Parallel mechanisms of visual memory formation across distinct regions of the honey bee brain

Arián Avalos[1], Ian M. Traniello[2,3], Eddie Perez Claudio[4], Tugrul Giray[5 *].

Affiliations:

1. United States Department of Agriculture, Agricultural Research Service, Honey Bee Breeding, Genetics and Physiology Research, Baton Rouge, LA.

2. Neuroscience Program, University of Illinois at Urbana-Champaign (UIUC), Urbana, IL.

3. Carl R. Woese Institute for Genomic Biology, UIUC, Urbana, IL

4. Department of Biology, University of Puerto Rico (UPR), Rio Piedras Campus, San Juan, Puerto Rico.

5. Institute of Neurobiology, UPR, Medical Sciences Campus, San Juan, Puerto Rico.

*Author for correspondence**:** tugrul.giray@upr.edu

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Author Contributions

AA and TG designed experiments; AA and EP performed behavioral and molecular analyses; AA and IMT performed statistical analyses and wrote the manuscript; AA, IMT and TG revised the manuscript.

Abstract

Visual learning is important to the behavioral ecology of the Western honey bee (*Apis mellifera*). Despite its importance in behaviors like orientation, foraging, and nest site selection, how visual memories are mapped to the brain remains poorly understood. We collected bees that successfully learned to avoid one visual stimulus over another in a conditioned aversion paradigm and compared gene expression correlates of memory formation between sensory transduction and learning centers of the brain. We looked at two classical genetic markers of learning and one gene specifically associated with punishment learning in vertebrates. We report substantial involvement of the mushroom bodies for all three markers and demonstrate a parallel involvement of the optic lobes across a similar time course. Our findings imply the molecular involvement of a sensory neuropil during visual associative learning parallel to a higher-order brain region, furthering our understanding of how a tiny brain processes environmental signals.

Introduction

Visual learning is vital to honey bee behavioral ecology. Honey bees use visual cues to perform orientation flights (Cartwright and Collett, 1983; Menzel et al., 2005), locate floral resources (Chittka and Raine, 2006; Giurfa et al., 1995; Lehrer et al., 1995; Sen Sarma et al., 2010), and possibly to assess and select new nest sites (Seeley and Visscher, 2003; Visscher, 2007). However, the process of how visual memories are established in sensory- and learning-related compartments of the brain remains poorly understood. To this end, we utilized expression analysis of established learning and memory genes in the primary visual neuropil and a higher-order processing center following a visual learning event. By analyzing brain region-specific gene expression

following a visual retention task, our study will inform an understanding of the spatiotemporal dynamics learning and memory.

The honey bee brain consists of ~1,000,000 neurons, about 340,000 of which are Kenyon cells composing mushroom bodies (MB), centers of sensory integration, learning and memory (Heisenberg, 1998; Strausfeld, 2002; Witthöft, 1967). Visual stimulus perception and transduction are carried by adjacent, distinctly compartmentalized regions, the optic lobes (OL), which project visual input to the collar and basal ring of the MB. By capitalizing on the contrast of cellular function in these discrete anatomic subunits, we can begin to understand the path of transduction of visual environmental information to sensory integration and processing.

In honey bees, long term memory (LTM) formation has been characterized using classical conditioning of the proboscis extension response (PER) to olfactory stimuli (Bitterman et al., 1983; Menzel, 1999). Like in other behavioral systems, LTM in honey bees involves activation of the calcium/calmodulin kinases (CAMK) (Kamikouchi et al., 2000; Perisse et al., 2009). Phosphorylation of CaMKII leads to the subsequent activation of cyclic AMP response element-binding protein (CREB), which becomes active and modulates transcription for the long-term maintenance of newly formed associations (Eisenhardt et al., 2003; Kamikouchi et al., 2000; Kandel, 2001; Kandel, 2012; Matsumoto and Mizunami, 2002). The downstream activation of CREB via phosphorylation induces its function as a transcription factor in LTM processes (Bito et al., 1996; Kandel, 2001; Kandel, 2012; Lakhina et al., 2015). Our approach used expression profiles of CaMKII and CREB, established markers of the LTM process in the honey bee and other systems (Eisenhardt et al., 2003; Kamikouchi et al., 2000;

Kandel, 2001; Kandel, 2012; Matsumoto et al., 2009; Menzel and Giurfa, 2001; Pasch et al., 2011; Perisse et al., 2009), as precursor signals of downstream LTM. The principal assumption is that detectable changes in the expression of these target genes over a short time course following learning can serve as a molecular marker for downstream LTM formation.

