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Original research article

Memory-related Signaling Pathways Revealed by Transcriptomic and Structural Analyses in *Apis mellifera*

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Abstract Honey bees (*Apis mellifera*) are essential pollinators with advanced cognitive abilities, but the molecular basis of their learning and memory remains poorly defined. In this study, we integrated brain RNA-seq data and comparative *in silico* analyses to characterize *A. mellifera* orthologs of canonical insect memory-related genes. Candidate genes were identified from a *de novo* assembled brain transcriptome by BLASTx, full-length coding sequences and proteins were retrieved from the *A. mellifera* reference genome, and their expression was confirmed by RT-PCR. Physicochemical properties were computed using ProtParam, protein-protein interaction networks were inferred via STRING and visualized in Cytoscape, and three-dimensional structural models of representative proteins were generated with SWISS-MODEL. Memory-related proteins segregated into two major groups: large, hydrophilic, and intrinsically unstable transcriptional/signaling regulators, and smaller, more stable and/or hydrophobic receptors and kinases. Network analysis highlighted hub nodes such as CrebB, CaMKII, dnc, Dop1R1, NF1, and Syn, forming conserved dopaminergic, cAMP-PKA-Creb, and Ca^{2+}-dependent synaptic plasticity modules analogous to those described in *Drosophila*. These results indicate that core memory pathways are evolutionarily conserved in honey bees and provide a comparative molecular framework for future functional studies on honey bee cognition under environmental stress.

Keywords *Apis mellifera*, Brain, Cognition, Learning, Memory, RNA-seq

INTRODUCTION

Honey bees (*Apis mellifera* Linnaeus) are widely utilized as key pollinators in agricultural ecosystems worldwide (Ollerton *et al*., 2011; Sung *et al*., 2023; Choi and Park, 2024). They contribute to the pollination of approximately 70% of 124 major food crops that together account for 90% of global food production, and about one-third of all crop species rely on honey bee-mediated pollination (Klein *et al*., 2007; Gallai *et al*., 2009; Choi and Park, 2024). The economic value of pollination services provided by honey bees has been estimated to be approximately 200–500 billion US dollars per year (Lautenbach *et al*., 2012). Consequently, declines in pollinators such as honey bees can lead to yield reductions, deterioration of crop quality, and increases in the prices of pollination-dependent crops, thereby posing serious risks to food security and biodiversity through wide-ranging economic and societal impacts (IPBES, 2016).

Over the course of evolution, honey bees have developed highly sophisticated sensory systems and advanced capacities for learning and memory, and their basic neural mechanisms are broadly comparable to those of vertebrates (Giurfa, 2003; Zhang and Srinivasan, 2004; Dyer *et al*., 2005). Owing to these similarities, honey bees have attracted considerable attention as a suitable model organism for studies on the neurobiology of

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learning and memory (Kim and Jung, 2025). During foraging, honey bees must learn and remember not only the color and shape of flowers that provide nectar and pollen, but also the routes leading to these floral resources (Collett *et al*., 2003; Wehner, 2003). In particular, honey bees have evolved a high level of cognitive ability that enables them to rapidly learn, store, and recall complex information such as surrounding landmarks, flight paths, and the temporal availability of floral resources because the species of flowers in bloom vary with time and location (Gould and Gould, 1988; von Frisch, 1993).

Honey bee learning and memory are supported by a sense of time that allows individuals to modulate their responses to the same stimulus depending on the time of day. Studies on circadian memory rhythms have demonstrated that honey bees can learn and memorize odors and colors as time-linked cues and retain this information with a 24 h periodicity (Zhang *et al*., 2006; Gong *et al*., 2018). Such a circadian system enables organisms to measure time in a way that promotes adaptation to periodic environmental changes (Moore-Ede *et al*., 1982). Honey bees synchronize their foraging activity with the daily flowering rhythms of plants and typically forage only when nectar and pollen concentrations are highest, remaining inside the hive at other times to minimize energy expenditure associated with flight (Moore, 2001).

In the social insect, honey bee workers form short-term memories (STMs) and consolidate them into stable long-term memories (LTMs) (Menzel and Müller, 1996). This capacity varies with the age and task of the worker, and is enhanced when mature workers (around 20 days old) begin foraging activity (Winston, 1987; Ichikawa and Sasaki, 2003). Although the exact duration of LTM has not been fully clarified, however, it may be maintained throughout the individual's lifespan (approximately 33–45 days) (Winston, 1987). Such LTMs in honey bees are reported to persist over winter or even across the entire life history of the individual (Gong *et al*., 2018). The maintenance of LTM depends on *de novo* protein synthesis (Menzel, 2001) and DNA methylation (Wang *et al*., 2006).

