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Bisphenol a affects neurodevelopmental gene expression, cognitive function, and neuromuscular synaptic morphology in *Drosophila melanogaster*

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ABSTRACT

Bisphenol A (BPA) is an environmentally prevalent endocrine disrupting chemical that can impact human health and may be an environmental risk factor for neurodevelopmental disorders. BPA has been associated with behavioral impairment in children and a variety of neurodevelopmental phenotypes in model organisms. We used *Drosophila melanogaster* to explore the consequences of developmental BPA exposure on gene expression, cognitive function, and synapse development. Our transcriptome analysis indicated neurodevelopmentally relevant genes were predominantly downregulated by BPA. Among the misregulated genes were those with roles in learning, memory, and synapse development, as well as orthologs of human genes associated with neurodevelopmental and neuropsychiatric disorders. To examine how gene expression data corresponded to behavioral and cellular phenotypes, we first used a predator-response behavioral paradigm and found that BPA disrupts visual perception. Further analysis using conditioned courtship suppression showed that BPA impairs associative learning. Finally, we examined synapse morphology within the larval neuromuscular junction and found that BPA significantly increased the number of axonal branches. Given that our findings align with studies of BPA in mammalian model organisms, this data indicates that BPA impairs neurodevelopmental pathways that are functionally conserved from invertebrates to mammals. Further, because *Drosophila* do not possess classic estrogen receptors or estrogen, this research suggests that BPA can impact neurodevelopment by molecular mechanisms distinct from its role as an estrogen mimic.

1. Introduction

Bisphenol A (BPA) is a ubiquitous environmental chemical used in the production of polycarbonate plastics and epoxy resins. Globally, 7.7 million metric tons of BPA were produced in 2015 and production is predicted to reach 10.6 million metric tons by 2022 (Almeida et al., 2018a). BPA is found in a wide range of common products, including aluminum can linings, plastic utensils, thermal receipt paper, and dental composites (Vandenberg et al., 2010; Almeida et al., 2018b). The most common route of human exposure is oral—BPA can depolymerize and leach from containers into the food or liquid contained within them (Nerín et al., 2003; Brede et al., 2003). Exposure can also occur via dermal contact of BPA-containing products and inhalation of air and dust (Konieczna et al., 2015).

In addition to its prevalence, BPA is a chemical of concern because of its potential to disrupt multiple physiological systems, including reproductive (Czubacka et al., 2021), metabolic (Valentino et al., 2016), cardiovascular (Fu et al., 2020), and immune (Aljadeff et al., 2018). Further, BPA is a lipophilic molecule that can permeate cell membranes, as well as placental and fetal blood brain-barriers (Ikezuki et al., 2002;

Abbreviations: BPA, Bisphenol A; NDD, Neurodevelopmental Disorder; ASD, Autism Spectrum Disorder; GO, Gene Ontology; RNA-seq, RNA Sequencing.

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Balakrishnan et al., 2010), which facilitates its ability to affect development (O'Shaughnessy et al., 2021). Analysis of developmental impacts in mammalian model organisms has shown BPA can elicit a range of neurodevelopmental and behavioral phenotypes, including impaired synaptogenesis (Xu et al., 2013), altered neural stem cell (NSC) proliferation (Tiwari et al., 2015), hyperactivity (Zhou et al., 2011; Ishido et al., 2011), learning and memory deficits (Johnson et al., 2016), anxiety-like behavior (Matsuda et al., 2012; Gioiosa et al., 2013), depressive-like behavior (Fujimoto et al., 2013), and reduced attention (Zhou et al., 2011). Epidemiological studies in humans support the notion that BPA alters brain development and may be a risk factor for neurodevelopmental disorders. Mothers exposed to high levels of BPA during pregnancy gave birth to children who were more likely to exhibit behavioral impairments (Miodovnik et al., 2011), and prenatal BPA exposure has been associated with an increased risk of autism spectrum disorder (ASD) (Hansen et al., 2021). Of particular concern, analysis of free versus conjugated BPA in human fetal samples demonstrated a reduced capacity of the fetus to metabolize BPA (Nahar et al., 2013), suggesting this stage may be more susceptible to deleterious consequences of BPA exposure. Given the ubiquity of BPA in our environment, it is critical to understand the developmental consequences of BPA exposure and to delineate the molecular mechanisms that underpin its ability to impair neurodevelopment.

The common fruit fly, *Drosophila melanogaster,* is increasingly being used as a model for neurodevelopmental toxicology given their simple maintenance and multitude of relatively inexpensive behavioral and cellular assays (Kaur et al., 2015; Musachio et al., 2021; Nguyen et al., 2021). Though more evolutionarily distant from humans than mammalian models, many of the signaling networks that govern neural development are conserved in *Drosophila* (Nichols, 2006). Fruit flies also possess functionally conserved orthologs of risk genes associated with human neurodevelopmental and neuropsychiatric disorders (Tian et al., 2017; Maurer et al., 2020; Pandey et al., 2017). Previous research has used *Drosophila* for investigating BPA toxicity (Kaur et al., 2015; Nguyen et al., 2021; Musachio et al., 2020; Vimal et al., 2019), though analysis of global transcriptomic impacts on development in fruit flies has not yet been explored.

The objective of this study was to determine how developmental exposure to BPA would affect gene expression using RNA-sequencing (RNA-seq) of whole larvae, and to validate those findings by examining phenotypes predicted by gene set enrichment analysis (GSEA). Our transcriptome analysis suggested that BPA exposure downregulates genes critical for neurodevelopment. The top twenty leading-edge genes included genes associated with some behavioral and neuronal phenotypes previously shown to be affected by BPA in *Drosophila*, including courtship behavior, locomotor behavior, axogenesis, and neuroblast development (Kaur et al., 2015; Musachio et al., 2021; Nguyen et al., 2021). In this study, we examined behavioral and neuronal phenotypes predicted by the leading-edge genes that had not yet been explored in studies of BPA in fruit flies. Our analysis identified deficits in visual perception, associative learning, and synaptogenesis, which corroborated the RNA-seq and GSEA findings. These results add to a growing body of research indicating BPA can disrupt neurodevelopment and further establishes *Drosophila* as a relevant model for BPA toxicology.

2. Materials and methods

2.1. Fly husbandry

The *Drosophila w1118* strain obtained from the Bloomington Stock Center was used for all experiments. Flies were reared on a standard cornmeal-yeast-agar medium recipe that was adapted from a Bloomington *Drosophila* Stock Center recipe and were maintained in a humidified incubator at 25 °C on a 12 -h light/dark cycle.

2.2. Chemical exposure

To prepare the BPA stock solution, 250 mg of BPA (Sigma-Aldrich, ≥99 %, No. 239,658) was added to 1 L water and stirred for 24 h at 50 °C, then filtered with a 0.22 μm bottle-top filter. To measure the final concentration of BPA in the stock solutions, diluted stock solutions were analyzed by High Performance Liquid Chromatography—tandem Mass Spectrometer (HPLC-MS/MS) on an Agilent 1260/6460 HPLC-MS/MS (Agilent Technologies, Santa Clara, CA, USA). Stock concentrations were found to contain 1.10–1.11 ± 0.04 mM BPA and were stable at room temperature for at least four months (no stocks older than four months were tested). To expose flies, the 0.25 mg/mL BPA solution was used instead of water to make fly food. Virgin females were collected and transferred to BPA treatment vials for four days before introducing males. BPA-treatment of the parental (P1) generation females ensured embryonic exposure to BPA. First filial (F1) larvae remained in the vials for exposure during larval development. F1 late third instar larvae were used for RNA sequencing and synapse studies. Because we were specifically interested in developmental BPA exposure, F1 adults used for behavioral analyses were collected as newborn virgins post-eclosion and transferred to vials with control food made without BPA. F1 females were used for oviposition and phototaxis. F1 adult males were used for conditioned courtship. Control larvae and adults were never treated with BPA.

