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ORIGINAL ARTICLE

Forager age and foraging state, but not cumulative foraging activity, affect biogenic amine receptor gene expression in the honeybee mushroom bodies

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Abstract

Foraging behavior is crucial for the development of a honeybee colony. Biogenic amines are key mediators of learning and the transition from in-hive tasks to foraging. Foragers vary considerably in their behavior, but whether and how this behavioral diversity depends on biogenic amines is not yet well understood. For example, forager age, cumulative foraging activity or foraging state may all be linked to biogenic amine signaling. Furthermore, expression levels may fluctuate depending on daytime. We tested if these intrinsic and extrinsic factors are linked to biogenic amine signaling by quantifying the expression of octopamine, dopamine and tyramine receptor genes in the mushroom bodies, important tissues for learning and memory. We found that older foragers had a significantly higher expression of *Amdop1*, *Amdop2*, *AmoctαR1*, and *AmoctβR1* compared to younger foragers, whereas *Amtar1* showed the opposite pattern. Surprisingly, our measures of cumulative foraging activity were not related to the expression of the same receptor genes in the mushroom bodies. Furthermore, we trained foragers to collect sucrose solution at a specific time of day and tested if the foraging state of time-trained foragers affected receptor gene expression. Bees engaged in foraging had a higher expression of *Amdop1* and *AmoctβR3/4* than inactive foragers. Finally, the expression of *Amdop1*, *Amdop3*, *AmoctαR1*, and *Amtar1* also varied with daytime. Our results show that receptor gene expression in forager mushroom bodies is complex and depends on both intrinsic and extrinsic factors.

KEYWORDS
age, biogenic amine receptor, daytime, experience, foragers, motivation

1 | INTRODUCTION

In the honeybee *Apis mellifera*, like in most other social insects, division of labor is partly age-dependent.[1,2] During the first 2 to 3 weeks after emergence, worker bees perform tasks in the hive like feeding the brood, building honey combs and processing nectar. After this time, they start to forage until they die at the age of approx. 5 to 7 weeks.[3-5] However, task performance is not tightly linked to a specific age, but can change according to a colony's needs.[6-8] The final task of foraging is cognitively demanding, as foragers have to learn food locations, odors, floral shapes, and colors.[9-13] Because flowers often bloom at specific times of the day, foragers also have to learn at which time of the day particular nectar and pollen sources are available.[14-16]

Biogenic amines are important for both division of labor and learning.[17-21] They act as neurotransmitters, neurohormones, and neuromodulators in the central nervous system (CNS) of animals. In insects, they mediate different physiological states and behaviors.[22] Dopamine (DA), for example, plays a key role in learning and memory, particularly in aversive learning.[23-28] In addition, DA is linked to motor behavior and activity level in insects in general and honeybees in particular.[29,30] Octopamine (OA) has an arousing effect and mediates the reward information during classical appetitive conditioning in honeybees.[31] This function as a reward signal is likely to explain why OA stimulates waggle dancing and site-fidelity in foragers.[32,104] OA and DA levels in the brain of bees are also related to division of labor.[20,33,34] Compared with young nurse bees, for example, foragers have higher brain levels of OA and DA[18,20] and the age of first foraging is reduced in OA treated bees.[19,35] Traditionally, octopaminergic and dopaminergic pathways were considered to be functionally separated, with OA being involved in reward signaling and DA in aversive signaling. However, more recent research in honeybees suggests that the dopaminergic pathway is also involved in appetitive memory formation.[36] Tyramine (TYR) is a chemical precursor of OA and recent studies have shown that it also affects the behavior of bees.[21,37] TYR, like OA, affects reward perception as it increases the sucrose responsiveness of nectar foragers to the level of pollen foragers.[35] Furthermore, the level of TYR in the brain of foragers is higher than nurse bees.[21] These studies show that biogenic amines affect whether and when workers transition from in-hive activities to foraging in honeybees.