Transcriptome-based studies in honey bees have been conducted across a wide range of environmental stressors and pathogenic infections. Dickey *et al*. (2023) examined the effects of acaricides commonly used for *Varroa* mite control, together with widely applied agricultural fungicides and insecticides, on queen bees, and suggested that pesticide exposure may induce neurotoxicity and behavioral alterations. In addition, Zhang *et al*. (2023) analyzed tissue-specific (head, thorax, abdomen, legs, and wings) transcriptomic responses of worker bees to heat stress and identified candidate genes involved in thermo-tolerance. Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) revealed prominent enrichment of pathways related to protein processing in the endoplasmic reticulum and lifespan regulation. Infection with *Nosema ceranae*, which increases the energetic demands of honey bees and reduces their survival, was shown to disrupt amino acid metabolism by down-regulating genes associated with antimicrobial peptides, cuticle formation, and odorant-binding proteins (Badaoui *et al*., 2017). Shah *et al*. (2009) reported that the transcriptomic responses of the mushroom body (MB), higher-order brain centers associated with cognitive function, in the brains of honey bees infected with deformed wing virus (DWV). Interestingly, DWV infection led to increased expression of immune-related genes (e.g., hymenoptacin, apidaecin) and significant differential expression of noncoding RNAs involved in gene regulatory networks, although a clear association between viral infection and cognitive function could not be established. Comparative analyses of micro RNA (miRNA) expression in the brain have identified several miRNA targets that may influence olfactory learning behavior (Huang *et al*., 2023). Previous study has focused on electrophysiological analyses of the neural basis of learning and memory in the mushroom bodies of honey bees (Plath *et al*., 2017). However, transcriptome-level analyses of these processes remain limited.

In this study, RNA-seq analysis of honey bee brain tissue under environmental stress conditions was performed. We aim to elucidate the molecular mechanisms underlying learning and memory in honey bees by identifying genes associated with the formation of STM and LTM.

MATERIALS AND METHODS

1. Honey bee transcriptome analysis

Total RNA extraction, cDNA library construction, Illumina NovaSeq sequencing, *de novo* assembly, read

mapping, and gene annotation analysis were performed following the transcriptomic pipeline described by Choi and Park (2025). This workflow was applied to *A. mellifera* and resulted in a *de novo* assembled transcriptome, which was deposited under GenBank accession PRJIVA1356701.

2. *In silico* identification of memory-associated candidate genes

Memory-related candidate genes were screened from the assembled *A. mellifera* transcriptome using BLASTx searches against the NCBI non-redundant (nr) protein database (Table 1). Several transcripts showed significant similarity to known memory-related genes; however, full-length open reading frames (ORFs) could not be reconstructed because of incomplete transcript coverage and low expression levels. To address this, the corresponding full-length coding sequences (CDS) and amino acid (aa) sequences for each gene were retrieved from the *A. mellifera* reference genome (GenBank accession GCF_003254395.2). These reference-derived canonical protein sequences were used for subsequent physicochemical characterization and protein-protein interaction analyses, ensuring that all downstream analyses were based on database-validated sequences.

3. Verification of memory-related gene expression

Adult honey bees (*Apis mellifera*) used in this study were kindly provided by Prof. Chuleui Jung from the experimental apiary of Gyeongkuk National University, Andong, Korea. To verify the expression of the selected memory-related genes, total RNA was extracted from the brains of various adult stages (newly emerged bee, nursing bee, and forager bee) using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For each adult stage, 10 honey bees were used and the experiments were conducted in three independent biological replicates. The reaction was performed at 45°C for 1 h, followed by enzyme inactivation at 95°C for 5 min. PCR amplification was performed to verify the expression of memory-related genes identified from the transcriptome. Each reaction was carried out in a final volume of 20 μL using AccuPower® HotStart PCR PreMix (Bioneer, Daejeon, Korea), 100 ng of synthesized cDNA, and 10 pmol of each gene-specific primer set. The thermal cycling conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52–57°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. Details of the primer sequences are presented in Table 2.

4. Analysis based on sequence features

Physicochemical properties of protein sequences derived from the reference genome were calculated using ExPASy ProtParam database (Gasteiger *et al*., 2005). The computed parameters included molecular weight, theoretical pI (Schuurmans *et al*., 2008), instability index (Guruprasad *et al*., 1990), aliphatic index (Ikai, 1980), and grand average of hydropathy (GRAVY, Kyte and Doolittle, 1982) score. The three-dimensional structural models of key memory-related proteins were generated using SWISS-MODEL (https://swissmodel.expasy.org, Waterhouse *et al*., 2018).

5. Protein-protein interaction (PPI) network

To characterize functional associations among memory-related proteins, we used the STRING (https://string-db.org, version 12.0) database with the organism parameter set to *Drosophila melanogaster*. Reference protein IDs (RefSeq/UniProt) corresponding to each gene were used to ensure compatibility with STRING's curated and predicted interaction datasets. The resulting PPI networks were visualized and analyzed in Cytoscape (v3.9), and hub genes were identified using degree and betweenness centrality metrics.

RESULTS AND DISCUSSION

1. Memory-related gene expression

The transcriptomic analysis results for honey bees have been reported previously (Choi and Park, 2024). Briefly, we identified 2,838 and 3,634 differentially expressed genes (DEGs) in honey bees under low (10°C)- and high (38°C)-temperature stress, respectively.