In addition to established targets of the LTM process (CaMKII, CREB) we also explored the expression profile of the gene flap structure-specific endonuclease 1 (fen-1). Though not yet described in honey bees, fen-1 has been previously associated with aversive conditioning in vertebrate models (Saavedra-Rodríguez et al., 2009; Wang et al., 2003), thus making it a relevant target gene for comparative analyses of aversive conditioning in the brain.

Considering both target expression and neural pathway input, our two primary hypotheses are: 1) gene expression differences will be present in the MB but not in the OL or PB, as mechanisms of synaptic plasticity may not be necessary in regions of sensory transduction; alternatively, 2) gene expression will follow the anatomical pathway visual input must take from sensory transduction to higher-order sensory processing with OL signal detected earlier than MB.

Results

In the OL, where the initial process of sensory transduction occurs, we observed significant upregulation CaMKII in L_E only at the 80 min time point ($t = 3.899$, $p = 0.004$; Fig. 3). Both CREB and fen-1 trended upward at 80 min, paralleling gene expression in the MB, but this relationship was not significant (Fig. 3). No other treatment group or timepoint showed a significant upregulation in this region, however both CaMKII and

CREB were significantly downregulated in C_C and S_C relative to N_C at 20 (C_C, t = -2.851, p = 0.04; S_C, t = -6.154, p << 0.001) and 80 min post-trial (C_C, t = -6.042, p << 0.001; S_C, t = -4.006, p = 0.004). In contrast to the OL, gene expression in the MB, where visual stimuli are contextualized, showed dramatic changes both relative to N_C and across timepoints (Fig. 3). Each gene was significantly upregulated 80 min post-trial in the MB of L_E (CaMKII, t = 3.844, p = 0.003; CREB, t = 3.29, p = 0.01; fen-1, t = 2.02, p = 0.05). In addition, CaMKII and fen-1 were significantly increased at 80 min relative to the 20 min timepoint (CaMKII, 20 vs. 80 min, t = -2.26, p = 0.0005; fen-1, 20 vs. 80 min, t = -2.11, p = 0.05). Tested genes in the PB showed no significant upregulation though significant downregulation was observed in at the 80 min C_C group and both S_C groups relative to N_C group for CaMKII and CREB (CaMKII, Naive vs. Context 80 min, t = -5.02, p < 0.001; CaMKII, Naive vs. Shock 20 min, t = -7.40, p < 0.001; CaMKII, Naive vs. Shock 80 min, t = -4.74, p < 0.001; CREB, Naive vs. Context 80 min, t = -5.59, p < 0.001; CREB, Naive vs. Shock 20 min, t = -6.10, p < 0.001; CREB, Naive vs. Shock 80 min, t = -5.60, p < 0.001; Fig. 3).

Discussion

Though the MB have received considerable attention as the seat of learning and memory in the insect brain (Heisenberg, 1998; Heisenberg, 2003; Strausfeld, 2012), we demonstrate activation of the OL, a first-order sensory neuropil, beyond visual sensory transduction. This finding is evidence against Hypothesis 1, which predicted that signal would be absent outside of the MB. Rather, molecular signatures of learning and memory following aversion learning show similar patterns of gene expression in both the OL and MB, supporting parallel mechanisms of learning and memory in both

tissues. This is supported by a recent study which investigated immediate early gene (IEG) expression in the honey bee brain and suggested that both sensory and higher-order brain regions express IEGs across a similar time course following an aggressive encounter (Traniello et al., 2019).