Biogenic amine receptors belong to the G-protein coupled receptor (GPCR) family that are heavily expressed in different parts of the bee brain, particularly in the mushroom bodies.[38-41] The mushroom bodies are an important center for various cognitive functions, such as sensory integration, memory formation and the organization of complex behaviors.[13,42,43] The observation that (i) mushroom bodies increase in size and change in anatomical features during the foraging period and when bees are older[40,44,45] and that (ii) biogenic amine receptor genes show higher expression in the mushroom bodies of foragers than in bees performing in-hive tasks[21,46,47] further highlights the importance of the mushroom bodies for foraging.

While the role of biogenic amines for the transition from in-hive to foraging tasks is relatively well studied, much less is known about the role of biogenic amines after bees have transitioned to foraging. For example, biogenic amines are likely to be important to understand the diversity of foraging-related behaviors and states, such as the tendency to be a scout or a nonscout or the likelihood to collect pollen instead of nectar.[17,48-50] Biogenic amine receptor gene expression has been shown to be a good indicator of behavioral states.[46,47,50,51] However, little is known about how forager age, foraging activity, or the foraging state of foragers are linked to biogenic amine signaling. Here, we explore these putative links between biogenic amine signaling and forager age and cumulative foraging activity. Foraging experience is not only reflected in the cumulative amount of foraging (eg, the number of foraging trips or the time spent foraging), but also in the immediate foraging state, which may vary during the course of a day.[52] Naeger et al, for example, found that inactive foragers differ in their whole-brain gene expression from bees that are expecting to forage in the immediate future.[53] The latter study did not include bees that were actively engaged in the foraging process and it is, thus, unclear if active foraging is accompanied by changes in gene expression of biogenic amine receptors. In addition, it remains unclear whether biogenic amine receptor gene expression differences linked to different foraging states can be found in the mushroom bodies of honeybees. Thus, we trained bees to collect food at particular times of the day and compared foragers that were inactive with bees that were engaged in foraging. Finally, gene expression might depend on the time of day as, for instance, cell adhesion genes that may be involved in learning and memory processes have been found to vary with time of day in honeybees.[53] Furthermore, the *foraging* gene is more expressed during the daylight hours when foragers of the ant *Pogonomyrmex occidentalis* are foraging outside the nests.[54] Thus, we tested if biogenic amine receptor genes expression fluctuates with the time of the day.

2 | MATERIALS AND METHODS

2.1 | Study species and study site

We used nine *Apis mellifera carnica* colonies (three for each of the three experiments; see below) housed in three-frame observation hives on the campus of the Johannes-Gutenberg University in Mainz, Germany. Each observation hive had ~3000 to 4000 workers, brood, honey, and a naturally mated queen. We kept the observation hives in a wooden shed for protection against the weather and sun exposure. The observation hives were made from a wooden casing comprising a translucent glass pane on each side and a transparent tube serving as an exit/entrance reaching the outside of the shed. The bees were allowed to adapt to the new environment for at least 1 week before further manipulations were performed.

2.2 | Experimental procedure

The objective of this study was to explore whether forager age (Experiment 1), cumulative foraging activity (Experiment 2), and foraging state (Experiment 3) are linked to the expression levels of biogenic amine receptor genes in the mushroom bodies of honeybee foragers. In experiment 1, the effect of the time of the day was also studied (Experiment1: daytime).

2.3 | Experiment 1: Does the expression of biogenic amine receptor genes depend on forager age?

The following procedure was performed for each observation hive, one at a time. Two frames of capped late-stage brood (shortly before eclosion) were removed from the original full-sized colony (from

which the observation hive was built) and stored in a humidified climate chamber (35°C) overnight. The next day the newly emerged honeybees were removed from the brood frames and marked with colored Opalith number plates to the thorax of bees. Then, the newly emerged, marked bees were introduced into the observation hive. Each colony received 100 newly emerged bees of this age group, which constituted the "old forager" group. To obtain the "young foragers," the marking procedure was repeated exactly 2 weeks after the introduction of the first marked honeybees, using newly emerged bees from two different brood combs, but from the same original full-sized colony. This time Opalith number plates of a different color were used to separate the two age-groups.

