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SHORT COMMUNICATION

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

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ABSTRACT

Visual learning is vital to the behavioral ecology of the Western honey bee (*Apis mellifera*). Honey bee workers forage for floral resources, a behavior that requires the learning and long-term memory of visual landmarks, but how these memories are mapped to the brain remains poorly understood. To address this gap in our understanding, we collected bees that successfully learned visual associations in a conditioned aversion paradigm and compared gene expression correlates of memory formation in the mushroom bodies, a higher-order sensory integration center classically thought to contribute to learning, as well as the optic lobes, the primary visual neuropil responsible for sensory transduction of visual information. We quantified expression of *CREB* and *CaMKII*, two classical genetic markers of learning, and *fen-1*, a gene specifically associated with punishment learning in vertebrates. As expected, we found substantial involvement of the mushroom bodies for all three markers but additionally report the 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.

KEY WORDS: *Apis mellifera*, Mushroom bodies, Aversive learning

INTRODUCTION

Visual learning is necessary for the survival and growth of honey bee societies. Honey bee foragers, bees that locate and gather resources for the colony, use visual cues to perform orientation flights (Cartwright and Collett, 1983; Menzel et al., 2005), to 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). Despite its importance, the neuroanatomical basis of visual memory formation remains poorly understood. Recently, it has been shown that in ant color learning, multiple brain levels, from sensory to central, are involved, as evidenced by volume and synaptic changes following associative color experiences (Yilmaz et al., 2019). We set out to explore the spatial and temporal aspects of learning in anatomically distinct sensory- and learning-associated regions of the honey bee brain, examining expression of genes associated with learning and memory formation following associative color experiences.

The honey bee brain consists of ∼1,000,000 neurons, about 340,000 of which are Kenyon cells comprising mushroom bodies (MB), centers of sensory integration, learning and memory (Heisenberg, 1998; Strausfeld, 2002; Witthöft, 1967). Visual stimulus perception and signal transduction are carried out by adjacent, distinctly compartmentalized regions, the optic lobes (OL), which project visual input to the MB. Previous work has identified volumetric changes in the OL, but not MB, associated with visual learning (Yilmaz et al., 2019), although the MB has other known roles in this process (Müller, 2012). However, it is not known whether molecular activity in the earliest phases of memory acquisition is shared or distinct across neuropils. By capitalizing on the contrast of cellular function in these discrete anatomical subunits, we can characterize the path of signal transduction of visual environmental information to sensory integration and processing while testing the hypothesis that sensory regions engage spatially or temporally distinct patterns of molecular activity in the form of gene expression. 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.

In honey bees, long-term memory (LTM) formation has been best characterized using classical conditioning of the proboscis extension response (PER) to olfactory stimuli (Bitterman et al., 1983; Menzel, 1999), with similar memory phases described following aversive conditioning (Agarwal et al., 2011; Nouvian and Galizia, 2019). As 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, 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, 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, 2012; Matsumoto et al., 2009; Menzel and Giurfa, 2001; Pasch et al., 2011; Perisse et al., 2009). Detectable changes in the expression of these genes following learning can serve as a predictive indicator 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

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associated with aversive conditioning in vertebrate models (Saavedra-Rodríguez et al., 2009; Wang et al., 2003), thus making it a promising target gene for comparative analyses of aversive conditioning in the honey bee brain.

Considering both target expression and neural pathway input, we hypothesized that: (1) gene expression differences will be present in the MB alone, as mechanisms underlying memory formation may not be necessary in regions of sensory signal transduction; or, alternatively, (2) gene expression will follow the anatomical pathway that visual input must take from sensory signal transduction to higher-order sensory processing, with OL signal detected earlier than MB signal. By analyzing brain region-specific gene expression following a visual retention task, we can begin to inform the spatial and temporal transcriptional dynamics of learning and memory.