Interestingly, significant CaMKII upregulation in L_E in both MB and OL at 80 min, but not 20 min, post-learning implies a similar time course of activation across visual neuropil and higher-order processing centers in the honey bee brain. Furthermore, both CREB and fen-1 showed increases in expression in the OL. Though a nonsignificant trend, these responses were only seen at 80 min post-learning, and only in the L_E bees, further supporting collateral mechanisms which contrast with the predictions of Hypothesis 2. This gene expression time course further contrasts with spatiotemporal mapping of gene expression in larger vertebrate brains, where specific regions may be genetically activated relative to their place in a signal transduction pathway (Saul et al., 2018). This difference may be related to spatial and metabolic constraints intrinsic to the arthropod brain (Chittka and Niven, 2009; Niven and Farris, 2012).

In addition, our results show fen-1 expression increased in a region-specific manner. Initially, we considered that fen-1 expression is not associated with learning but instead could be a response to electric shock-induced oxidative damage to DNA (Adachi et al., 1993; Lee et al., 2000). Our finding that fen-1 exhibited a significant, localized increase in the MB and upward trend in the OL following aversive learning but not shock alone offers evidence of a specific association with LTM. This suggests a highly conserved function of fen-1 in aversive learning, which has previously only been described in vertebrate systems (Saavedra-Rodríguez et al., 2009; Wang et al., 2003).Our study

relates neuroanatomical substrates to conserved molecular processes associated with visual memory formation. We show that distinct compartments of the honey bee brain are activated across a similar time course independent of their location in a neural circuit involved in learning. Further studies will be necessary to dissect peaks of upregulation for each gene in each region to determine if, for example, levels of gene expression in the OL are comparable to those in the MB, but peak at distinct times following the learning assay. Here, we demonstrate involvement of a sensory region not typically associated with learning in the molecular response to learning and memory. This observation implies that the biological embedding of environmental information is distributed across distinct anatomical regions of the tiny bee brain.

Materials and Methods

Collections. We collected returning foraging worker honey bees at the research apiary at Gurabo Agricultural Research Station of the University of Puerto Rico in Gurabo, Puerto Rico. All workers were collected during peak foraging hours (8:00-17:00) (Mattu et al., 2012) by blocking the colony entrance with a wire mesh screen (6.32 mm^2 aperture), then using a modified collection vacuum (Model 5911, Type 1, 12V DC; BioQuip, Rancho Dominguez, CA) to safely aspirate workers into a collection vessel. Immediately following collection, the wire mesh was removed, and the collection cage was extracted from the vacuum and sealed. Collected bees were provided with 50% sugar solution and transported to our research laboratory at the University of Puerto Rico, San Juan. Foragers were quickly placed in a rearing cage (Bug Dorm 1 Rearing Cage; BioQuip, Rancho Dominguez, CA) with food provided *ad libitum* and left overnight in a dark incubator set at 34^0C.

163 *Electric Shock Avoidance assay.* We used the Electric Shock Avoidance (ESA) assay to
164 examine color-learning. This assay is a free-operant experimental paradigm that
165 selectively isolates visual learning (Agarwal et al., 2011; Avalos et al., 2017). Foraging
166 workers collected and transported to our research laboratory were quickly placed in a
167 rearing cage one day following collection, and groups of 10 bees were sequentially
168 extracted for the ESA (Avalos et al., 2017).

169 A simplified version of the ESA assay was used to train individual bees. The protocol
170 presented the color using a Styrofoam^{TM} block with equal halves of its surfaced lined
171 with blue and yellow construction paper. The cassette was placed on top of the block
172 during shock presentation, aligning the electrified area with the selected color of the
173 grid. During recovery periods between the 5 min trials and short-term memory test, the
174 cassette was placed in a dark incubator. Both color and position of shock were
175 counterbalanced between groups of bees trained to avoid spatial learning independent
176 of color.

177 Four experimental groups were sampled in this study: Naïve control (N_C), Context
178 control (C_C), Shock control (S_C), and Learned (L_E). N_C bees were collected directly from
179 the colony, acclimated in the incubator and anesthetized with a 15 s exposure to CO_2
180 and flash-frozen 15 min later, upon recovery. This group therefore controls for baseline
181 gene expression of a honey bee forager during experimental handling.