Thirty-five days after the introduction of the first age cohort, we captured both young and old foragers. Thus, young foragers were 21 days old, whereas old foragers were 35 days old. Bees usually transition to foraging between the age of 2 to 3 weeks.[2,3] However, they can also start to forage at a younger age if the weather is consistently good.[40] In our study, video analysis (see details in Experiment 2) confirmed that all bees captured for Experiment 2 performed one or more field excursions when reaching the age of 21 days, indicating the commencement of foraging prior to this age. Also, bees were caught at four different times of the day: in the morning (~9:00), around noon (~12:00), in the afternoon (~16:00) and at night (~22:00). To catch the bees, the windows of the observation hives were carefully and slowly removed to access all the numbered bees inside the hive. Bees were collected individually with 5 mL Eppendorf tubes and immediately put into liquid nitrogen to maintain the state of gene expression in the mushroom bodies. Afterwards the samples were transferred to a −80°C freezer. For each time period, in total 12 bees were caught (60 bees in total, 20 from each of the three observation hives). The number of captured young foragers was the same as the number of old foragers. This experiment was performed between May and July 2017.

2.4 | Experiment 2: Does cumulative foraging activity affect the expression of biogenic amine receptor genes?

In Experiment 1, we studied if the age could affect receptor genes expression, however, the cumulative foraging activity of foragers was not controlled for. For example, a previous study has shown that long durations of foraging could cause a cognitive decline in honeybees,[55] which might also influence receptor gene expression. Therefore, we repeated the experiment the following year using video cameras to quantify the cumulative foraging activity of foragers that had the same age. We used three different observation hives and introduced 150 newly emerged, marked bees from the original full-sized colony (from which the observation hive was built) into the observation hive, as described for Experiment 1 (450 bees in total). When the marked bees were 10 days old and until the age of 21 days, the entrance to the hives were filmed (JVC, model GZ-GX1BE) daily from 7 am to 7 pm in order to quantify the foraging activity of marked bees. To catch the bees on day 22, the same method was used as described above. The marked bees were caught between 12:30 and 13:30 using Eppendorf tubes and killed immediately with liquid nitrogen. For further storage, the bees were placed in a −80°C freezer.

When reviewing the video material, the time of exit and return of the marked bees were noted. From these recordings, we calculated the following parameters: the total (ie, cumulative) number of foraging trips performed over the entire observation period, the total cumulative foraging time, the total foraging days and the average duration of foraging trips. We excluded hive excursions of less than 4 min because they are unlikely to represent foraging trips. The total cumulative foraging time was the sum of the times of all trips one bee completed during the filming period. The total foraging days were calculated by counting the number of days a bee performed foraging trips. The average duration of foraging trips was determined by dividing the total cumulative foraging time of a bee by its number of cumulative foraging trips. These parameters indicated how much foraging activity bees had accumulated by the time of capture.

Among all the captured bees, 15 bees were selected from each observation hive for the qPCR analysis. The bees were selected so that they showed variation in their cumulative foraging activity (total number of cumulative foraging trips, total cumulative foraging time, total foraging days, and average duration of foraging trips). This experiment was performed between May and July 2018.

2.5 | Experiment 3: Does the expression of biogenic amine receptor genes depend on the foraging-related state?

We used three different observation hives (see above for description), one hive at a time. We trained two different groups of bees from an observation hive to collect food from two different feeders. Both feeders were 100 m from the hive, but in opposite directions. One group of bees was trained in the morning (MO bees), between 9:30 and 11:30. The other group was trained in the afternoon (AF bees) from 15:30 to 17:30. To establish a training group, we used standard procedures to train a group of 30 to 50 foragers to a feeder offering unscented 50% sucrose solution.[9] By doing so, the bees would learn the location of their respective artificial feeder.

One day after the two groups of foragers were established, the bees trained to the feeders were numbered with Opalith number plates for individual identification. Training continued for 12 days to maintain an adequate number ($N \approx 30$) of trained and marked bees that only appeared at a single training time at the respective feeder. During this training time, both feeders offered 30% unscented sucrose solution and bees could acquire a time-place memory for the respective feeder and training time. This reward represents an average food source,[56] which reduced the likelihood that bees would perform dances and recruit nestmates from the other training group. Meanwhile, the arrival of foragers was recorded for each foraging trip to make sure foragers only visited their own training feeder. Most bees exhibited allegiance to a single training time, the remaining bees

TABLE 1 The design of Experiment 3 and the six behavioral groups analyzed in this study

	States of bees	
Time of collection	Morning trained (MO bees)	Afternoon trained (AF bees)
10:45-11:00	Foraging	Inactive
16:45-17:00	Inactive	Foraging

(31.5% ± 1.9%) that visited both feeders were removed before the sample collection phase.