2.3. RNA sequencing

RNA-seq data was generated from four biological replicates from each condition—four samples of control larvae and four samples of BPA-treated larvae. Each sample included 30 late third instar larvae. RNA was extracted using the Direct-zol Miniprep Plus Kit (Zymo Research). Samples were sequenced on the HiSeq4000 platform at 100bp reads to generate single end data (SR100) at the University of California, Davis (UC Davis) Genome Center. In the preprocessing step, FastQC (0.11.8) was used to evaluate raw sequencing data quality. FastQC output was used to identify Illumina adapters for trimming via Trimmomatic (0.39), used in single-end read mode. A phred quality score threshold of 33 was used to ensure high-quality reads post-trimming, which were re-evaluated using FastQC to ensure proper trimming of adapters, validate read quality, and ensure sufficient reads were present for downstream analysis.

A reference genome fasta file and genome annotation file in the form of general transfer format (gtf) were obtained from Flybase, for the FB2020_04, dmel.r6.35 version (Larkin et al. (2021)). HiSAT2 (2.1.0) was used for genome indexing provided the genome fasta file and genome annotation file. HiSAT2 was used for read mapping with the newly indexed reference genome and trimmed reads output by Trimmomatic. For HiSAT2, the –mp flag was adjusted to 4,2 the maximum and minimum penalties for mismatches. Resulting. sam files were sorted and converted into the binary BAM file format using Samtools (1.4.1). The Picard tool, MarkDuplicates (2.21.8), was used on the mapped BAM files output by Samtools; sequencing and PCR duplicates were removed.

For read feature quantification, HTSeq count (0.11.3) was used on deduplicated mapped BAM files created by Picard. To manage reads that aligned with more than one feature, the union mode of HTSeq count was used. The input genome annotation file (gtf) contained Ensembl annotated transcripts to associate reads with known transcripts in the reference genome (Yates et al. (2020)). HTSeq count generated count files for each known transcript per sample. Count files were used as input for differential expression (DE) analysis, which was performed using the DESeq2 package (1.28.1) in R. DESeq2 converted count files into statistically significant datasets based on an adjusted P values (p-adj) threshold of 0.01.

GSEA was performed using the fgsea package (1.14.0) in R. The ranked gene list was based on the DESeq2 output; the log2 fold change was used as the ranking metric. Duplicates were removed from the

ranked gene list when multiple transcripts mapped to the same gene. The msigdb package was used to gather pathways and their corresponding genes listed for *Drosophila melanogaster* from the Gene Ontology (GO) collection within the Cellular Component (CC) and Biological Processes (BP) subcategories. The fgsea algorithm was used after being provided the ranked gene list from DESeq2 results and gene sets for *D. melanogaster* as input for the GO collection, CC and BP subcategories. A gene set enrichment table was created showing top misregulated pathways, their statistical significance, and a list of leading-edge genes.

Heatmaps were generated using normalized count data from leading-edge genes identified within the top misregulated pathways from fgsea results from GO subcategories. Python modules Seaborn and matplotlib were used for visualizations. Normalized count matrices were generated using DESeq2 from raw count files. The DESeq2 function "vst" (variance stabilizing transformation) was used to generate normalized count matrices, which were narrowed for each pathway using leading-edge genes. The normalized counts were Z-score scaled per gene across all samples to enhance the visualization in the heatmaps.

2.4. Wasp culturing

Leptopilina heterotoma (strain 14) endoparasitoid wasps were a generous gift from Dr. Todd Schlenke (University of Arizona, Tucson, AZ). The wasps were maintained at room temperature and cultured using *Drosophila* as a host. Host flies laid their eggs for 2–3 days and were then removed from the vials before introducing wasps (10 females and 6 males). New wasps eclosed from pupal cases within 3–4 weeks.

2.5. Oviposition behavior

Performed partly as described in Kacsoh, B.Z., et al., 2015 (Kacsoh et al., 2015a), with the primary modification being that we halted the experiment following the 24 h acute response period and did not add naïve student flies. Wasps aged 3–7 days post-eclosion and flies aged 3–5 days post-eclosion were used for all experiments. Yeast paste was added to the center of fresh grape juice agar made in 60 mm x 15 mm culture plates. Embryo collection chambers were used to house the flies and wasps during experiments. We collected four groups of flies (each group initially consisting of 5 virgin females and 1 naïve male) and aged them together for 3–5 days. The four groups were used to examine the following conditions: (1) untreated females, no wasps, (2) untreated females, + wasps, (3) BPA-treated females, no wasps, and (4) BPA-treated females, + wasps. None of the male flies used in this experiment were treated with BPA. Following the 3–5 day aging period, flies were either introduced to 3 female wasps for 24 h (for the "+ wasp" conditions) or, as controls, were incubated without wasps for 24 h (for the "no wasp" conditions). The extent of oviposition was determined for all four groups by counting embryos laid following the 24 -h acute response period, either in the presence or absence of wasps. To move forward with the full procedure described by Kacsoh, B.Z., et al., 2015, wasp-exposed females must exhibit depressed oviposition to be competent "teachers" for naïve "student" flies. We stopped at the acute response stage because the BPA-treated females did not depress their oviposition behavior meaning we could not use these flies as "teachers" or measure associative learning in "student" flies.

2.6. Phototaxis

Performed as described in Vang et al., 2012 (Vang et al., 2012). Female flies aged 3–5 days were placed in an apparatus consisting of a glass *Drosophila* vial connected to a glass test tube (2.5 × 20 cm) that was marked to indicate evenly divided quartiles. A light source was placed 15 cm from the end of the fly vial-test tube apparatus to create a gradient of light across the tube, with the first quartile being the darkest and fourth quartile being the brightest. The first quartile of the glass tube was also surrounded by foil to create a darker environment. Flies were gently tapped into the first quartile prior to illumination. Following one, two, three, and four minutes of illumination, flies were counted in each quartile. Flies were acclimated to the dark for 30 min prior to each trial. Three trials with 29–30 flies were conducted.

2.7. Conditioned courtship suppression

BPA-treated and untreated (control) naïve males were collected post-eclosion and aged in isolation chambers with control food for 5–6 days. Unreceptive, pre-mated females were created by collecting unexposed virgin females and housing them with males at a ratio of 1:3 males to females for 5–6 days. To assess conditioned courtship suppression, each naïve male was placed in a courtship chamber with an unreceptive female for an hour. Flies were recorded for the initial ten minutes (0−10 min) and final ten minutes (50−60 min) of the hour-long interaction period. Videos of BPA-treated and untreated males were then examined, in a blinded manner, to determine the time each male spent engaging in courtship behaviors during the initial and final ten-minute intervals. The percent time a male fly participates in courtship behaviors over the duration of an assay is referred to as the courtship index (CI). To measure learning, the CI of the initial ten-minute interval was compared to the CI of the final ten-minute interval. Learning is indicated by a significant reduction in CI during the final ten minutes compared to the initial ten minutes.

2.8. NMJ synapse morphology

Performed as described in Karim et al., 2011 (Karim and Moore, 2011), with minor modifications. Late third instar larvae were dissected (< 7 min) in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature, washed 3 × 20 min in PBS with 0.2 % Triton X-100 (PBST), incubated at room temperature in blocking buffer (5% normal goat serum (NGS) in PBST), then incubated overnight at 4 ° C with anti-Discs Large (DLG; Developmental Studies Hybridoma Bank) at 1:500 in 5% NGS/PBST. Larva pelts were next washed 3 × 10 min in PBST, incubated at room temperature for 2 h with goat-anti-mouse Alexa488 (Jackson ImmunoResearch) at 1:250 in 5% NGS/PBST, then washed 5 × 15 min with PBST, followed by an overnight incubation at 4 ° C with Cy3-conjugated anti-horseradish peroxidase (HRP; Jackson ImmunoResearch) at 1:100 in 5% NGS/PBST. Larval pelts were washed 5 × 15 min with PBST and mounted in VECTASHIELD (Vector Labs) mounting medium. Images of muscle group 4, abdominal segment 3 were captured with an Olympus Fluoview FV10i confocal microscope. Synaptic boutons and axonal branches were quantified in a blinded manner. An axonal projection with at least two synaptic boutons was counted as an axonal branch.