182 L_E bees were exposed to the same handling process as N_C bees, but, following
183 recovery from CO_2, were then subjected to training. In the training assay, we paired one
184 of two colors with a mild shock (CS^+) over two 5 min trial presentations. These two
185 presentations were separated by a 10 min inter-trial interval (ITI) spent in a dark

incubator to remove visual stimuli and avoid possible memory extinction in the absence of shock. Following training, L_E bees were again placed in the incubator for 20 min, then exposed to a one min short term memory (STM) test in which color but no shock was provided. C_C group bees went through the same process as learning group bees, but during the 5 min trials no shock was provided to either side. This group therefore provides a control of potential effects from bees being placed in the training arena.

For the S_C group, we also assayed 10 individuals at a time. We used a yoked control design in which one bee was designated "master" and experienced the same training as the L_E individuals, while the remaining nine bees were designated "yoked," experiencing the same proportion of shock events and duration as the master bee but disassociated from the visual stimulus. In this way S_C individuals served as controls experiencing noxious stimuli in absence of color context.

For all groups, behavioral response was measured and cataloged by two observers via scan sampling. One observer scanned the grid every 15 seconds and conveyed the presence/absence of each bee on the shock side of the apparatus to the cataloguing participant. Response data were used to categorize individuals (*see below*). Individual bees were collected and flash frozen in liquid nitrogen immediately following recovery (N_C group) or at 20 or 80 min following the last presentation trial (all other groups). Individuals were kept at -80^{0}C to await sample selection and gene expression analysis.

Sample selection for molecular analysis. Across all samples, we screened for survival of handling (all), adequate interaction with the arena (C_C, S_C), and in the case of the L_E group, association of shock stimulus with color. To be suitable for gene expression analysis, all individuals needed to have survived handling and CO_2 anesthetization. For

those groups experiencing the apparatus, they were required to have shuttled between color regions at least three times. For L_E we additionally required that 1) they spend more than 50% of the last 2.5 min of the second trial on the safe side, and also 2) on two of the possible four 2.5 min time blocks (*described below*). This selection scheme allowed us to identify bees that correctly associated shock with color and therefore experience learning. Criterion 1 focused on improvement: better-than-average performance in this time period suggests retention of learned information (Agarwal et al., 2011; Avalos et al., 2017). Criterion 2 assured that acquisition occurred throughout the assay. These additional criteria in L_E assured we identified bees that correctly formed an association between color and punishment, i.e. learned avoidance (Fig. 1). Any bee not meeting selection criteria was excluded from further analysis resulting in the following per-group sample sizes: 6 (N_C), 16 (C_C), 24 (S_C), 18 (L_E).

Gene expression analysis. Head capsules were chipped on dry ice to expose the brain, glands, and optic lobe pigment, and the whole head was submerged in RNAlater® ICE (Thermo Fisher Scientific, Waltham, MA) at -20°C for 16 hr (Fig. 2a). Brains were fully extracted on wet ice and regions of interest were dissected (Fig. 2b). We performed region-specific analysis aided by the honey bee brain atlas (Brandt et al., 2005; Rybak et al., 2010), dissecting out the MB and OL specifically. We also utilized the remaining tissue, composed of the protocerebrum, subesophageal ganglion (SOG), and AL as a conglomerate we reference here as peripheral bodies (PB) (Fig. 2c). We used the PB as a contrasting physiological control as they contain regions likely involved with signal transduction during visual LTM (e.g. protocerebrum) but also those not (e.g. SOG, AL). Regions were re-suspended in RNAlater® ICE solution for later analysis. To obtain

sufficient genetic material for analysis, we pooled brain regions from two individual bees randomly chosen from each behavioral group. This resulted in the final per-individual sample sizes of: N_C n = 3, C_C 20 min n = 3, C_C 80 min n = 5, S_C 20 min n = 7, S_C 80 min n = 5, L_E 20 min n = 5, L_E 80 min n = 4, with each individual contributing three regions (MB, OL, PB).