On collection day, the feeders were set up with 30% unscented sucrose solution during the respective training time. We collected two types of bees: foraging bees who foraged during the training time and inactive bees whose catching time was at least 4 hours earlier/later than the training time (Table 1). Inactive bees were taken from the top frame, which contained most of the honey stores. They were captured when in an immobile state, never carried pollen nor performed waggle dances before collection.[9,53,57] They were unlikely to be active nectar foragers because nectar is not stored in cells by the collecting foragers themselves, but nectar is transferred to nectar processors close to the nest entrance.[58,59] Foraging bees were also immediately caught inside the observation hive near the entrance after they returned from the feeder. Using the number tags, we made sure that these bees were actively engaged in the foraging cycle, which includes hive-stays to unload food.[60] Bees engaged in foraging were caught less than a minute after they left the feeder. It is, therefore, unlikely that biogenic amine receptor transcription levels changed significantly since the bees left the feeder (see eg, for the temporal dynamics of expression changes of immediate early genes [IEGs] in bumble bees after fresh air stimulation[61]).

Thus, all bees used for the qPCR analysis were collected while they were inside the hive and the bees were used for qPCR analysis only if they successfully trained to their corresponding feeder location for at least 7 days to make sure that they had learned the time of food availability.[62] The method for catching bees was the same as described above. Thus, in total four different behavioral groups of bees were analyzed in Experiment 3: actively foraging and inactive bees, both for the MO and the AF group (Table 1). At least 12 bees from each of the four behavioral groups (four from each hive, a total of three hives) were used for molecular analysis. This experiment was carried out between July and September 2018.

2.6 | Brain dissections, RNA isolation and cDNA synthesis

Heads were removed from the body and fixed with dental wax on an ice-cooled petri-dish to dissect the mushroom body calyces with cooled bee saline (154 mM NaCl, 2 mM NaH_2PO_4, 5.5 mM Na_2HPO_4, pH 7.2) over ice as quickly as possible (dissection lasted less than 3 minutes). We used sharp tweezers (FST, Canada) to remove the calyces of mushroom bodies along the bottom of the calyces (see Sarma et al, their Figure 1).[63] While the paired mushroom bodies are made up of the pedunculus connected to the two cup-like calyces (a lateral and a medial calyx), we only used the calyces of the mushroom bodies because of the difficulty to completely remove the mushroom bodies from other brain parts. The calyces contain the intrinsic Kenyon cells, where a large part of mushroom body transcription takes place and the calyces are often used in mushroom body gene expression studies.[63,64] The calyces were directly transferred into 100 μL TRIzol (Invitrogen, Massachusetts) for RNA extraction. According to the manual, RNA was extracted from isolated calyces using RNeasy Mini Kit (Qiagen, Germany). Samples obtained with our method have high-RNA integrity numbers (≥6.0) for analysis.[47] The Quanti Tect Reverse Transcription Kit (Qiagen, Germany) was used to synthesize the cDNA. Before we synthesized the cDNA, the DNase digestion step took place according to the manufacturer instructions. For cDNA synthesis, we used 10 ng total RNA for each reaction.

2.7 | Real-time quantitative PCR

Until now, three DA receptors, five OA receptors and two TYR receptors have been identified in the honeybee,[65-70] all of which were included in our study. AmOCTαR1 is a α-type OA receptor, which mediates Ca^{2+} signaling. Four AmOCTβ receptors belong to the OA β-receptors, which are cAMP-coupled receptors.[71,72] DA receptors have been categorized into two groups in the honeybee. AmDOP1 and AmDOP2 belong to D1-like receptors. Activation of D1-like receptors leads to an increase in intracellular cAMP levels.[73] AmDOP3 is a D2-like receptor that reduces intracellular cAMP when it is activated.[46,67,73] AmOCTβR3 and−βR4 receptors are two splicing variants of the same gene and we used AmOCTβR3/4 to represent the AmOCTβR3 and−βR4 receptors in our study.[51,68] TYR activates AmTAR1 that leads to the inhibition of adenylyl cyclase, resulting in a decrease of cAMP.[69] Activation of AmTAR2 causes cAMP production by combination with nanomolar concentrations of TYR or micromolar concentrations of OA.[70]