MATERIALS AND METHODS

Collections

We collected returning foraging worker honey bees, *Apis mellifera* Linnaeus 1758, 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 (08:00–17:00 h) (Mattu et al., 2012) in the summer of 2013 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, 12 V DC, BioQuip, Rancho Dominguez, CA, USA) 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) with food provided *ad libitum* and left overnight in a dark incubator set at 34°C.

Electric shock avoidance assay

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

A simplified version of the ESA assay was used to train individual bees, which involved a learning apparatus or 'cassette' comprising individual lanes in a wire grid with a color background (see Avalos et al., 2017). The protocol presented the color using a Styrofoam™ block with equal halves of its surfaced lined with blue and yellow construction paper. The cassette was placed on top of the block during shock presentation, aligning the electrified area with the selected color of the grid. During recovery periods between the 5 min trials and short-term memory (STM) test, the cassette was placed in a dark incubator. Both the color and position of the shock were counterbalanced between groups of bees trained to avoid spatial learning independent of color.

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

L_E bees were exposed to the same handling process as N_C bees, but, following recovery from CO_2, were then subjected to training. In the training assay, we paired one of two colors with a mild shock (CS^+) over two 5 min trial presentations. These two 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 1 min STM test in which color but no shock was provided. C_C group bees went through the same process as L_E 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 the absence of a color context.

For all groups, behavioral response was measured and cataloged by two observers via scan sampling. One observer scanned the grid every 15 s 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°C until 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 anesthesia. For those groups experiencing the apparatus, they were required to have shuttled between color regions at least 3 times. For the L_E group, 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 (2) were on the safe side 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 experienced 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 ensured that acquisition occurred throughout the assay. These additional criteria in L_E ensured we identified bees that correctly formed an association between color and punishment, i.e. learned avoidance (Fig. S1). Any bee not meeting the selection criteria was excluded from further analysis, resulting in the following per-group sample sizes: N_C n=6, C_C n=16, S_C n=24, L_E n=18.

Gene expression analysis

Head capsules were chipped on dry ice to expose the brain, glands and OL pigment, and the whole head was submerged in RNAlater® ICE (Thermo Fisher Scientific, Waltham, MA, USA) at −20°C for 16 h (Fig. S2A). Brains were fully extracted on wet ice and regions of interest were dissected (Fig. S2B). 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 and antennal lobes as a conglomerate we reference here as the central brain (CB) (Fig. S2C). We used the CB as a contrasting physiological control as it contains regions likely to be involved with signal transduction during visual LTM (e.g. protocerebrum) but also those that are not (e.g. subesophageal ganglion, antennal lobes). Brain 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, CB).

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, USA). RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany), which included a DNase treatment step. The resulting RNA material was checked using gel electrophoresis with a 1% agarose gel to ensure no genomic DNA contamination was present. Quality and relative quantity were assessed using a NanoDrop® (Thermo Fisher Scientific), and resulting quantity measures were further verified using a GloMax® Luminometer (Promega, Madison, WI, USA). 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, USA). The resulting 96-well plate with cDNA was used for quantitative PCR (qPCR) analysis (see below).

Primer design

We used *Ribosomal Protein S5* (*rpS5*) as a reference gene (Evans, 2004, 2006; Evans and Wheeler, 2001), and three target genes: *CREB*, *CaMKII* and *fen-1*. Reference gene primer sequences (Table 1) were obtained from previously published sources implementing *rpS5* as a reference gene given its expression stability (Evans, 2004, 2006; Evans and Wheeler, 2001), which we also confirmed across tissue and time points (linear model of *rps5* C_t time point×tissue $F_{2,3}$=0.8993, P=0.4845). The *fen-1* primers used in our study (Table 1) 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 to isoforms known to be specific to the brain (Eisenhardt et al., 2003).

qPCR

Optimized primer sets (Table 1) were used in conjunction with iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and aliquots of our samples to conduct our qPCR 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 melting temperature (T_m) deviated by 1°C from that of the expected amplicon and other resulting T_m values to avoid mis-priming or primer artifacts during amplification.