Table 1. Memory-related genes in *Apis mellifera*

Gene	Contig	NT Annotation	TPM*	Memory formation
CrebB (1)	c179255_g1_i23	XM_043940341 PREDICTED: *Apis laboriosa* cyclic AMP-responsive element-binding protein 1 (LOC122716872), transcript variant X7, mRNA	23.10	Long term
sna (2)	c172384_g1_i1	XM_393944 PREDICTED: *Apis mellifera* protein escargot (LOC410464), mRNA	0.53	Long term
	c193927_g6_i1	XM_026441938 PREDICTED: *Apis mellifera* protein snail (LOC725190), transcript variant X2, mRNA	1.54	
sens (2)	c193559_g2_i7	XM_026445124 PREDICTED: *Apis mellifera* zinc finger protein 300-like (LOC102655131), mRNA	4.05	Long term
	c200471_g9_i2	XR_003305961 PREDICTED: *Apis mellifera* fez family zinc finger protein 1 (LOC100577543), transcript variant X2, misc_RNA	1.05	
CaMKII (8)	c185332_g2_i1	XM_006560540 PREDICTED: *Apis mellifera* calcium/calmodulin-dependent protein kinase II (Camkii), transcript variant X42, mRNA	10.32	Long term
	c192325_g3_i1	XM_006560540 PREDICTED: *Apis mellifera* calcium/calmodulin-dependent protein kinase II (Camkii), transcript variant X42, mRNA	18.67	
	c197804_g10_i2	XM_006560540 PREDICTED: *Apis mellifera* calcium/calmodulin-dependent protein kinase II (Camkii), transcript variant X42, mRNA	4.28	
	c198008_g1_i1	XM_006560540 PREDICTED: *Apis mellifera* calcium/calmodulin-dependent protein kinase II (Camkii), transcript variant X42, mRNA	11.67	
	c198800_g2_i1	XM_026439968 PREDICTED: *Apis mellifera* calcium/calmodulin-dependent protein kinase II (Camkii), transcript variant X20, mRNA	8.58	
	c199365_g6_i8	XM_006560528 PREDICTED: *Apis mellifera* calcium/calmodulin-dependent protein kinase II (Camkii), transcript variant X30, mRNA	5.02	
	c202275_g5_i1	XM_006560540 PREDICTED: *Apis mellifera* calcium/calmodulin-dependent protein kinase II (Camkii), transcript variant X42, mRNA	18.90	
	c203231_g5_i2	XM_006560514 PREDICTED: *Apis mellifera* calcium/calmodulin-dependent protein kinase II (Camkii), transcript variant X17, mRNA	7.26	
orb2 (3)	c184233_g1_i1	XM_006620050 PREDICTED: *Apis dorsata* translational regulator orb2 (LOC102680533), transcript variant X3, mRNA	18.92	Long term
	c198187_g12_i2	XM_006620048 PREDICTED: *Apis dorsata* translational regulator orb2 (LOC102680533), transcript variant X2, mRNA	46.16	
	c198187_g13_i1	XM_006558511 PREDICTED: *Apis mellifera* transmembrane protein 132C (LOC102654165), transcript variant X2, mRNA	7.84	

Table 1. Continued

Gene	Contig	NT Annotation	TPM*	Memory formation
rg (6)	c834_g1_i1	XM_043935073 PREDICTED: *Apis laboriosa* adenylyl cyclase 78C (LOC122714062), transcript variant X5, mRNA	8.61	Long term
	c189135_g1_i6	XM_026443915 PREDICTED: *Apis mellifera* adenylate cyclase type 9 (LOC552535), transcript variant X1, mRNA	30.33	
	c189135_g2_i1	HG995272 Bombus pascuorum genome assembly, chromosome: 5	0.21	
	c193844_g2_i9	XM_006569465 PREDICTED: *Apis mellifera* adenylate cyclase type 2 (LOC551461), transcript variant X3, mRNA	18.82	
	c199192_g5_i3	NM_001327964 *Apis mellifera* adenylate cyclase type 8-like (LOC726262), mRNA <gi|390397300|emb|HE611296.1| *Apis mellifera* mRNA for adenylyl cyclase (ac8 gene)	4.89	
	c200982_g5_i6	XM_624593 PREDICTED: *Apis mellifera* adenylate cyclase type 2 (LOC552216), mRNA	6.77	
5-HT1A (1)	c196236_g4_i7	XM_006560054 PREDICTED: *Apis mellifera* serotonin receptor (5-HT1), transcript variant X3, mRNA	1.30	Long term
Syn (12)	c136618_g1_i1	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	6.57	Long term
	c175491_g2_i1	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	0.65	
	c179011_g4_i1	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	5.18	
	c184458_g1_i1	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	17.38	
	c184960_g2_i1	XM_028669731 PREDICTED: Apis cerana synapsin (LOC108003836), mRNA	0.23	
	c190554_g3_i1	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	0.74	
	c192695_g3_i1	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	4.20	
	c194694_g1_i2	XR_001705188 PREDICTED: *Apis mellifera* uncharacterized LOC107965336 (LOC107965336), transcript variant X2, ncRNA	0.88	
	c194757_g2_i1	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	4.44	
	c198401_g1_i3	XR_001705187 PREDICTED: *Apis mellifera* uncharacterized LOC107965335 (LOC107965335), ncRNA	2.23	
	c200378_g6_i7	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	5.04	
	c253748_g1_i1	XM_006562252 PREDICTED: *Apis mellifera* synapsin (LOC551737), transcript variant X2, mRNA	6.24	
Dop1R1 (3)	c101011_g1_i1	NM_001011595 *Apis mellifera* dopamine receptor, D1 (Dop1), mRNA <gi|2661768|emb|Y13429.1| *Apis mellifera* mRNA for dopamine receptor D1	0.38	Short term
	c171608_g1_i1	NM_001011595 *Apis mellifera* dopamine receptor, D1 (Dop1), mRNA <gi|2661768|emb|Y13429.1| *Apis mellifera* mRNA for dopamine receptor D1	2.76	
	c191685_g5_i3	XM_026445262 PREDICTED: *Apis mellifera* dopamine receptor, D1 (Dop1), transcript variant X2, mRNA	0.40	

