in voluntary motor behavior or exercise (Kempermann et al., 1997; van Praag et al., 1999a). In crowded larval cultures, larvae were exposed to increased social interactions, but with few changes in activity. We also noticed that flies were inactive unless disturbed in both flight cages and bottles. Most of the time, flies merely remained inside or at the edge of food bottles. The lack of stimulation of exploratory movement or voluntary exercise might be one of the reasons that adult living space is not a sufficient enrichment by itself to induce significant responses in brain structures and behavior in *Drosophila*.

Combination of environmental influences on MB development

Larval crowding in *Drosophila* has been reported to induce heat shock protein 70 expression and increase adult longevity and adult thermal stress resistance (Sørensen and Loeschcke, 2001). We combined larval crowding and heat stress to determine whether these environmental factors are compounding or offsetting. Under these conditions we observed a significant interaction effect between rearing density and heat stress on AL volume but not on MB calyx volume. In 20 larvae cm^{-3} cultures, AL volume in HS flies was significantly decreased in comparison with the control group (Fig. 1C; Tukey's test, $P<0.05$). In 40 and 60 larvae cm^{-3} cultures, there was no significant difference in AL volume between control and HS flies. At high larval densities, AL development might have gained elevated thermal stress resistance from larval crowding, or heat stress may have alleviated some negative effects of larval crowding. We observed that daily heat stress (39.5°C for 35 min) was so deleterious that it caused about 60% larval mortality during development. That is, this heat stress would reduce a culture from ∼60 larvae cm^{-3} to close to 20 larvae cm^{-3}, and from ∼40 larvae cm^{-3} to close to 14 larvae cm^{-3}. Therefore, AL volume in HS group flies cultured at 60 larvae cm^{-3} was actually that of flies cultured at 20 larvae cm^{-3} by the end of development. Indeed, AL volume of HS group flies cultured at 60 larvae cm^{-3} (20 larvae cm^{-3} final density) was smaller than that of flies in the control 20 (and 6) larvae cm^{-3} group (Fig. 1C; Tukey's test, $P<0.05$); and AL volume in HS group flies cultured at 40 larvae cm^{-3} (∼14 larvae cm^{-3} final density) was smaller than that of flies in the control 20 (and 6) larvae cm^{-3} group (Fig. 1C; Tukey's test, $P<0.05$). High larval density did not mitigate the harmful effects of daily hyperthermic stress. Instead, heat stress may have alleviated high larval density-induced developmental pressure of malnutrition and competition through increased larval mortality (i.e. decreasing larval density), although it still disrupted AL development. Nevertheless, MB development was not sensitive to overcrowding, and thus the benefits of heat stress-induced reduction in larval density were not realized as a significant increase in MB calyx volume.

Conclusions

The environmental sensitivity of insect brain development varies depending on specific brain regions and the type of environmental input or stress. In this study, larval rearing cultures ranging from sparse (1 larva per vial, ∼1 larva cm^{-3}) to modest (∼2, ∼6 larvae cm^{-3}), crowding (∼20, 40 larvae cm^{-3}) and extreme crowding (∼60 larvae cm^{-3}) had no significant effects on adult MB calyx volume in *Drosophila*, although extreme larval crowding impaired the volumetric development of AL, OL and CCX. Adult

living conditions, including flies that were singly isolated in vials, grouped in small bottles or maintained as populations in cages (with and without olfactory enrichment), did not affect MB calyx volume either. Conversely, heat stress during pre-adult stages, regardless of larval rearing density, strongly decreased MB calyx volume, but had little or no effect on AL, OL and CCX volume. Furthermore, neither larval crowding nor an enriched early adult rearing space significantly enhanced olfactory associative learning performance in flies. These results show that although some brain structures and behaviors are especially sensitive to certain environmental factors, many traits are invariant to them. The brain tends to retain its authenticity in genetically determined development and function under a certain range of situations. Likewise, laboratory rearing of snails did not reduce their capability to form memories compared with nurturing in the wild (Orr et al., 2008). These authors proposed that either their laboratory rearing conditions were not sufficiently impoverished to affect brain development and memory ability or the behavior they examined may have been resistant to environmental challenges during development. In humans, there is an abundance of data showing that stress, such as severe malnutrition at an early age, causes delayed brain development and decreased intelligence (Ivanovic et al., 2004). However, it has been reported that accommodations can be made by the brain itself in response to retarding growth conditions to maintain successful neuronal development and later cognitive performance (Martyn et al., 1996). Thus, brain plasticity encompasses a capacity not only to change but also to adapt and maintain developmental and behavioral fidelity in response to a variety of environmental factors.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: X.W., J.S.d.B., S.P.R.; Methodology: X.W.; Data curation: X.W., A.A.; Writing - original draft: X.W.; Writing - review & editing: J.S.d.B., S.P.R.; Supervision: J.S.d.B., S.P.R.; Project administration: S.P.R.; Funding acquisition: J.S.d.B., S.P.R.

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