Following dissection, total RNA was extracted from the sample pools. Each pool was homogenized using a 2-mercaptoethanol lysing solution and a 21-gauge, 1 mL sterile syringe (BD; Franklin Lakes, NJ). RNA was extracted using the RNeasy Micro Kit (QIAGEN, Hilden, Germany), which included a DNase treatment step. Resulting RNA material was checked using gel electrophoresis with a 1% agarose gel to assure no genomic DNA contamination was present. Quality and relative quantity were assessed using a NanoDrop® (Thermo Fisher Scientific, Waltham, MA), and resulting quantity measures were further verified using GloMax® Luminometer (Promega, Madison, WI). Following extraction, aliquots of the samples were organized in a 96-well PCR plate and reverse transcribed to cDNA using the iScript™ Reverse Transcription Supermix kit and protocol (Bio-Rad Laboratories, Hercules, CA). The resulting 96-well plate with cDNA was used as source quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Primer design. We used Ribosomal Protein S5 (rpS5) as a reference gene (Evans, 2004; Evans, 2006; Evans and Wheeler, 2001), and three target genes: CREB CaMKII, and fen-1. Reference gene primer sequences were obtained from previously published sources implementing rpS5 as a reference gene given its expression stability (Evans, 2004; Evans, 2006; Evans and Wheeler, 2001), which we also confirmed across tissue

and time points (*data not shown*). The fen-1 primers used in our study were previously developed and validated as part of the University of Puerto Rico at Rio Piedras 2010 Topicos Graduate Course (*data not shown*). For CREB, we developed primers specific to isoforms known to be specific in the brain (Eisenhardt et al., 2003).

qRT PCR. Optimized primer sets were used in conjunction with iTaq™ Universal SYBR® Green Supermix (BioRad, Hercules, CA) and aliquots of our samples to conduct our qRT-PCR analysis. For each gene three 96-well PCR plates were run in a Stratagene™ MX3005P qPCR system. Resulting cycle thresholds (C_t) were checked and samples that did not produce at least two consistent values across the three plates were discarded from the study. Replicates were discarded if the product T_m deviated by one degree from the expected amplicon and other resulting T_m values to avoid mis-priming or primer artifacts during amplification.

Data analysis. We used -ΔΔCt to analyze resulting qRT-PCR expression data using the N_C group as the calibrator. Gene expression differences were examined gene-by-region between N_C and all other groups using a one-way ANOVA which combined treatment and timepoints into a single variable. This approach identified significant changes in expression related to experimental manipulation, with individual pairwise differences identified via a post-hoc Dunnett's test using the N_C group as control. Significant changes in gene expression over time and between treatment groups were determined via a two-way ANOVA of C_C, S_C, and L_E groups and individual pairwise differences were identified via a Tukey's post-hoc test. All statistical analyses were conducted using the R software (R Core Team, 2016).

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Tables and Figures

Gene	Accession Number	Strand	Primer Sequence
AmCaMKII	NM_001134950	Sense	5'-GACAAGAGACTGTGGATTGC-3'
		Antisense	5'-TGATGCTCCGACTGGAAA-3'
Amfen-1	XP_006559671.1	Sense	5'-GCTCAACTTACCTCCGTAGATGGT-3'
		Antisense	5'- TGCATTTCCAGCTTCTTCTGCTGC-3'
AmCREB*	AJ430462.1, AJ430463.2, AJ430466.2	Sense	5'-CTGTTGACCCATTGTCTG-3'
		Antisense	5'-GAGTTTGCTGCTGTGTTC-3'
AmrpS5	XP_006570300.1, XP_006570299.1	Sense	5'- AATTATTTGGTCGCTGGAATTG-3'
		Antisense	5'- TGCATTTCCAGCTTCTTCTGCTGC-3'

*** Brain-specific CREB variant (see text)**

Table 1. Primer sets used for each of the target candidate genes. This table reports primer sequences for the panel of genes used. Specifically provided are the genes' name, GenBank accession number, primer strand read direction, and actual sequence.