Table 1. Continued

Gene	Contig	NT Annotation	TPM*	Memory formation
dnc (2)	c200065_g3_i2	XM_006568534 PREDICTED: *Apis mellifera* cAMP-specific 3′,5′-cyclic phosphodiesterase (LOC411288), transcript variant X7, mRNA	31.52	Short term
	c200355_g2_i3	XR_003305178 PREDICTED: *Apis mellifera* uncharacterized LOC113218944 (LOC113218944), ncRNA	1.31	
NF1 (1)	c191871_g1_i4	XM_026446394 PREDICTED: *Apis mellifera* neurofibromin (LOC552370), mRNA	38.55	Short term
ple (1)	c195866_g7_i2	XM_006565075 PREDICTED: *Apis mellifera* tyrosine hydroxylase (TyHyd), transcript variant X1, mRNA	15.95	Short term
sra (1)	c128910_g2_i1	XM_006618225 PREDICTED: Apis dorsata protein sra (LOC102672716), mRNA	15.30	Short term
gish (1)	c189202_g4_i10	XM_006569004 PREDICTED: *Apis mellifera* casein kinase I (LOC410831), transcript variant X5, mRNA	17.70	Short term
rg (2)	c202710_g4_i1	OU342926 *Bombus terrestris* genome assembly, chromosome: 6	0.24	Short term
	c203200_g5_i1	XM_026441536 PREDICTED: *Apis mellifera* neurobeachin (LOC724172), transcript variant X18, mRNA	5.68	
elm (1)	c119111_g1_i1	XM_006567378 PREDICTED: *Apis mellifera* calcineurin B homologous protein 1 (LOC408982), mRNA	26.47	Short term
arouser (2)	c180846_g2_i4	XM_016910689 PREDICTED: *Apis mellifera* epidermal growth factor receptor kinase substrate 8 (LOC408693), transcript variant X9, mRNA	0.24	Short term
	c190520_g4_i8	XM_016910686 PREDICTED: *Apis mellifera* epidermal growth factor receptor kinase substrate 8 (LOC408693), transcript variant X2, mRNA	8.32	
trbl (3)	c181248_g1_i2	XM_043938126 PREDICTED: *Apis laboriosa* tribbles homolog 2 (LOC122715720), transcript variant X3, mRNA	0.79	Short term
	c192379_g5_i1	XM_043727054 PREDICTED: *Bombus pyrosoma* tribbles homolog 2 (LOC122567906), mRNA	20.95	
	c194447_g5_i1	XM_043938124 PREDICTED: *Apis laboriosa* tribbles homolog 2 (LOC122715720), transcript variant X1, mRNA	71.06	

*Transcripts Per Million

Table 2. Primers for RT-PCR of memory-related genes in *Apis mellifera*

Gene	Primer	Sequence (5′ to 3′)	Annealing temp (°C)	Product length (bp)
CrebB	Crt1_F Crt1_R	GGA AAA CGT CGG CCA TTT TG ACA TCT TCC TTA AGG CGC CA	52	320
sens	Sens_F Sens_R	CAA TCC AAC AAA TCT TGA GTC C TTT GCT GGT TGG TAA CCC TC	52	339
Syn	Syn_F Syn_R	AGG AAG GAA GGA AGG AAG GA ACG ATC TTG TTA GCC ATC CG	52	335
CaMKII	CaMKII_F CaMKII_R	AGA GAG AGA GAG AGA GAG AG AAA TGG CGC GGG GAT TTT AG	52	379
orb2	Orb2_F Orb2_R	CAC GGC GAC ATA GAC AAG AG AAT TAG GGA CAA CGC AGC TG	52	348
Dop1R1	Dop1R1_F Dop1R1_R	TTT ATC CAC GGA CGA TGA AAA G CGT AGA TGC GCT ATT AAC ATG	52	328
dnc	Dnc_F Dnc_R	AGA GAG AGA GAA AGA GAA AGA G GTC CGT GTT TCG ATA CGT GT	54	324
ple	Pale_F Pale_R	GGC AAC GTT TAT ATG CGG CG AAC TCT TCT AGC CCC GAA GA	52	397
sra	Sra_F Sra_R	ATG GAG AAG AAA CAT GGT TCT G GCT GCT GCA TTT GGA GAA CT	52	359
gish	Gish_F Gish_R	CTC GTA ACA AAT CTC ACC CA ACA TGC ACT TTG CGA GCT TC	52	320
rg	Rug_F Rug_R	ATT TCT TAA ACG GCT GGA AAG G GTA AGG GAG AAG AAA GTT TCG	52	324
elm	Elm_F Elm_R	ATG GGT AAT AGA TCT AGC CTT C TTG CAC CAA CCA TCA TAT GCA	52	418
trbl	Trbl_F Trbl_R	ACA CAC GAG GAT ACC ATA TAA C GAG GAC TGG ATA ATT CGA AAG	52	331
sna	Sna_F Sna_R	GGG ATA CAC ATA CAC ACA TAC AAT GGC CGC CAA GAA TTC GA	57	335
5-HT1A	5-HT_F 5-HT_R	TTT TAT CGA TTG TTC ACG CGT C ACC AAT GTT CCG TTA TTC GGA	52	334
NF1	Nf_F Nf_R	TCC CCA ACG GGA GAG AAA GA GAC TGG ATA TCA CAA GAG AGA	57	322