2.9. Statistical analyses

All data was first examined for homoscedasticity using SPSS (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). All subsequent statistical analyses were performed using Prism 9 (GraphPad Software, San Diego, CA). Normality was tested using the Shapiro-Wilk test. Parametric data was analyzed using the Student's *t*-test. Nonparametric data was measured using the Mann-Whitney *U* test (for unpaired analysis of two groups), Wilcoxon signed-rank test (for paired analysis of two groups), and Kruskal-Wallis test with Dunn's multiple comparison test (for analysis of more than two groups). Figures were prepared using Prism 9 and BioRender.com. On graphs, bars represent the mean and error bars indicate the standard error of the mean (SEM).

3. Results & discussion

3.1. BPA predominantly downregulates genes associated with neural development and function

Given that *Drosophila melanogaster* is increasingly being used as a model for toxicological risk assessment of BPA, we wanted to examine how developmental exposure to BPA would affect gene expression in fruit flies. To enable comparison with previous studies (Kaur et al., 2015; Musachio et al., 2021; Nguyen et al., 2021), we exposed *Drosophila* to a similar dose of BPA (0.25 mg/mL) during embryonic and larval development. RNA from age-matched third instar larvae was used for RNA-seq. Differential expression (DE) analysis with an adjusted P value (p-adj) threshold of 0.01 led to the identification of 1040 genes that were differentially expressed in BPA-exposed larvae (Supplementary Fig. 1). To identify biological functions associated with misregulated genes, we used GSEA with log2 fold change in expression as the ranking metric. We performed a comparison across molecular processes from the Gene Ontology (GO) collection within the Cellular Component (CC) and Biological Processes (BP) subcategories. A summary of the top ten downregulated and upregulated pathways revealed BPA predominantly downregulated genes within the most impacted GO pathways (Table 1)—the p-adj was below 0.05 for all top downregulated pathways in both CC and BP subcategories, but not a single upregulated pathway met this criterion. GO processes associated with neurodevelopment and neural function were prominently represented among the top downregulated pathways, including GO_Synapse, GO_Presynapse, GO_ Postsynapse, GO_Dendritic_Tree, GO_Glutamatergic_Synapse, and GO_Neuronal_Process. In addition, GO_Chromatin and GO_Positive_Regulation_of_Transcription _By_RNA_Polymerase_II were among the top downregulated regulated processes in the CC and BP annotations, respectively. Although not specific to neurodevelopment, misregulated transcription is conspicuously associated with neurodevelopmental disorders, including ASD and intellectual disability (Ayhan and Konopka, 2019; Gabriele et al., 2018).

Heatmaps were generated to visualize the DE patterns of genes from the CC and BP subsets, which highlight the pronounced BPA-mediated downregulation (Fig. 1A-D, Supplementary Fig. 2). The BPA-induced downregulation of genes associated with the positive regulation of transcription (Fig. 1D) could at least partially account for the repression observed in other GO subsets. However, review of the complete list of DE genes showed that 42.7 % were upregulated by BPA, so the marked downregulation of neurodevelopmentally relevant GO processes and leading-edge genes may indicate preferential downregulation by BPA in neural tissues. We are unable to decipher tissue-specific mechanisms from our RNA-seq data set because we isolated RNA from whole larvae.

The top twenty leading-edge genes were organized into a table based upon frequency in GO pathway gene sets (Table 2). Leading-edge genes are those that contributed most to the enrichment score of each pathway within the CC and BP subsets. Previously determined functions associated with the top twenty leading-edge genes include a range of neurodevelopmental and/or behavioral functions in *Drosophila.* Some of these functions—including courtship behavior, locomotor activity, mushroom body development, axon growth and guidance, and neuroblast development—correspond to mutant phenotypes attributed to BPA exposure in previous studies (Kaur et al., 2015; Musachio et al., 2021; Nguyen et al., 2021). Additionally, many of the downregulated leading-edge genes have human orthologs for which loss-of-function mutations are implicated in ASD, intellectual disability, epilepsy, schizophrenia, and bipolar disorder, including DLG, CHD8, KCNA2, GSK3A, GSK3B, and TRAK1—a specific subset of genes previously identified in a toxicogenomic analysis of BPA exposure in human tissues (Wang and G.R.a. W.D., 2017). Given that GO annotation of RNA-seq data from both *Drosophila* and human tissues has found that BPA preferentially disrupts expression of genes critical for neurodevelopment and yielded a group of overlapping genes underscores that (1) BPA can likely interrupt neurodevelopmental pathways in a multitude of organisms ranging from invertebrates to mammals, and (2) *Drosophila* is a useful model for the toxicological risk assessment of BPA.

When using *Drosophila* to study the biological impacts of BPA, an important distinction between fruit flies and mammals to consider is that *Drosophila* do not have classic estrogen receptors (ERs) or estrogens. Though it is becoming increasingly clear that BPA can bind a broader array of receptors (MacKay and Abizaid, 2018), toxicity studies of BPA in vertebrates have largely centered on its ability to agonize multiple ER subtypes (Wetherill et al., 2007; Bonefeld-Jorgensen et al., 2007). Instead of classic ERs, *Drosophila* express an estrogen-related receptor (dERR), an orphan receptor that belongs to the same nuclear receptor superfamily as ERs but does not bind estrogen (Tennessen et al., 2011; Giguere, 2002). dERR may have the ability to bind BPA according to simulated molecular docking analysis (Wang et al., 2021), but actual binding studies are lacking. Regardless of whether dERR can bind BPA, comparison of our RNA-seq data to studies of dERR activity does not support this receptor as being critical for BPA-mediated transcriptional impacts within the nervous system. RNA-seq and GO analysis of dERR mutants show significant enrichment of metabolic processes, including carbohydrate metabolism and lipid metabolism; however, unlike our analysis of BPA-treated *Drosophila*, neurodevelopmentally related GO subsets were not significantly affected in dERR mutants (Beebe et al., 2020). Functional analyses have also found dERR to be critical for glycolysis and lipogenesis (Tennessen et al., 2011; Li et al., 2017), as well as testicular development (Misra et al., 2017). Our GO analysis did not indicate a BPA-associated enrichment in glycolytic or lipogenic gene subsets (Table 1), and there was no significant change in expression of testicular genes known to be affected by dERR activity, including *aly, mia, bruce, bam, bgcn, fzo, and eya* (Supplementary Fig. 1). While it is possible that a more sensitive approach, like single-cell RNA-seq, could reveal overlapping gene subsets enriched by BPA and dERR, current evidence suggests that BPA affects neurodevelopmental gene expression in a dERR-independent manner. Determining the receptors through which BPA impairs neurodevelopment in *Drosophila* will be a critical next step in defining its mode of action and could shed light on human health risks that are unrelated to the ability of BPA to dysregulate estrogen signaling.

3.2. Predator-induced oviposition behavior is impaired by BPA

We wanted to examine phenotypes associated with leading-edge genes that had not yet been explored by previous studies of BPA in *Drosophila.* Of the top leading-edge genes identified as being downregulated by BPA, seven are associated with learning and/or memory in fruit flies (Table 2). Of that subset, studies examining loss of function of *Pp1−87B, shaker, shaggy, CASK,* and *kismet* have reported diminished learning and memory in mutant flies (Asztalos et al., 1993; Lee et al., 2008; Wolf et al., 2007; Malik et al., 2013; Malik and Hodge, 2014; Melicharek et al., 2010). Thus, we hypothesized that BPA treatment would reduce learning and memory. We initially sought to measure these behaviors using the predator-induced non-associative learning and memory recall paradigm described by Kacsoh, Bozler et al. (Kacsoh et al., 2015b). The endoparasitoid wasp, *Leptopilina heterotoma,* infects and lays their eggs in *Drosophila* larvae and then consumes their host upon hatching. Female *Drosophila* recognize and physiologically respond to this threat by depressing their rate of oviposition when wasps are present (Kacsoh et al., 2015b). Further, when wasp-exposed female flies are introduced to naïve female flies that have never encountered wasps, the naïve females learn this oviposition behavior from the wasp-exposed females and subsequently depress their egg laying, despite never being exposed to wasps (Kacsoh et al., 2015b). The naïve females also exhibit memory recall by maintaining reduced oviposition for days following acquisition of the learned behavior (Kacsoh et al., 2015b). While it is unclear how wasp-exposed females recognize wasps as a threat to their offspring to trigger reduced egg-laying, this is an

Table 1
Top misregulated pathways by BPA within the Cellular Component (CC) and Biological Process (BP) subsets of the gene ontology (GO) collection. The top ten downregulated and top ten upregulated pathways from the CC and BP subsets of the GO collection. The P value (p-val), adjusted P value (p-adj), enrichment score (ES), normalized enrichment score (NES), and size of each gene set are listed.