Data analysis

We used $-\Delta\Delta C_t$ to analyze resulting qPCR 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 time points 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 a 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 R software (http://www.R-project.org/).

RESULTS AND DISCUSSION

Gene expression in the MB, where visual stimuli are processed and contextualized, showed dramatic changes both relative to N_C and across time points (Fig. 1; data provided in Table S1). 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* expression was significantly increased at 80 min relative to the 20 min time point (*CaMKII*, 20 versus 80 min, t=−2.26, P=0.0005; *fen-1*, 20 versus 80 min, t=−2.11, P=0.05).

Similarly, in the OL, where the initial process of sensory signal transduction occurs, we observed significant upregulation *CaMKII* in L_E at the 80 min time point (t=3.899, P=0.004; Fig. 1). Both *CREB* and *fen-1* were also elevated at 80 min, paralleling gene expression in the MB, but this relationship was not significant (Fig. 1). No other treatment group or time point showed a significant upregulation in this region; however, both *CaMKII* and *CREB* were significantly downregulated in the C_C and S_C groups relative to the N_C group at 20 min (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). These results suggest learning-associated gene expression patterns in the OL that parallel those in the MB.

The CB region served as an experimental control as we do not anticipate that visual learning specifically activates the olfactory or gustatory system. The CB showed no significant upregulation of expression, though significant downregulation was observed in the

Table 1. Primer sets used for each of the target candidate genes

Gene	Accession no.	Strand	Primer sequence
CaMKII	NM_001134950	Sense	5′-GACAAGAGACTGTGGATTGC-3′
		Antisense	5′-TGATGCTCCGACTGGAAA-3′
fen-1	XP_006559671.1	Sense	5′-GCTCAACTTACCTCCGTAGATGGT-3′
		Antisense	5′-TGCATTTCCAGCTTCTTCTGCTGC-3′
*CREB**	AJ430462.1, AJ430463.2, AJ430466.2	Sense	5′-CTGTTGACCCATTGTCTG-3′
		Antisense	5′-GAGTTTGCTGCTGTGTTC-3′
rpS5	XP_006570300.1, XP_006570299.1	Sense	5′-AATTATTTGGTCGCTGGAATTG-3′
		Antisense	5′-TGCATTTCCAGCTTCTTCTGCTGC-3′

*Brain-specific CREB variant (see Materials and Methods).