Among these, *HSP70* and *HSP60* genes showed marked expression changes under 38°C condition, whereas *ApoID*, *HR38*, and *ABRA* genes exhibited pronounced differential expression under 10°C condition compared with the normothermic condition (25°C).

We screened candidate memory-related genes from the transcriptomic analysis results described above. RT-PCR analysis confirmed that all of these genes are expressed in adult honey bees (Fig. 1). Clear amplicons of the expected sizes were obtained for *CrebB* (Cyclic-AMP response element binding protein B), *sens* (senseless), *Syn* (Synapsin), *CaMKII* (Ca^{2+}/calmodulin-dependent protein kinase II), *orb2* (Cytoplasmic polyadenylation element-binding protein 2), *Dop1R1* (Dopamine receptor 1), *dnc* (Dunce, cAMP phosphodiesterase), *ple* (Pale, tyrosine hydroxylase), *sra* (Sarah), *gish* (Gilgamesh, casein kinase 1γ), *rg* (rugose), *elm* (Ethanol sensitive with low memory), *trbl* (tribbles), *sna* (snail), 5-*HT1A* (5-hydroxytryptamine (serotonin) receptor 1A), and *NF1* (Neurofibromin 1) in all three behavioral groups. These results indicate that the core components of cAMP signaling, monoaminergic modulation, and synaptic plasticity are constitutively present in the brains of newly emerged bees, nurse bees, and forager bees. No differences in expression levels were observed across the brains of the different adult stages.

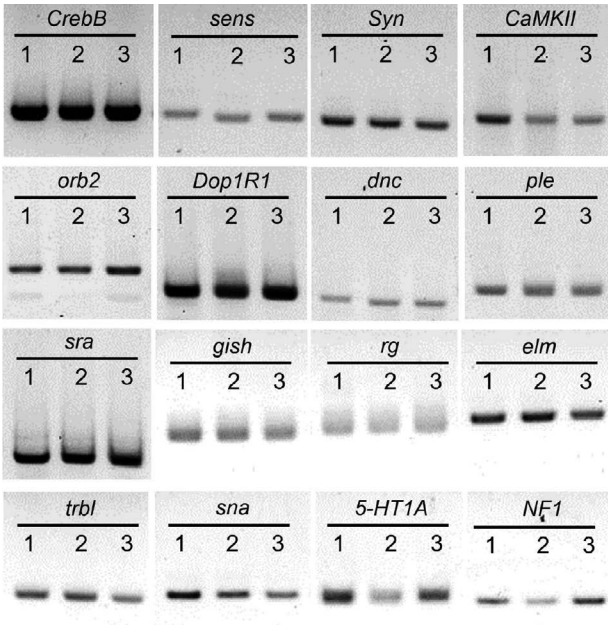

Fig. 1. RT-PCR validation of candidate memory-related genes in *A. mellifera* worker at different behavioral stages. Amplicons correspond to *CrebB* (Cyclic-AMP response element binding protein B), *sens* (senseless), *Syn* (Synapsin), *CaMKII* (Ca^{2+}/calmodulin-dependent protein kinase II), *orb2* (cytoplasmic polyadenylation element-binding protein 2), *Dop1R1* (dopamine receptor 1), *dnc* (cAMP phosphodiesterase), *ple* (tyrosine hydroxylase), *sra* (sarah), *gish* (gilgamesh, casein kinase 1γ), *rg* (rugose), *elm* (ethanol sensitive with low memory), *trbl* (tribbles), *sna* (snail), *5-HT1A* (5-hydroxytryptamine receptor 1), and *NF1* (neurofibromin 1). Lanes 1-3 represent cDNA from newly emerged bees, nurse bees, and forager bees, respectively.

2. *In silico* analysis of sequence-derived features

The physicochemical properties of memory-related gene orthologs identified in the *A. mellifera* transcriptome (Davis, 2023) were analyzed and are summarized in Table 3. Protein lengths ranged from 189 aa for elm to 3,344 aa for rg, corresponding to molecular weights of approximately 21.7−375.5 kDa. Large multi domain proteins such as NF1 and rg exhibited high molecular weights, consistent with their roles in neuronal regulation and intracellular signaling (Yohay, 2006; Volders *et al*., 2012). By contrast, small proteins such as elm and sra are likely to function as regulatory or adaptor proteins that fine-tune upstream signaling pathways, including calcineurin-dependent cascades (Takeo *et al*., 2006).