CELLULAR COMPONENT (CC) PATHWAYS											
DOWNREGULATED PATHWAYS, TOP 10	p-val	p-adj	ES	NES	Size	UPREGULATED PATHWAYS, TOP10	p-val	p-adj	ES	NES	Size
GO_SYNAPSE	5.18E-07	2.73E-04	−0.4994	−2.6915	45	GO_CILIARY_PLASM	6.80E-03	0.1022	0.5559	1.8089	12
GO_NUCLEAR_BODY	2.36E-06	6.22E-04	−0.5008	−2.6343	42	GO_TIM23_MITOCHONDRIAL_IMPORT_INNER_MEMBRANE_TRANSLOCASE_COMPLEX	0.013	0.1483	0.9147	1.5187	2
GO_PRESYNAPSE	1.22E-05	2.15E-03	−0.6826	−2.5304	15	GO_TERTIARY_GRANULE_LUMEN	0.0258	0.2303	0.9922	1.3136	1
GO_CHROMATIN	1.54E-04	0.0177	−0.445	−2.3186	40	GO_DEUTEROSOME	0.0329	0.2501	0.9882	1.3084	1
GO_NUCLEAR_CHROMOSOME	1.68E-04	0.0177	−0.4494	−2.2899	38	GO_XY_BODY	0.0329	0.2501	0.9882	1.3084	1
GO_NUCLEAR_SPECK	3.86E-04	0.029	−0.4722	−2.1788	28	GO_SPINDLE_POLE	0.0471	0.2847	0.7077	1.518	4
GO_POSTSYNAPSE	3.41E-04	0.029	−0.5042	−2.2086	23	GO_RADIAL_SPOKE	0.0568	0.3115	0.7655	1.4592	3
GO_CELL_CORTEX	9.48E-04	0.0453	−0.6528	−2.1643	11	GO_INNER_MITOCHONDRIAL_MEMBRANE_PROTEIN_COMPLEX	0.0598	0.3178	0.647	1.5083	5
GO_DENDRITIC_TREE	9.33E-04	0.0453	−0.4907	−2.1816	24	GO_CILIARY_TIP	0.0646	0.3205	0.9745	1.2902	1
GO_GLUTAMATERGIC_SYNAPSE	8.55E-04	0.0453	−0.5391	−2.1386	18	GO_DENDRITE_TERMINUS	0.0646	0.3205	0.9745	1.2902	1

BIOLOGICAL PROCESS (BP) PATHWAYS											
DOWNREGULATED PATHWAYS, TOP 10	p-val	p-adj	ES	NES	Size	UPREGULATED PATHWAYS (TOP 10)	p-val	p-adj	ES	NES	Size
GO_NERVOUS_SYSTEM_PROCESS	3.32E-06	7.29E-03	−0.582	−2.5861	26	GO_CELLULAR_METABOLIC_ COMPOUND_SALVAGE	0.0235	0.2531	0.9922	1.3341	1
GO_POSITIVE_REGULATION_OF_RNA_ BIOSYNTHETIC_PROCESS	2.47E-06	7.29E-03	−0.4318	−2.4764	59	GO_GLYCOSYL_COMPOUND_ BIOSYNTHETIC_PROCESS	0.0235	0.2531	0.9922	1.3341	1
GO_REGULATION_OF_TRANSPORT	6.53E-06	9.57E-03	−0.4565	−2.4578	47	GO_GLYCOSYL_COMPOUND_ CATABOLIC_PROCESS	0.0235	0.2531	0.9922	1.3341	1
GO_POSITIVE_REGULATION_OF_ TRANSCRIPTION_BY_RNA_ POLYMERASE_II	1.14E-05	0.0105	−0.4579	−2.4144	44	GO_NUCLEOBASE_CONTAINING_ SMALL_MOLECULE_BIOSYNTHETIC_ PROCESS	0.0235	0.2531	0.9922	1.3341	1
GO_SENSORY_ORGAN_ DEVELOPMENT	1.19E-05	0.0105	−0.6041	−2.4668	21	GO_NUCLEOBASE_CONTAINING_ SMALL_MOLECULE_CATABOLIC_ PROCESS	0.0235	0.2531	0.9922	1.3341	1
GO_NEGATIVE_REGULATION_OF_ MOLECULAR_FUNCTION	2.09E-05	0.0115	−0.4237	−2.298	49	GO_NUCLEOBASE_METABOLIC_ PROCESS	0.0235	0.2531	0.9922	1.3341	1
GO_POSITIVE_REGULATION_OF_ CELLULAR_BIOSYNTHETIC_PROCESS	2.00E-05	0.0115	−0.3916	−2.3096	65	GO_NUCLEOSIDE_CATABOLIC_ PROCESS	0.0235	0.2531	0.9922	1.3341	1
GO_POSITIVE_REGULATION_OF_ NUCLEOBASE_CONTAINING_ COMPOUND_METABOLIC_PROCESS	1.99E-05	0.0115	−0.387	−2.3142	68	GO_NUCLEOSIDE_SALVAGE	0.0235	0.2531	0.9922	1.3341	1
GO_BIOLOGICAL_ADHESION	4.08E-05	0.018	−0.4649	−2.3236	37	GO_PYRIMIDINE_CONTAINING_ COMPOUND_BIOSYNTHETIC_ PROCESS	0.0235	0.2531	0.9922	1.3341	1
GO_REGULATION_OF_CELLULAR_ LOCALIZATION	3.96E-05	0.018	−0.511	−2.3967	31	GO_PYRIMIDINE_CONTAINING_ COMPOUND_CATABOLIC_PROCESS	0.0235	0.2531	0.9922	1.3341	1