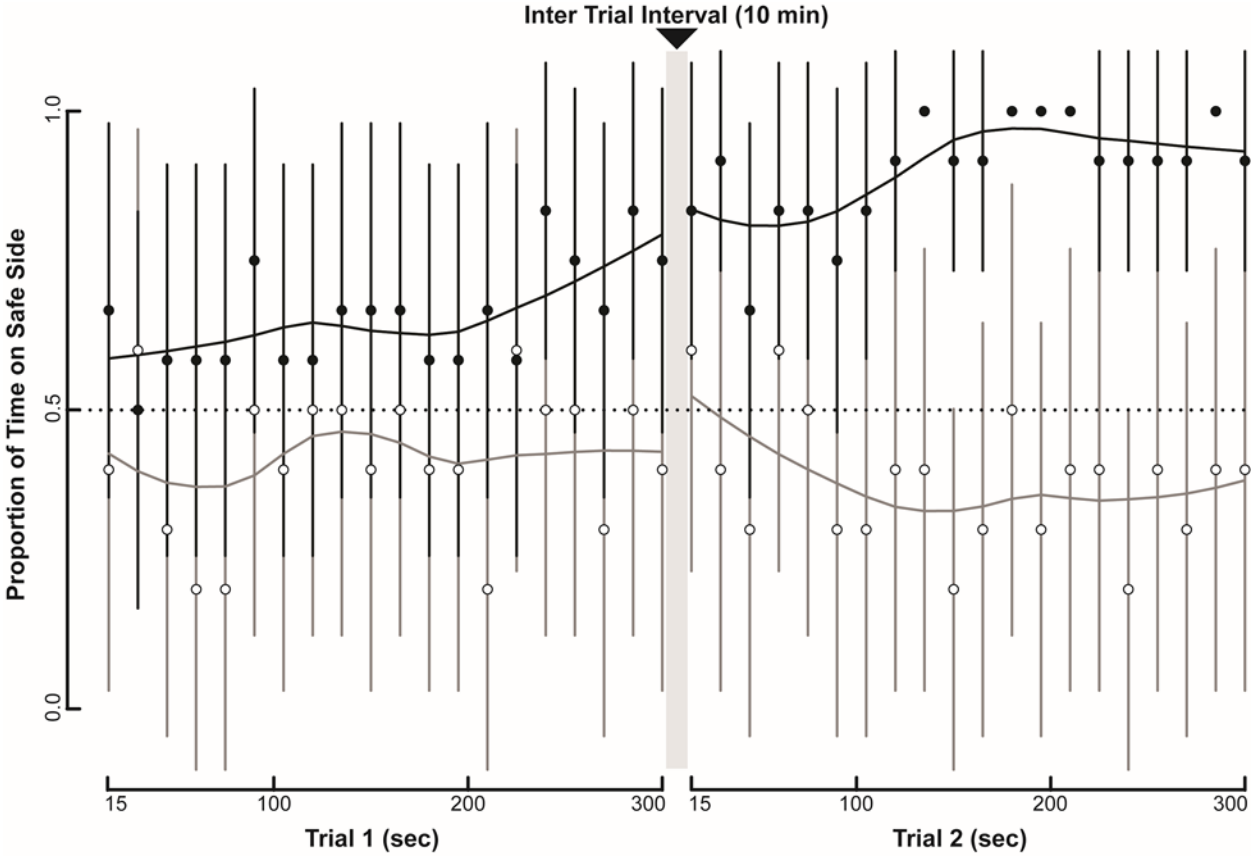

Figure 1. Summary of behavioral response used for sample selection. The plot represents learning response of test bees. Dark circles identify the group mean for individuals that met sample selection criteria and were included in the L_E (n = 18), light circles identify cohorts that did not meet selection criteria and were not considered (n = 8, *see Methods*). Vertical dark and light bars represent the 95% confidence interval for each corresponding colored group. A Loess smoothing curve is also provided to visualize the learning trend for each group and each Trial.