The theoretical pI values ranged from 5.05 to 9.39, demonstrating diverse charge properties. Acidic proteins (sra, pale and elm) contain abundant negatively charged residues, facilitating interactions with basic protein regions or nucleic acids. Basic proteins such as Dop1R1, gish, and sens exhibited high pI values, suggesting potential nuclear localization and DNA-binding capacity. Notably, Dop1R1 and gish act as upstream regulators of transcriptional signaling, whereas sens is a DNA-binding transcription factor involved in neuronal differentiation and the regulation of memory-associated genes (Wu *et al*., 2025). Instability indices indicated that only CaMKII, Dop1R1 and gish were predicted to be stable (Guruprasad *et al*., 1990), whereas the other proteins were classified as unstable. The predominance of instability suggests that most memory-related proteins function within dynamic multi protein complexes or require stabilizing interactions with membranes or molecular chaperones. Such intrinsic instability is considered advantageous for synaptic plasticity, as it allows rapid protein turnover during memory formation and remodeling (Alvarez-Castelao and Schuman, 2015).

The aliphatic index values ranged from 56.38 (sna) to 101.14 (Dop1R1), reflecting variation in thermal stability. Dop1R1 and 5-HT1A showed particularly high aliphatic indices, indicating resilience under fluctuating temperatures. These characteristics may contribute to neuronal tolerance to environmental stress and support adaptive maintenance of memory processes under variable thermal conditions (Kagias *et al*., 2012). Most proteins exhibited negative GRAVY values, indicating hydrophilicity and potential solubility, except for the more hydrophobic Dop1R1, and 5-HT1A. The higher hydrophobicity of Dop1R1 aligns with its function as a membrane-bound dopamine receptor, whereas strongly hydrophilic proteins such as CrebB and orb2 are characteristic of cytosolic or nuclear regulators with broad roles in signaling and transcriptional control. Hydrophilic regulators are widely implicated in pathways governing synaptic plasticity and memory formation (Rosenberg *et al*., 2014).

Taken together, these physicochemical properties indicate that honey bee memory-related proteins can be broadly divided into two functional groups: large, hydrophilic, and intrinsically unstable transcriptional or signaling regulators, and smaller, relatively stable and/

Table 3. Physicochemical properties of memory-related proteins in *Apis mellifera* predicted using the ProtParam

Gene name	Protein length (aa)	Molecular weight (Da)	Theoretical pI*	Instability index	Aliphatic index	GRAVY**	Predicted stability
CrebB	302	32,408.21	5.59	57.21	86.19	−0.424	Unstable
sna	390	42,955.33	8.49	71.67	56.38	−0.587	Unstable
sens	541	61,306.06	8.74	70.77	56.14	−1.019	Unstable
Syn	1,025	107,538.66	7.15	53.16	59.05	−0.539	Unstable
CaMKII	490	55,482.04	6.28	39.47	82.86	−0.423	Stable
orb2	704	74,504.03	7.37	55.01	60.99	−0.509	Unstable
Dop1R1	511	56,170.24	8.63	33.30	101.14	0.365	Stable
dnc	1,209	129,413.49	5.71	50.70	69.65	−0.538	Unstable
pale	579	65,995.98	5.05	56.22	79.53	−0.522	Unstable
sra	292	31,423.68	5.02	50.77	68.97	−0.626	Unstable
gish	474	53,276.54	9.39	33.00	71.60	−0.604	Stable
rg	3,466	384,513.15	5.22	48.73	87.06	−0.321	Unstable
elm	189	21,995.99	5.08	42.28	85.66	−0.443	Unstable
trbl	484	54,077.21	5.25	49.44	85.45	−0.354	Unstable
5-HT1A	834	89,520.82	5.56	46.31	83.94	−0.195	Untable
NF1	2,764	312,938.62	6.35	46.91	97.01	−0.117	Unstable

*Isoelectric Point, **Grand average of hydropathy

or more hydrophobic proteins, including receptors and kinases.

3. Protein-protein interaction network analysis

A protein-protein interaction (PPI) network was constructed using honey bee memory-related protein sequences queried against *D. melanogaster* orthologs in the STRING database (Fig. 2). STRING-based protein-protein interaction analysis (Szklarczyk *et al*., 2023) revealed a high-confidence network underlying learning and memory in the honey bee, integrating neuromodulatory signaling, second-messenger regulation, transcriptional control, and synaptic effectors (Davis, 2023). Based on this network, six genes were identified as core components linking intracellular signaling to transcriptional regulation and synaptic plasticity during memory formation. At the center of the network, dnc, NF1, CaMKII, CrebB, Dop1R1 and Syn formed a densely connected core. The hub positioning of dnc and NF1 supports a model in which tight regulation of cAMP dynamics is critical for memory-related signaling, consistent with insect learning circuits centered on mushroom body function (Georganta *et al*., 2021).