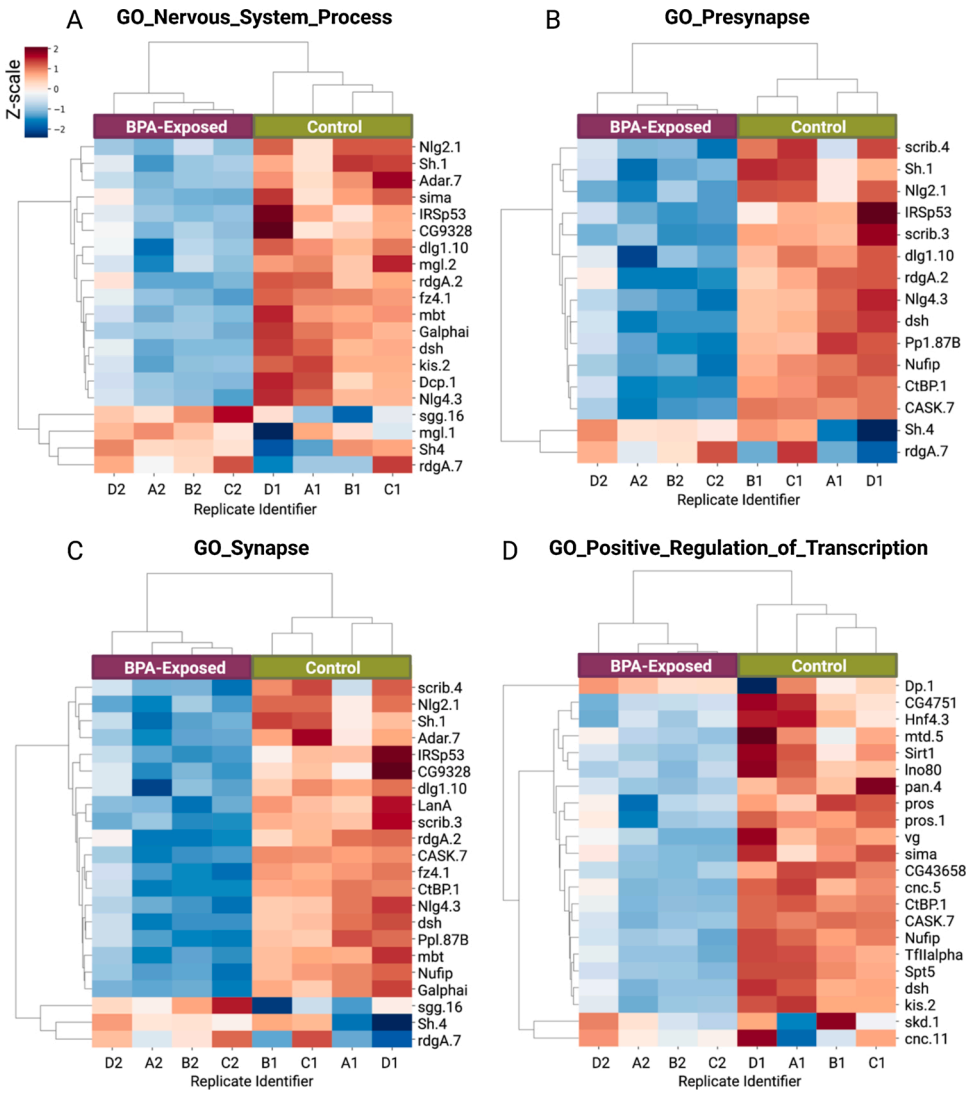

Fig. 1. Heatmaps based on GO enrichment analysis of BPA-exposed versus control larvae. Heatmaps display z-scaled values to reflect expression differences between samples. On the x-axis, BPA-exposed replicates (A2, B2, C2, D2) are on the left, and unexposed control replicates (A1, B1, C1, and D1) are on the right. Gene symbols are on the right y-axis; symbols followed by a number separated by a dot are transcript isoforms. (A) GO_Nervous System Process (B) GO_Presynapse, (C) GO_Synapse, and (D) GO_Positive Regulation of Transcription [by RNA Polymerase II].

innate response that requires visual perception (Kacsoh et al., 2013) and caspase-dependent apoptosis in the ovary (Kacsoh et al., 2015b). For the nonassociative learning and memory of this behavior to occur in the naïve flies, neural circuits involved in learning and memory are also required (Kacsoh et al., 2015b, c).

In this experimental paradigm, egg-laying behavior is first measured after an acute wasp-exposure period to ensure the innate response to wasps is intact (Fig. 2A). During this time the flies must visually perceive the wasps, which involves integrating the sensory information and triggering the physiological response of reduced egg-laying via apoptosis. Our control flies responded to wasp exposure as expected by significantly reducing the number of eggs deposited from an average of 131.3 eggs pre-wasp exposure to 39.4 eggs post-wasp exposure ($P < 0.0001$; Fig. 2B). However, we found that BPA-treated female flies did not significantly reduce their oviposition behavior in response to wasp-exposure—they deposited an average of 155.3 eggs pre-wasp exposure and 140.1 eggs post-wasp exposure ($P > 0.999$; Fig. 2B). The lack of significant decline in oviposition meant BPA-treatment compromised the innate response to wasps and that BPA-treated flies could not be used as "teachers." While this result indicated we could not use this experimental paradigm to measure non-associative learning and memory, it also suggested that BPA may interfere with the process of visual perception.

Visual perception is a cognitive process that involves reception, recognition, and response to visual stimuli. We wondered if BPA might be preventing reception of visual stimuli by causing blindness. Because flies that are blind do not exhibit positive phototactic behavior (Dushay et al., 1989), we measured this behavioral response using a simple phototaxis assay (Fig. 2C) (Vang et al., 2012). Flies were placed into a glass tube divided into four quartiles. A light source was positioned to create a gradient of light such that the first quartile was the darkest and the fourth quartile was the brightest. We found that BPA-treated flies moved toward the light stimulus to the same extent as control flies—an average of 61.1 % of control flies were found in the fourth quartile following one minute of light exposure compared to an average of 70.1 % of BPA-treated flies ($P = 0.743$; Fig. 2D). Because flies exposed to BPA during development have previously been shown to be hyperactive as adults (Kaur et al., 2015; Musachio et al., 2021), we also measured flies after two, three, and four minutes of light exposure to ensure the response at one minute was not merely due to increased locomotor activity. BPA-treated flies exhibited phototactic behavior that was indistinguishable from control flies at all timepoints (Supplementary Fig. 3). Therefore, at least some aspects of the visual light capture system and motion-related behavioral responses were intact in BPA-treated flies.

We propose that BPA interferes with the cognitive process of visual perception by disrupting the neural circuitry required for recognizing the wasps as a threat or for triggering the physiological response of reduced oviposition. In the absence of wasps, we did not observe a

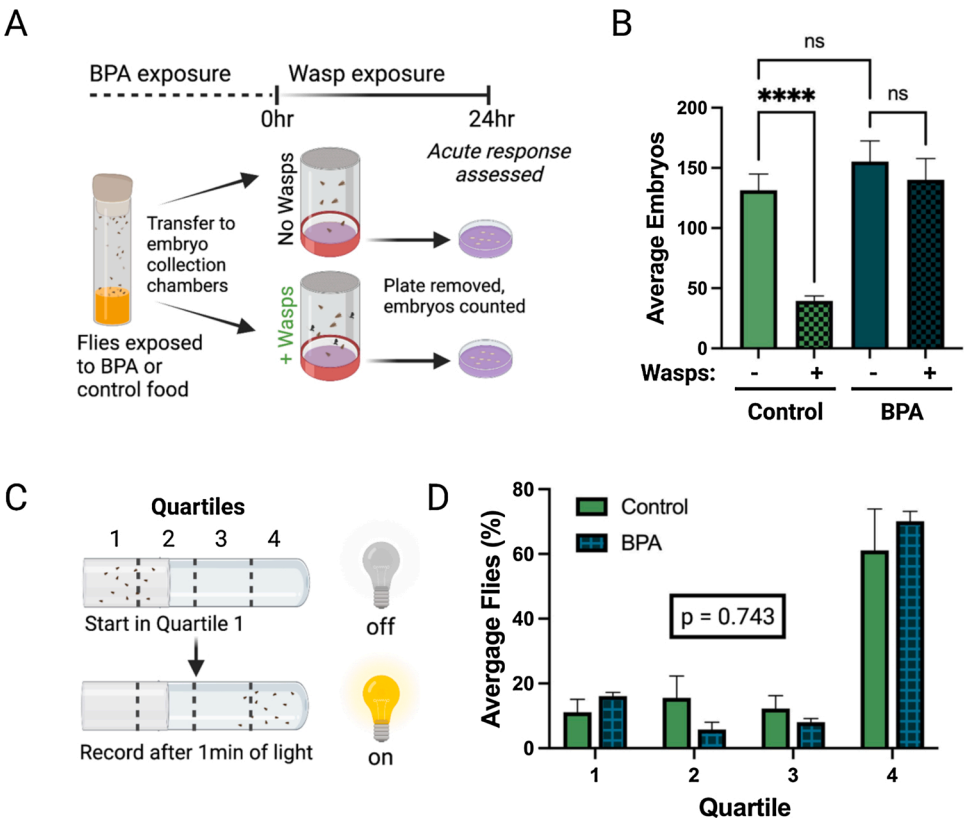

Fig. 2. BPA impairs wasp-induced oviposition behavior without causing blindness. (A) Experimental scheme for predator-induced oviposition depression assay. Females were exposed to wasps for 24 h before eggs were counted. Control females not exposed to wasps were used for comparison. (B) Control females significantly depressed their oviposition rate following wasp exposure. BPA-exposed flies did not have a significantly different rate of oviposition compared to control flies or when exposed to wasps. (**** $P < 0.0001$; ns = not significant; n = 18-20 trials of 5 females per trial; Kruskal-Wallis and Dunn's multiple comparison tests.) (C) The phototaxis assay involves placing flies in a glass tube divided into quartiles. A gradient of light is created by placing a light source adjacent to the fourth quartile. Flies began in the first quartile in the dark. Following one minute of light exposure the quartile location of flies was recorded. (D) BPA-exposed flies exhibited positive phototactic behavior that was not significantly different from control flies. A Mann-Whitney *U* test was used to compare the percent of control versus BPA-exposed flies located within each quartile ($P > 0.743$); the graph shows grouped, summary data of three trials of n = 29-30 female flies.

significant change in oviposition following BPA treatment (Fig. 2B), a finding that aligns with a separate study (Musachio et al., 2021). Thus, BPA does not appear to impact the process of oviposition; rather, BPA interferes with the process by which oviposition is reduced following exposure to wasps. Based on our RNA-seq findings, BPA does not affect the expression of genes required for apoptosis in the ovary, including *cyt-c, Dronc, Dredd, Drice,* or *Dcp-1* (Supplementary Fig. 1); although, this data does not exclude the possibility of BPA affecting apoptosis in a transcription-independent manner. BPA exposure did cause a significant downregulation of *pebbled* and *prospero (pros)*, two leading-edge genes (Table 2) that are both involved in photoreceptor cell axon guidance (Oliva et al., 2015; Pickup et al., 2002; Morey et al., 2008). Though further investigation is warranted, the downregulation of *pebbled* and *pros* in BPA-treated flies could disrupt the neural connectivity of retinal photoreceptors to distal brain regions required for visual interpretation of the wasps and initiation of the innate behavioral response.