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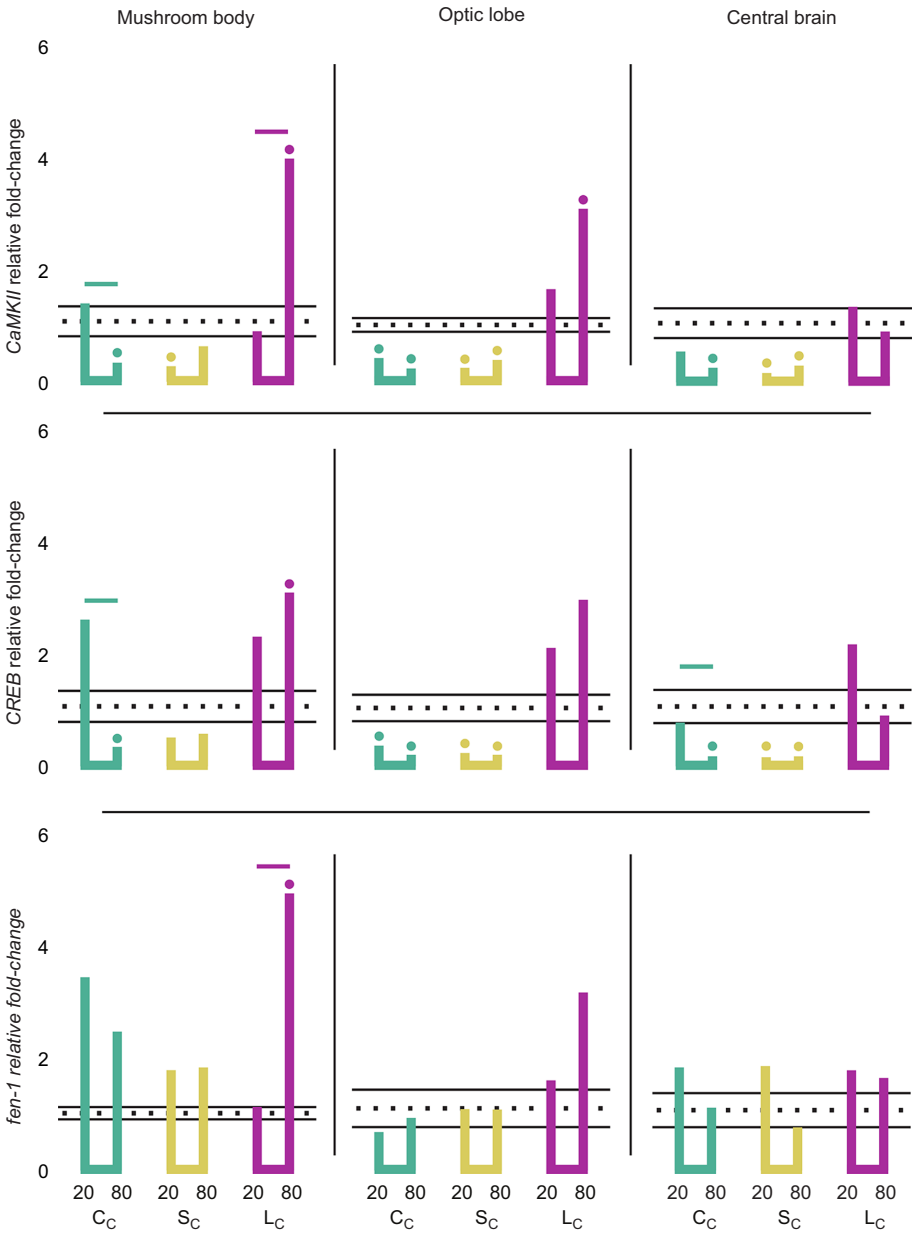

Fig. 1. Expression profile of target genes following punishment learning. Each row corresponds to a target gene, each column to a broad brain region. The simplified bar graphs show the mean relative fold-change in expression for each group (C_C, context control; S_C, shock control; L_E, learned) and time point (20 or 80 min) combination. The horizontal dotted line represents the mean relative fold-change in expression of the naïve control (N_C) group, with the solid lines delimiting the range within 1 s.e.m. Significant differences from N_C group expression are indicated by colored circles. Significant expression level differences between time points within a group are indicated by horizontal colored lines.

80 min C_C group and both S_C groups relative to the N_C group for *CaMKII* and *CREB* (*CaMKII*: C_C 80 min, t=−5.02, P<0.001; S_C 20 min, t=−7.40, P<0.001; S_C 80 min, t=−4.74, P<0.001; *CREB*: C_C 80 min, t=−5.59, P<0.001; S_C 20 min, t=−6.10, P<0.001; S_C 80 min, t=−5.60, P<0.001; Fig. 1). This signal suggests that though the regions aggregated in the CB are still responding to the exposure, it is a distinct response from those observed in either the OL or MB.

Conclusions

Though the MB has received considerable attention as the seat of learning and memory in the insect brain (Heisenberg, 1998, 2003; Strausfeld, 2012), sensory neuropils such as the OL may also be a neuroanatomical substrate for learning (Müller, 2012; Yilmaz et al., 2019), and we describe here shared transcriptomic responses that follow a similar time course in the OL and MB. This finding is evidence against our first hypothesis, which predicted that transcriptomic signals would be absent outside of the MB following a task that demanded learning. Rather, gene expression following aversive learning showed similar patterns in the OL and MB, supporting parallel mechanisms of learning and memory in the two tissues. This pattern 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, *CaMKII* upregulation following learning in both the 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 non-significant, these responses were only seen at 80 min post-learning, further supporting collateral mechanisms, which contrasts 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). We suggest that this difference may be related to spatial and metabolic constraints intrinsic to the arthropod brain that influence distinct processing strategies (Chittka and Niven, 2009; Müller, 2012; Niven and Farris, 2012; Yilmaz et al., 2019).