The dopamine receptor Dop1R1 was embedded within this core, linking reinforcement-related neuromodulatory input to the cAMP-kinase-transcription axis. Such positioning suggests that dopaminergic signaling modulates memory formation by gating downstream intracellular pathways rather than acting as an isolated input (Schwaerzel *et al*., 2003; Yamazaki *et al*., 2023). Downstream of the signaling core, CaMKII and CrebB formed a convergence point between calcium-dependent kinase activity and transcriptional regulation, consistent with their established roles in committing transient neuronal activity to long-term molecular changes (Kandel *et al*., 2014; Davis, 2023). In addition, a synaptic effector submodule centered on Syn and orb2 was closely linked to the core network. Given the roles of Syn in synaptic vesicle dynamics and orb2 in post-transcriptional regulation required for LTM stabilization, this submodule likely represents the execution layer translating upstream signaling into persistent synaptic modification (Greengard *et al*., 1993; Kozlov *et al*., 2023; Stewart *et al*., 2024). Overall, the STRING-derived network supports a two-tiered model of honey bee memory, comprising a central regulatory core integrating neuromodulatory and second-messenger signaling and a downstream synaptic module responsible for translational and structural consolidation. The integration of multiple data

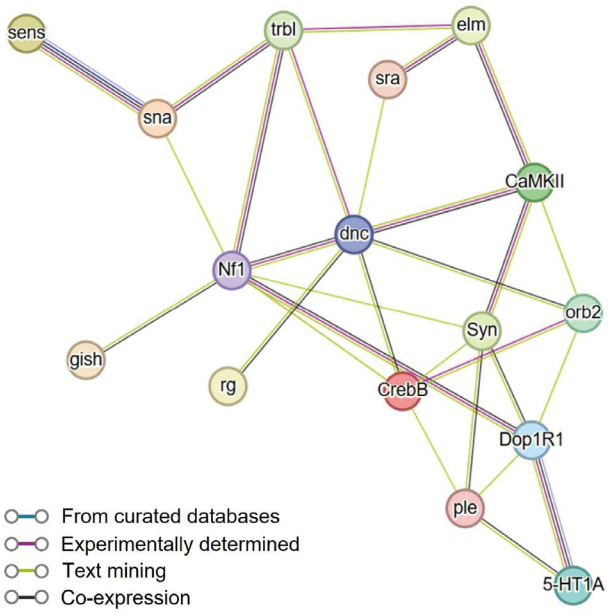

Fig. 2. STRING-based protein-protein interaction network illustrating potential functional relationships among memory-related proteins in *A. mellifera*, consisting of 16 nodes classified into five functional categories—cAMP signaling regulators (blue/purple), transcriptional regulators (red), synaptic plasticity proteins (green), neuromodulatory receptors (cyan/teal), and regulatory/modulatory proteins (beige)—with edges representing functional associations defined by the STRING database and supported by both experimentally validated interactions and database-derived predictive evidence (including curated pathways, co-expression, and text mining). This network is intended as an overview and is largely inferred from orthology-based evidence derived primarily from *Drosophila* datasets.

types in STRING suggests that this network represents a biologically meaningful and conserved framework for insect learning and memory.

4. Structural modeling of key memory-related proteins

To further explore structural characteristics, three-dimensional models of six representative memory-related proteins (CaMKII, CrebB, Dop1R1, orb2, NF1, and Syn) were generated using SWISS-MODEL (Fig. 3). Three-dimensional structural modeling revealed distinct yet complementary architectural features underlying their functional roles in learning and memory in the honey bee. Despite differences in size and domain composition, all proteins displayed conserved structural motifs consistent with their known molecular functions in neuronal signaling, transcriptional regulation, and synaptic plasticity. Structural modeling of six core memory-related proteins in the honey bee revealed a shared structural principle in which conserved functional cores are coupled with flexible regulatory regions to support memory-related signaling and plasticity (Jumper *et al*., 2021). Despite their distinct molecular roles, these proteins form a structurally coordinated system that enables efficient integration of neuromodulatory input, intracellular signaling, gene regulation, and synaptic function.

CaMKII and NF1 exhibited stable globular domains optimized for enzymatic and signaling regulation, consistent with their established roles in calcium-dependent phosphorylation and Ras-cAMP pathway modulation during learning and memory (Lisman *et al*., 2012; Wolman *et al*., 2014). In contrast, CrebB and orb2 displayed pronounced structural flexibility, reflecting their functions in activity-dependent transcriptional and post-transcriptional regulation underlying LTM formation and maintenance (Yin *et al*., 1995; Majumdar *et al*., 2012). orb2 included RNA-binding domains and a low-complexity, prion-like region known to facilitate protein aggregation associated with long term memory maintenance (Si *et al*., 2003). Dop1R1 adopted a canonical seven-transmembrane receptor architecture, structurally suited for translating dopaminergic signals into cAMP-mediated intracellular responses essential for associative learning (Schwaerzel *et al*., 2003). Syn contained an ATP-binding region and coiled-coil segments implicated in synaptic vesicle docking and neurotransmitter release (Kusick *et al*., 2022). The predicted three-dimensional structure of Syn reveals a conserved globular core domain surrounded by extended intrinsically disordered regions. The folded core likely mediates synaptic vesicle association and structural stability, whereas the flexible terminal regions may enable activity-dependent regulation of vesicle mobilization (Bertin *et al*., 2023) through phosphorylation-dependent conformational changes. This modular architecture is consistent with the established role of Syn in dynamic control of presynaptic vesicle pools during neurotransmission and memory expression. Collectively, these structural features indicate that honey bee memory formation relies on a modular protein architecture in which rigidity ensures functional specificity, while flexibility enables adaptive regulation. This structure-function