3.3. BPA diminishes associative learning in the conditioned courtship paradigm

To further explore the impact of BPA on learning, we chose a behavioral test called conditioned courtship suppression. This paradigm examines a form of associative learning and memory dependent on multiple sensory modalities, including olfactory and gustatory (Hall, 1994; Siegel and Hall, 1979). The assay involves introducing a naïve male fly to an unreceptive (recently mated) female that functions as an aversive stimulus. Upon repeated rejection of copulation attempts by the unreceptive female, the male will learn to reduce his courtship activity by the end of an hour-long training period (Siegel and Hall, 1979; Tompkins, 1984). Thus, associative learning is reflected by a significant decrease in male courtship activity during the final ten-minute interval compared to the initial ten-minute interval of the training period (Fig. 3A). If learning occurs, memory recall can be evaluated by introducing a receptive female to the male 1−3 hours after the training period and assessing whether male courtship activity remains depressed (Siegel and Hall, 1979; Tompkins, 1984).

Using conditioned courtship suppression, we found that control flies exhibited a significantly reduced courtship index (CI) in the final ten-minute interval (0.19 +/- 0.07) compared to the initial interval (0.33 +/- 0.07) of the training period ($P < 0.0001$; Fig. 3B), a change indicative of associative learning. However, the CI of BPA-treated flies during the initial (0.34 +/- 0.22) and final (0.38 +/- 0.23) ten-minute intervals were not significantly different ($P = 0.300$; Fig. 3C). This showed that BPA exposure impairs learning in *Drosophila*, consistent with the predicted behavioral outcome based on the RNA-seq expression data. In the absence of learning, memory cannot be independently measured, so we were unable to use this experimental approach to assess memory.

BPA likely impairs associative learning in *Drosophila* by disrupting synaptic patterns within regions of the brain required for associative learning and memory. One candidate region is the mushroom body (MB), a central brain structure with established roles in translating olfactory sensory information into learned behavioral responses (Heisenberg et al., 1985; Aso et al., 2014; Vogt et al., 2014; Sitnik et al., 2003). To integrate olfactory stimuli, antennal lobe projection neurons must synapse with Kenyon cell neurons of the MB (Aso et al., 2014). To elicit a response, the Kenyon cells must synapse with MB output neurons that extend their axons beyond the MB (Aso et al., 2014). Associative olfactory learning is also dependent on the modification of synapses between Kenyon cells and MB output neurons by modulatory dopaminergic neurons that innervate the MB (Burke et al., 2012; Liu et al., 2012). Thus, there are multiple MB synaptic connections required to enable the experience-dependent responses observed in the conditioned courtship paradigm. Developmental exposure to BPA has previously been shown to impair axon guidance within the MB (Nguyen et al., 2021), though the molecular underpinnings of this phenotype were not investigated. Among our identified leading-edge genes downregulated by BPA are those with roles in axogenesis and/or synapse development, including *CASK, Pp1−87B, shaker, pros, shaggy,* and *kismet* (Table 2)

Table 2
Top twenty leading-edge genes. The top twenty leading-edge genes, frequency within the GO subsets, BPA-induced log2 fold change in expression, adjusted P value, relevant experimentally determined biological functions (from Flybase.org), human orthologs (from Flybase.org), and disorders associated with the human orthologs (from genecards.org, medlineplus.org, OMIM.org, orpha.net, and/or sfari.org).

Drosophila Gene	Frequency	Log2 Fold Change	Adjusted P Value (p-adj)	Biological Functions	Human Ortholog(s)	Associated Human Disorder(s)
dishevelled (dsh)	16	−1.56	7.57E-12	Wingless/Wnt signaling; planar cell polarity	DVL1, DVL2, DVL3	Major Depressive Disorder (DVL3), Robinow syndrome (DVL1, DVL3)
CASK (CASK)	12	−2.23	4.34E-16	Memory; locomotor activity; courtship behavior; synapse development and plasticity	CASK	ASD, intellectual disability, epilepsy, schizophrenia
discs large 1 (dlg1)	12	−3.3	4.39E-09	Synapse development; courtship behavior; locomotor activity; anterior-posterior axis specification; neuronal differentiation and organization	DLG1	ASD, cleft lip/palate
Protein phosphatase 1 at 87B (Pp1−87B)	12	−1.47	1.33E-10	Olfactory and visual learning; locomotor behavior; axon guidance	PPP1CC	Intellectual disabilities, congenital heart disease
maleless (mle)	10	−2.7	1.17E-03	Axon outgrowth; courtship behavior; dosage compensation; mRNA processing	DHX9	Werner's syndrome
scribble (scrib)	9	−2.31	5.02E-05	Memory; olfactory behavior; apical/basal polarity; anterior/posterior axis specification	SCRIB	Neural tube defects
Shaker (Sh)	9	−1.51	2.30E-09	Learning and memory; courtship behavior; locomotor behavior; circadian behavior; axon outgrowth	KCNA1, KCNA2	Episodic ataxia (KCNA1); epileptic encephalopathy, intellectual disability (KCNA2)
cap-n-collar (cnc)	8	−2.31	3.18E-08	Dendrite morphogenesis; oxidative stress; endoplasmic reticulum stress; intestinal stem cell homeostasis	NFE2L1, NFE2L2	Immunodeficiency, developmental delay, and hypohomocysteinemia
G protein alpha i subunit (Galphai)	8	−1.55	1.90E-16	Asymmetric neuroblast division; axon ensheathment; glial blood-brain barrier development; calcium-mediated signaling	GNAI1	ASD, developmental delay, intellectual disability, hypotonia, epilepsy
Neuroligin 4 (Nlg4)	8	−2.95	1.30E-09	Synaptic transmission; neuron cell-cell adhesion; social behavior; circadian behavior	NLGN4	ASD, schizophrenia, Tourette syndrome
prospero (pros)	8	−1.57	2.85E-07	Memory; courtship behavior; neuroblast proliferation and differentiation; axon outgrowth and guidance; dendrite morphogenesis; glial cell differentiation; synapse development	PROX1	Potential role in demyelinating disorders
shaggy (sgg)	8	−2.04	3.97E-04	Learning; synapse development; circadian behavior; Wingless/Wnt signaling; Insulin receptor signaling; planar cell polarity	GSK3A, GSK3B	ASD, fragile X syndrome, Rett syndrome, bipolar disorder, schizophrenia, major depressive disorder, epilepsy, Alzheimer disease
domino (dom)	7	−1.93	4.35E-23	Dendrite morphogenesis; circadian behavior; chromatin organization	EP400	Epilepsy, ossifying fibromyxoid tumor
kismet (kis)	7	−2.33	1.48E-15	Memory; synapse development; axon guidance; courtship; neuron remodeling; intestinal stem cell development; locomotion; blastoderm segmentation; chromatin organization	CHD6, CHD7, CHD8, CHD9	ASD (CHD6−9), CHARGE syndrome (CHD7)
Lim3 (Lim3)	6	−3.36	1.01E-04	Axon guidance; transcriptional regulation	LHX4	Hypothyroidism, hypopituitarism
milton (milt)	6	−2.76	5.24E-03	Axon transport of mitochondrion (mitochondrion localization and distribution)	TRAK1, TRAK 2	Developmental and epileptic encephalopathy, schizophrenia (TRAK1), amyotrophic lateral sclerosis 2, juvenile (TRAK2)
Neuroligin 2 (Nlg2)	6	−3.63	9.58E-13	Social behavior; courtship behavior; locomotor behavior; synapse development, maintenance, and function	NLGN2	ASD, Tourette syndrome, seizure disorder, hepatic encephalopathy
myoglianin (myo)	5	−4.79	1.64E-03	Mushroom body development; adult lifespan; active receptor signaling	MSTN, GDF11	Muscle hypertrophy (MSTN), vertebral hypersegmentation and orofacial anomalies (GDF11)
poly(A) binding protein (pAbp)	5	−2.07	4.46E-13	Synaptic transmission; negative regulation of neuron death; dorsal-ventral pattern formation; oogenesis; spermatogenesis	PABPC4, PABPC1	Myotonic dystrophy 2 (PABPC1)
pebbled (peb)	5	−1.19	3.18E-08	Eye development; photoreceptor cell axon guidance; ommatidial rotation	RREB1	Digeorge syndrome, spitzoid melanoma