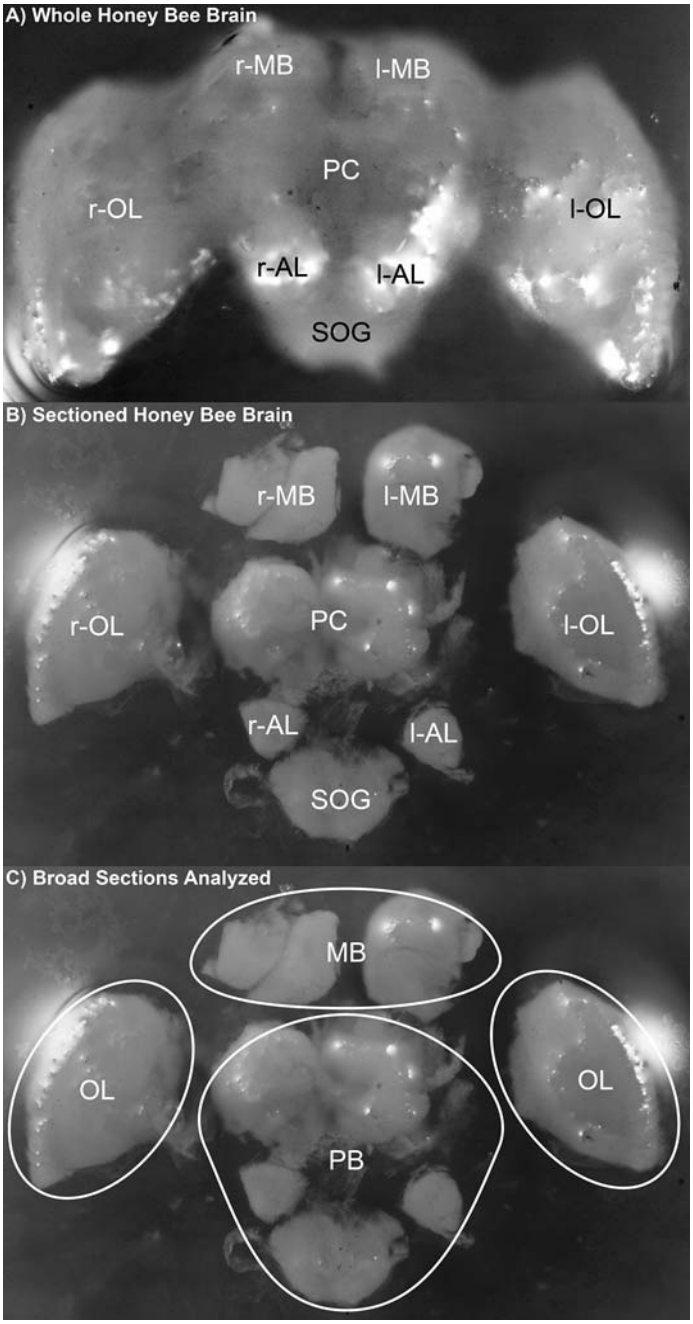

Figure 2. Dissected and sub-sectioned honey bee brain defining broad brain regions analyzed via qRT-PCR Methods. The figure demonstrates a dissected (a) honey bee worker brain with annotated gross anatomical regions that include right and left mushroom bodies (r-, l-MB), proto-cerebrum (PC), right and left optic lobes (r-, l-OL),

right and left antennal lobes (r-, l-AL), and the subesophageal ganglion (SOG). Further depicted is the same brain, sub-sectioned into these gross anatomical regions (b), and later those same gross anatomical regions highlighted (c) to denote the three broad brain regions used in the study, namely: mushroom body (MB), optic lobe (OL), and peripheral bodies (PB).