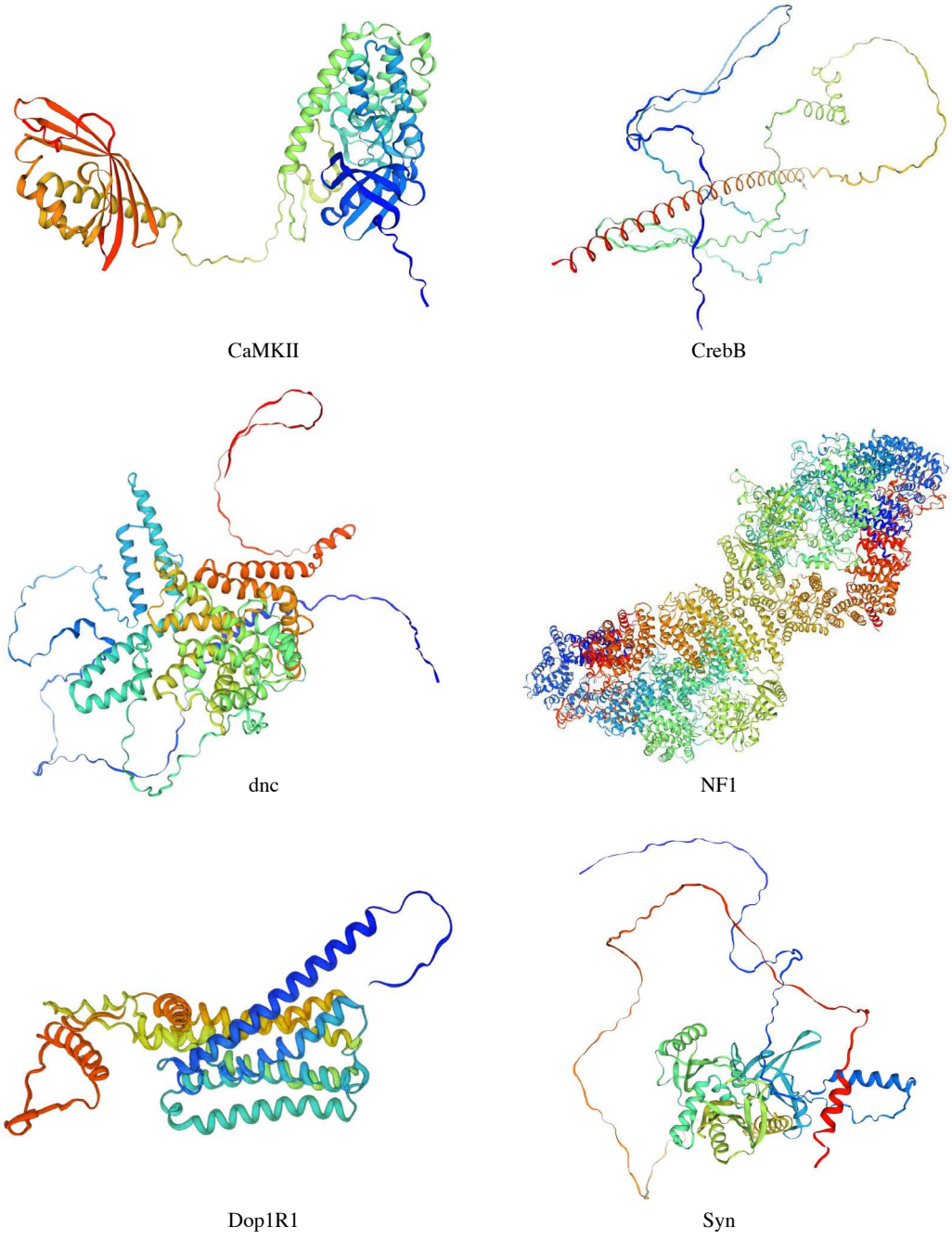

Fig. 3. Three-dimensional structural models of memory-related proteins in *A. mellifera* predicted by SWISS-MODEL. Each model is color-coded from the N-terminus (Blue) to the C-terminus (Red) to indicate the directionality of the polypeptide chain. The predicted tertiary structures illustrate diverse folding patterns and domain organizations, reflecting their distinct biochemical and regulatory roles in neuronal signaling, synaptic plasticity, and memory formation processes in the honeybee brain.

complementarity provides a mechanistic basis for coordinated memory processes spanning signal transduction, transcriptional control, and synaptic plasticity.

CONCLUSION

In this study, we used an integrated transcriptome-based approach to investigate conserved molecular

mechanisms underlying learning and memory in the honey bee brain, focusing on short- and long-term memory-related genes. RT-PCR confirmed consistent expression of key memory genes across worker behavioral stages, indicating that core memory machinery is maintained throughout adult life. PPI and structural analyses highlighted cAMP-dependent signaling-centered on *CaMKII*, *CrebB*, *dnc*, *Dop1R1*, *Nf1*, and *Syn*-as a conserved hub linking neuromodulation, transcription, and synaptic plasticity. Overall, our results provide a concise multi-level framework for honey bee memory pathways and their evolutionary conservation with *Drosophila* model.

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