(Melicharek et al., 2010; Chen and Featherstone, 2011; Babu et al., 2005; Grueber et al., 2007; Zhong and Wu, 2004; Franco et al., 2004). Each of these genes also have demonstrated roles in olfactory learning and/or memory in *Drosophila* (Asztalos et al., 1993; Wolf et al., 2007; Malik et al., 2013; Melicharek et al., 2010; Walkinshaw et al., 2015; Sokolowski, 2001). Thus, it is possible that the BPA-induced concomitant downregulation of *CASK, Pp1−87B, shaker, pros, shaggy,* and *kismet* impairs the MB neural network required for associative olfactory learning. However, given that conditioned courtship suppression involves other sensory modalities in addition to olfactory, it is also possible that neurodevelopmental defects in other brain regions contribute to the associative learning phenotype of BPA-treated flies.

3.4. BPA affects synaptic branching morphology at the larval neuromuscular junction

Our transcriptome analysis indicated BPA downregulates genes involved in synapse development at the larval neuromuscular junction (NMJ). Of these genes, *CASK*, *discs large 1 (dlg1)*, *Neuroligin 2* (*Nlg2*), and *Neuroligin 4* (*Nlg4*) have been shown to impact synaptic structure of the

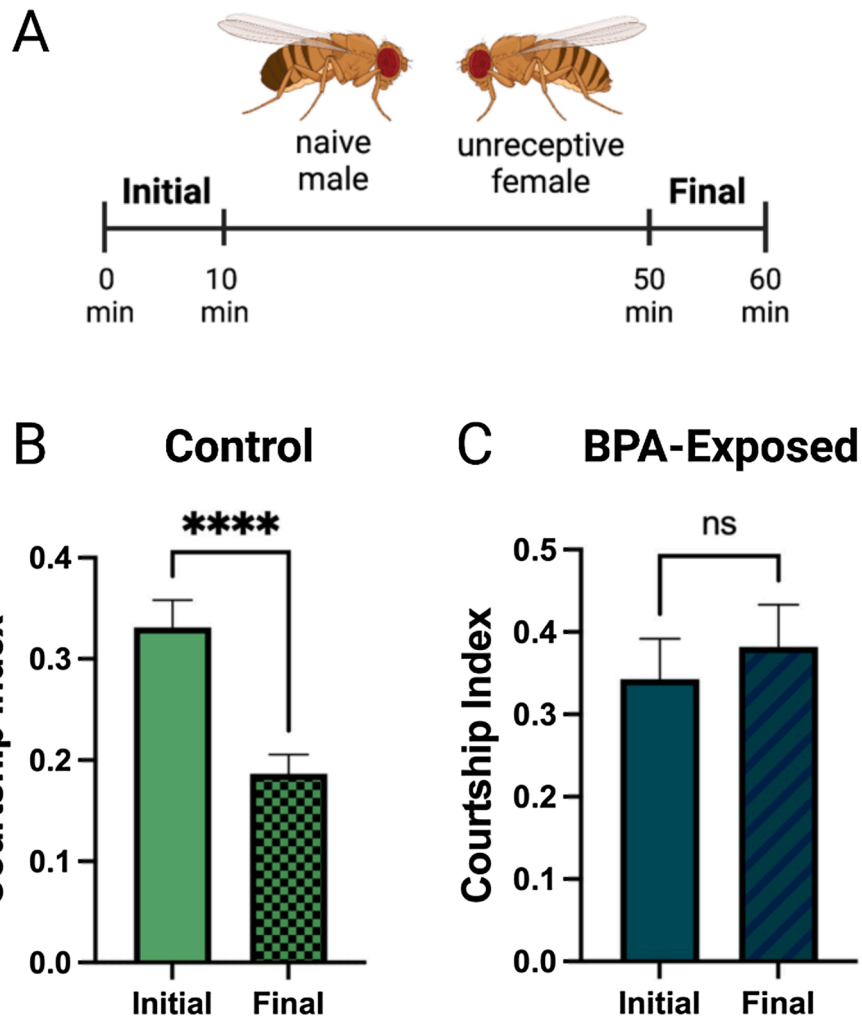

Fig. 3. Associative learning is suppressed by BPA. (A) Conditioned courtship suppression involves pairing a naïve male with an unreceptive female for a one-hour training period. The courtship index (CI) is measured for the initial and final ten minutes of the training period. (B) Control flies had a significantly lower CI in the final ten minutes compared to the initial ten minutes of the training period (**** $P < 0.0001$; $n = 18$, paired *t* test). (C) BPA-exposed flies did not have a significantly different CI in the final ten minutes compared to the initial ten minutes of the training period (ns = not significant; $n = 16$, Wilcoxon signed-rank test).

NMJ specifically by affecting the number of synaptic boutons and/or axonal branches (Chen and Featherstone, 2011; Arredondo et al., 1998; Zhang et al., 2017, 2007; Liu et al., 2017). Loss of *CASK*, *dlg1*, *Nlg2*, and *Nlg4* function causes fewer synaptic boutons to form within the NMJ (Chen and Featherstone, 2011; Zhang et al., 2017, 2007; Sun et al., 2011). *Nlg2* loss of function mutants also have reduced axonal branching in the NMJ (Sun et al., 2011). In contrast, *Nlg4* activity has been associated with restricting axonal branching (Liu et al., 2017), suggesting reduced *Nlg4* expression could lead to increased axonal branching. Given these findings, we hypothesized that BPA-treated larvae would have fewer synaptic boutons and dysregulated axonal branching; though, given the differing phenotypes associated with loss of *Nlg2* and *Nlg4* function, we were unable to predict if branching would be increased or decreased.

To examine NMJ synaptic morphology, we fluorescently labeled the neuronal and postsynaptic membranes and assessed the synaptic architecture within muscle group 4 of abdominal segment 3 of age-matched late third instar larvae (Fig. 4A-B). We found the number of synaptic boutons to be similar across the control (21.2+/- 5.2) and BPA-exposed (24.6 +/-6.2) conditions ($P = 0.153$; Fig. 4C). However, we did identify a significant increase in the number of axonal branches between control (2.6 +/- 0.8) and BPA-exposed (3.4 +/- 0.9) larvae ($P = 0.033$; Fig. 4D). Though modest, this disruption in NMJ synaptic architecture aligns with the synaptic dysregulation predicted by the gene expression data. Future investigations should focus on muscle groups with more elaborate synaptic architecture, like muscles 6/7, which are more suitable for revealing subtle phenotypes. Using electrophysiology to measure synaptic transmission would also help clarify how BPA influences synapse function.

The larval NMJ is part of a complex locomotor circuit involving sensory, inter, and motor neurons that coordinate movement (Hunter et al., 2021). Previous studies have indicated that developmental exposure to BPA causes hyperactive locomotor responses in both larvae (Nguyen et al., 2021) and adults (Kaur et al., 2015; ; Musachio et al., 2021). In stark contrast, adult exposure to BPA has been shown to reduce adult locomotor activity (Musachio et al., 2020), demonstrating that BPA can have different biological impacts contingent upon the developmental stage at which exposure occurs. Both increased synaptic boutons and axonal branches at the *Drosophila* NMJ have previously been connected to hyperactive locomotor responses (Kashima et al., 2017); therefore, the observed increase in axonal branches in BPA-treated larvae are consistent with the hyperactive locomotor responses observed in *Drosophila* exposed to BPA during development (Kaur et al., 2015; Nguyen et al., 2021).