In addition, our results show that *fen-1* expression increased in a region-specific manner. Initially, we considered that *fen-1* expression was not associated with learning but instead could be a response to shock-induced oxidative damage to DNA (Adachi et al., 1993; Lee et al., 2000). Our finding that *fen-1* was elevated in the MB and OL following aversive learning but not shock alone is evidence of a specific association with LTM. This suggests a conserved function of *fen-1* in aversive learning, previously described only 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 gene expression in distinct compartments of the honey bee brain is 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 whether, for example, expression levels in the OL are comparable with those in the MB, but peak at distinct times following the learning assay. Here, we provide further evidence of the involvement of a sensory region not typically associated with learning, thus implying that the memory of environmental experience is distributed across distinct anatomical regions of the honey bee brain.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.A., I.M.T., T.G.; Methodology: A.A., I.M.T., T.G.; Validation: A.A., I.M.T.; Formal analysis: A.A., I.M.T.; Investigation: A.A., E.P.C., T.G.; Resources: A.A., T.G.; Data curation: A.A., E.P.C.; Writing - original draft: A.A., I.M.T.; Writing - review & editing: A.A., I.M.T., E.P.C., T.G.; Visualization: A.A.; Supervision: A.A., T.G.; Project administration: T.G.; Funding acquisition: T.G.

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Data availability

Data for this study are available in Figshare: 11823753.

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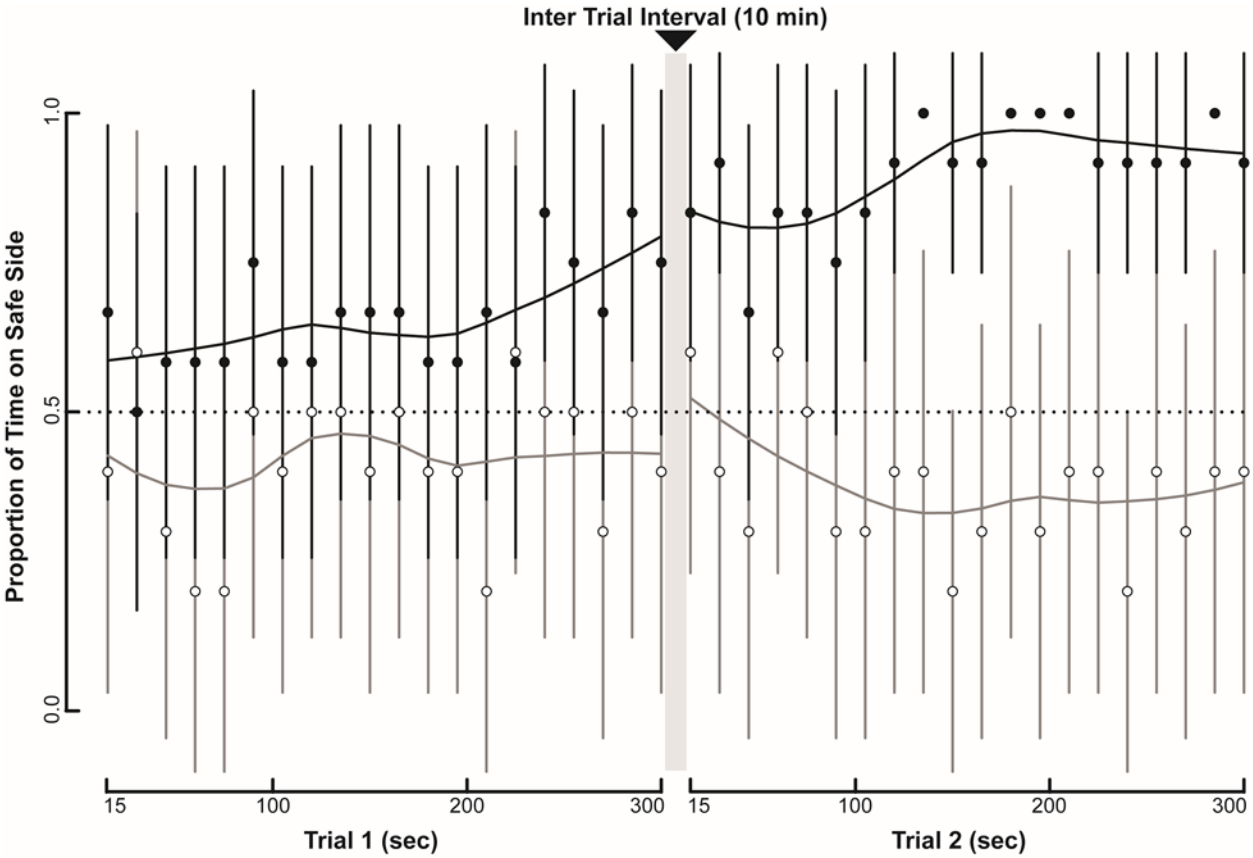