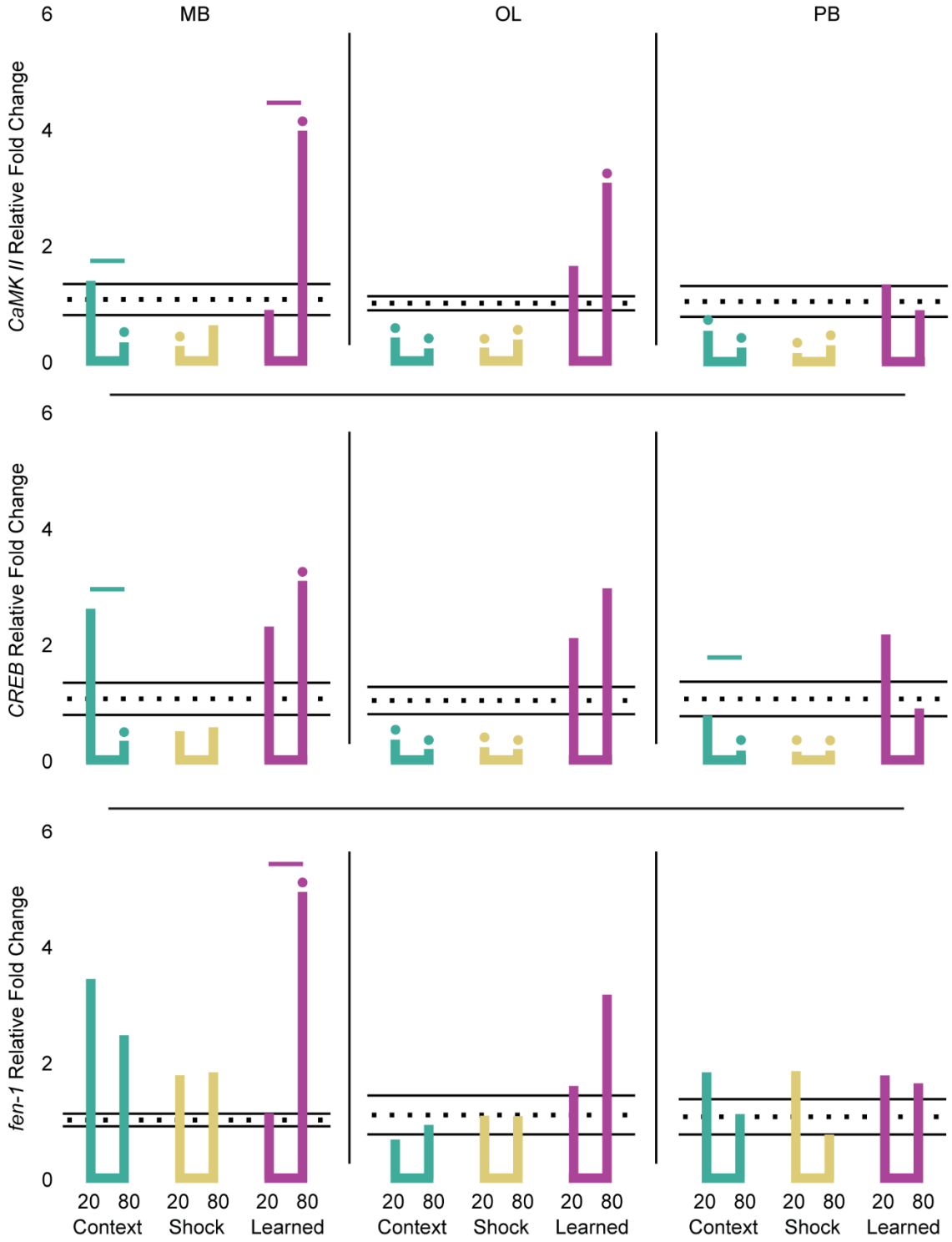

Figure 3. Expression profile of target genes following punishment learning. Each row in the figure corresponds to a target gene, each column to a broad brain region. In

each panel section, the simplified bar graph shows lines extending towards the mean relative fold change in expression for each group and timepoint combination. The horizontal dotted line represents the mean relative fold change in expression of the N_C group with the solid lines delimiting the range within 1 standard error around the mean. Significant differences from N_C group expression are indicated by the circles atop the line. Significant expression level differences between timepoints within a group are identified by horizontal lines matching the group's color.