In BPA-treated larvae, some leading-edge genes associated with synapse development at the larval NMJ, like *CASK, dlg1*, *Nlg2* and *Nlg4*, are also involved with synaptogenesis in the central nervous system (CNS) (Sun et al., 2011; Hodge et al., 2006; Lahey et al., 1994; Li et al., 2013). The dysregulated synaptic circuits in the brain caused by loss of *CASK, dlg1*, *Nlg2* and *Nlg4* are thought to underpin their behavioral phenotypes, including impaired memory *(CASK),* courtship *(CASK, dlg1, Nlg2),* and social behavior *(Nlg2, Nlg4)* (Malik and Hodge, 2014; Corthals et al., 2017; Mendoza-Topaz et al., 2008). Thus, genes involved with synapse formation at the NMJ often have overlapping roles

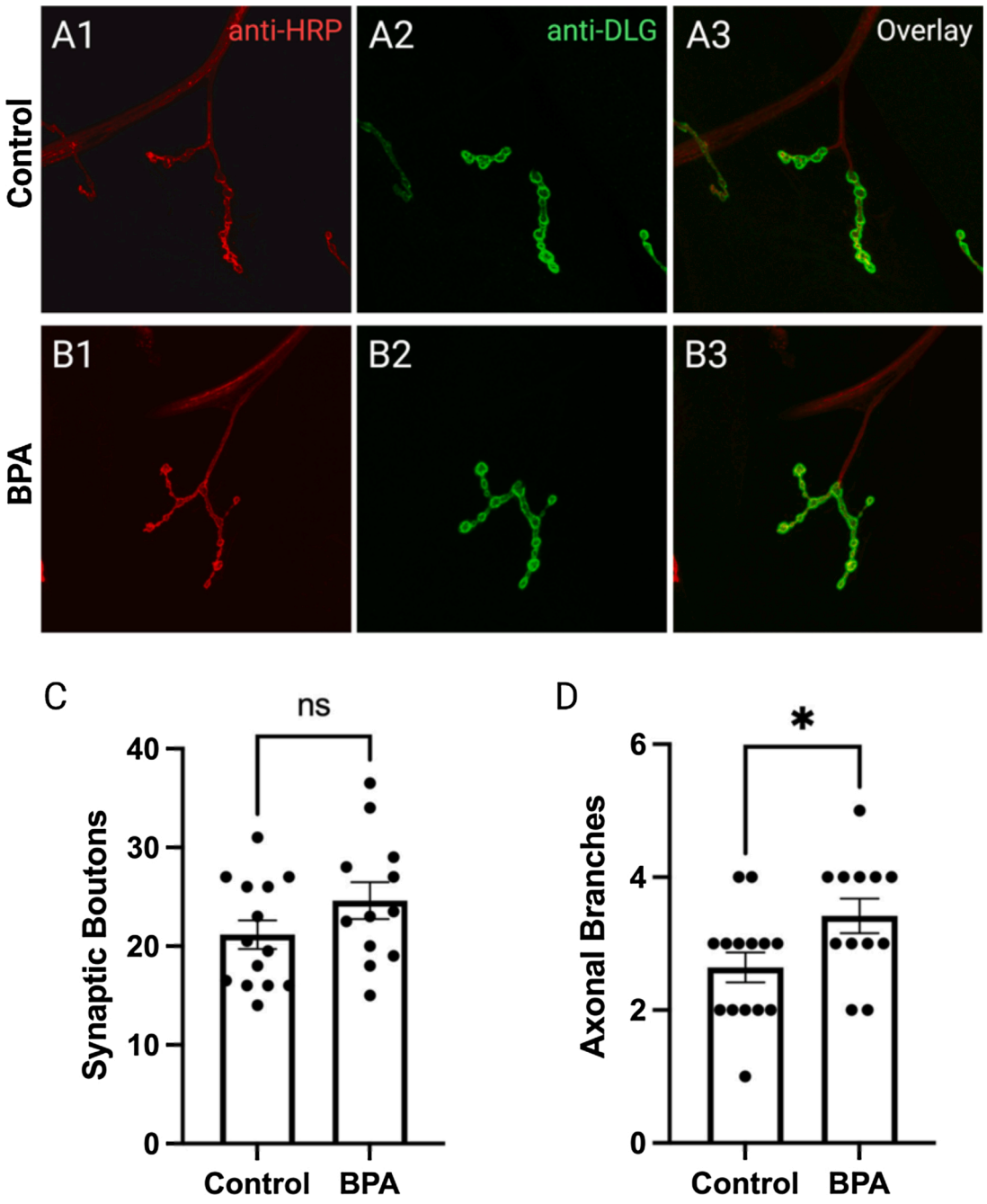

Fig. 4. BPA significantly increases the number of axonal branches, but not synaptic boutons. (A) Representative axon termini from control larvae. (B) Representative axon termini from BPA-exposed larvae. (A1, B1) Cy3-conjugated anti-horseradish peroxidase (HRP), marked the neuronal membrane in red. (A2, B2) Anti-discs large (DLG) and Alexa 488 anti-mouse marked the post-synaptic membrane in green. (A3, B3) Overlay. (C) The number of synaptic boutons in were not significantly different between control and BPA-exposed groups (n = 12 - 14; ns = not significant; Student's *t* test). (D) BPA-exposed larvae exhibited a significant increase in the number of axonal branches (n = 12 - 14; * $P < 0.05$; Student's *t* test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

regulating synapse development within the CNS, which can impact behavior. This implies that even though NMJ synaptogenesis is not the direct cause of the cognitive defects observed in this study, dysregulated NMJ synaptic architecture can be suggestive of synaptic defects elsewhere. Notably, because we did not use single-cell RNA-seq, we cannot pinpoint whether these leading-edge genes were downregulated in both the NMJ and the CNS. In the case of BPA-treatment, there were also downregulated leading-edge genes, like *pros, shaggy,* and *kismet,* that are exclusively required for synaptogenesis in the CNS, as well as for cognitive functions like learning and memory (Wolf et al., 2007; Melicharek et al., 2010; Walkinshaw et al., 2015). An interesting future direction would be to examine synapse formation within the CNS following BPA exposure, and to decipher the specific genes involved in BPA-associated synaptic changes and cognitive deficits.

4. Conclusion

BPA is a ubiquitous environmental chemical that has been shown to impact neurodevelopment in organisms spanning the animal kingdom, from fruit flies to humans. Given the implications for human neurodevelopmental disorders, as well as the potential harm to the broader ecosystem, the delineation of the molecular, cellular, and behavioral

consequences of BPA exposure is critical. Our study highlighted the utility of *Drosophila* as a model for examining the developmental neurotoxicity of BPA. We developed a bioinformatic pipeline for GSEA in *Drosophila* using GO annotation that showed BPA causes the misexpression of hundreds of genes, with the most prominent impact being the downregulation of genes associated with neurodevelopment and behavior—including orthologs of risk genes for human neurodevelopmental and neuropsychiatric disorders. A demonstrated advantage of using *Drosophila* is the repertoire of relatively simple and low-cost cellular and behavioral assays that can be used to substantiate RNA-seq data. A limitation of this study is that RNA-seq data can only be correlated with the observed cognitive and synaptic phenotypes; BPA may also contribute to neurodevelopmental and behavioral phenotypes in a transcription-independent manner not detected by RNA-seq. Nevertheless, our RNA-seq data and GSEA findings provided corroborating evidence for previous studies reporting neurodevelopmental consequences of BPA exposure, as well as motivated our novel discovery that BPA exposure impairs visual perception, learning, and NMJ synaptic morphology in *Drosophila*.

Data availability

Our open-access data is available via Zenodo (https://doi.org/10.5281/zenodo.5794358) but we were unable to link this data set in the \

Data will be made available on request.

CRedit authorship statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2022.01.006.

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