Fig. S1. Summary of behavioral response used for sample selection. The plot represents learning response of test bees. Dark circles identify the group mean for honey bees that met sample selection criteria and were included in the LE (n = 18), light circles identify cohorts that did not meet selection 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.

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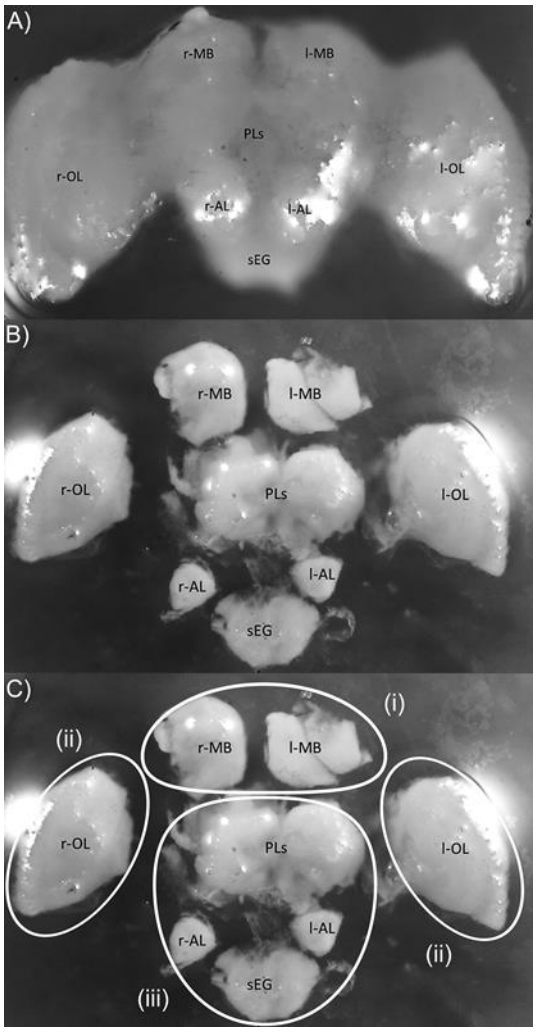

Fig. S2. Dissected and sub-sectioned honey bee brain defining broad brain regions analyzed via qRT-PCR Methods. The figure demonstrates a dissected (A) honey bee worker brain with annotated gross anatomical regions that include right and left mushroom bodies (r-, l-MB), proto-cerebrum (PC), right and left optic lobes (r-, l-OL), right and left antennal lobes (r-, l-AL), and the subesophageal ganglion (sEG). 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: (i) mushroom bodies (MB),(ii)optic lobes (OL), and (iii) central brain (CB).

Table S1.

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