Real-time quantitative PCR was performed on a mic qPCR cycler (Bio Molecular Systems, Australia) using the Blue S'Green qPCR mix Separate ROX (BioZyme, Missouri). Gene primers were based on published sequences and Primer Premier 3.0 (Table 2). All primers were synthesized by Biolegio (Netherlands). Each reaction volume of 20 μL contained 10 μL Blue S'Green qPCR mix, 0.25 μM of each primer, 2 μL cDNA and DNase/RNase free distilled water. The following cycling parameters were used: 95°C for 2 minutes; 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. The fluorescence signal was measured at the end of each extension step at 60°C. Quantification cycle (C_q) values were determined at the same fluorescent threshold for each gene by the micPCR Version2.6 software (Bio Molecular Systems, Australia). The transcript levels of the target genes were expressed as normalized transcript abundance using *GAPDH* and *eiF3-S8* as internal reference genes[74,75]. Using the software package NormFinder version 0.953.[76] we examined the stability of the

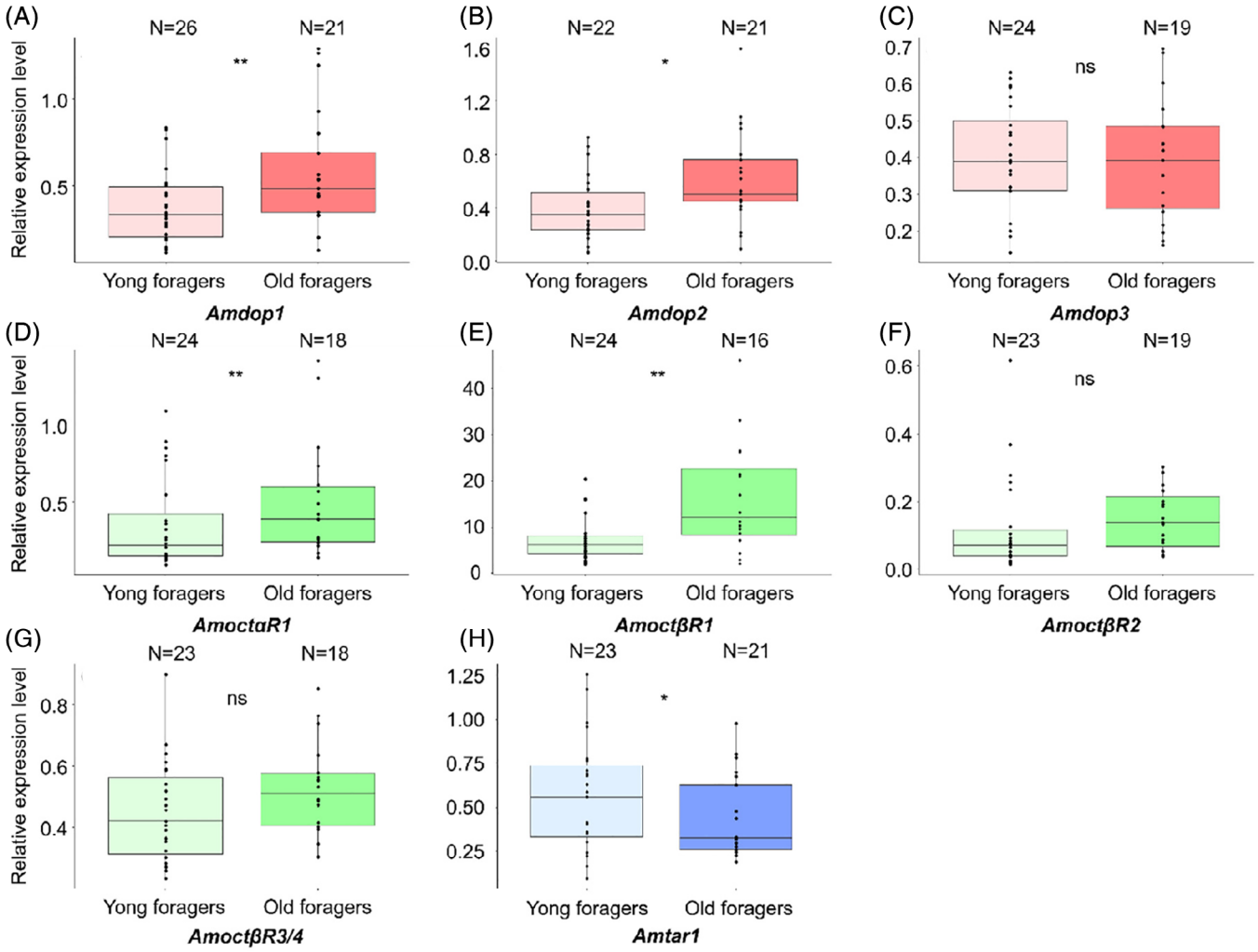

FIGURE 1 Biogenic amine receptor gene expression (A-E) in the mushroom bodies of young and old foragers. Boxplots show the medians, the 25%- and 75% quartiles of expression levels relative to the two reference genes (*GAPDH* and *eiF3-S8*) ± SE. Numbers above bars indicate sample size. Asterisks indicate significant differences between young and old foragers (*$P \leq .05$, **$P \leq .001$)

reference genes and found that combining the two reference genes was more stable than using a single reference gene. The relative gene expression was calculated using the $2^{-\Delta CT}$ method using the following formula: Normalized = $2^{-(CqTarger\text{-}Cq\ \ Reference)}$.[77] PCR efficiency (E) values were calculated by the software the micPCR Version2.6 software (Bio Molecular Systems, Australia) for each gene from the given slope after running standard curves and the following formulate $E = 2^{-1/slope}\text{-}1$.[78] We were unable to amplify *Amtar2*, as indicated by our melting curves that show no clear pattern and multiple peaks. This was the case with two different primers (Table 2). One explanation could be that *Amtar2* might be expressed only in small amounts in mushroom bodies.

2.8 | Statistical analysis

All data were analyzed using linear mixed-effects models (LME) with the nlme package 3.1 to 137 in the R environment version 3.4.4 (http://www.R-project.org/). The Shapiro-Wilk test was used to test for the normality of the residuals. If necessary, data were log- or square root transformed to achieve a Gaussian distribution of the model residuals. Colony ID was always included as random effect to account for the nonindependence of observations from the same colony.[79]

Experiment 1: To test the significance of age/daytime-dependent biogenic amine receptor gene expression, we explored the role of two fixed effects, age, and daytime. We removed the interaction between the fixed effects from the final model, because the interaction was never significant (all *P*-values > .05). To compare the expression of biogenic amine receptor genes between the four different daytimes, pairwise comparisons were performed and a sequential Bonferroni correction was applied to adjust *P*-values for multiple testing (multcomp package 1.4–8 in R).

Experiment 2: To test for relationships between forager activity and biogenic amine receptor gene expression, we again used LME's to explore the four measures of foraging activity (total number of

TABLE 2 Primers used in real-time RT-PCR

Primer name	Sequence (5′-3′)	Reference
Amdop1-F	ACA GAA TTC CGA GAA GCG TTC A	50
Amdop1-R	ATT CGC TAG TCG ACG GTT GAT TT	
Amdop2-F	ACA CGG AAT TGG TTC TCC ATC T	50
Amdop2-R	TCC CGT AAC CGG CTG TCA	
Amdop3-F	CGT TGC AAA CTG TCA CCA AT	103
Amdop3-R	GAC GTC CAT TGC GAT GTA AA	
AmoctαR1-F	ACG AAG GCG GCG AAG AC	50
AmoctαR1-R	CGC GCA CCA AGT ACA TTG TG	
AmoctβR1-F	CAG CAC CGT CTC CAT ACT CC	Primer primer 3
AmoctβR1-R	GAG GTG TTT CTC GGT GGT GT	
AmoctβR2-F	AGC GTT GGC CGA CAT GTT	50
AmoctβR2-R	AGC CAT TTG CCG GTC AAT T	
AmoctβR3/4-F	CAC TTC GAT ACG ACA ACA AAC G	Primer primer 3
AmoctβR3/4-R	GGT TCA GGG CGC TGT TGA	
Amtar1-F	CGA GGA CAT TGG GCG TGA TA	Primer primer 3
Amtar1-R	GTA GAT GAG CGG GTT GAG GG	
Amtar2-F-1	GTT ACT AAT TGT TTC GTG TCC AGC TT	70
Amtar2-R-1	GCA GTA CAG AGA AGA ATG TCG AGG	
Amtar2-F-2	AAG AGG TTG GCC GGT CTA AT	Primer primer 3
Amtar2-R-2	CCT CCC GTA AAC GTA AAG CA	
GAPDH-F	ACC TTC TGC AAA ATT ATG GCG A	75
GAPDH-R	CAC CTT TGC CAA GTC TAA CTG TTA AG	
eIF3-S8-F	TGA GTG TCT GCT ATG GAT TGC AA	74
eIF3-S8-R	TCG CGG CTC GTG GTA AA	

foraging trips, total foraging time, total foraging days, and average trip duration).

Experiment 3: To study how the foraging state of a forager was related to the biogenic amine receptor expression, we used foraging state and training group (MO and AF group) as fixed effects.

3 | RESULTS

3.1 | Age- and daytime effects on biogenic amine receptor gene expression

The expression of receptor genes *Amdop1*, *Amdop2*, *AmoctαR1*, *AmoctβR1* showed a significant difference among age-groups, with the old foragers having an up-regulated expression (LME, *Amdop1*: Likelihood-ratio test (*LRT*) = 11.26, *P* = .0008; *Amdop2*: *LRT* = 5.95, *P* = .015; *AmoctαR1*: *LRT* = 5.96, *P* = .015; *AmoctβR1*: *LRT* = 7.50, *P* = .0062, Figure 1A,B,D,E). *Amdop3*, *AmoctβR2*, *AmoctβR3/4* expression did not show a trend in the same direction (LME, *Amdop3*: *LRT* = 0.16, *P* = .68; *AmoctβR2*: *LRT* = 3.13, *P* = .077; *AmoctβR3/4*: *LRT* = 2.07, *P* = .15 Figure 1C,F,G). On the other hand, the expression of *Amtar1* was higher in young foragers (LME, *Amtry1*: *LRT* = 3.99, *P* = .046, Figure 1H).

We found that time of day affected gene expression in *Amdop1*, *Amdop3*, *AmoctαR1*, and *Amtar1* (LME, *Amdop1*: *LRT* = 30.75, *P* < .0001; *Amdop3*: *LRT* = 30.80, *P* < .0001; *AmoctαR1*: *LRT* = 36.46, *P* < .0001; *Amtar1*: *LRT* = 29.42, *P* < .0001). Pairwise comparison tests showed that levels of *Amdop1* were significantly lower in the morning (~9:00) and at night (~22:00) compared to the other periods (Table 3, Figure 2A). The expression of *Amdop3* was significantly lower in the morning (~9:00) compared to other periods (Table 3, Figure 2C). *AmoctαR1* and *Amtar1* transcript levels, however, were significantly higher in the morning (~9:00) compared to other periods (Table 3, Figure 2D,H). The expression of *Amdop2*, *AmoctβR1*, *AmoctβR2*, *AmoctβR3/4* did not change during the day (LME, *Amdop2*: LRT = 2.62, *P* = .45; *AmoctβR1*: LRT = 2.67, *P* = .45; *AmoctβR2*: LRT = 0.85, *P* = .84; *AmoctβR3/4*: LRT = 6.16, *P* = .10, Figure 2B,E-G).

3.2 | Cumulative foraging activity effects on biogenic amine receptor gene expression

From the recorded videos, we were able to determine the departure and return time of a tagged forager leaving the hive in 89.84% of all trips. This is considerably higher than in studies using RFID tags.[52] In total, 1494 trips (trip ≥ 4 minutes duration) performed by 199 marked bees were observed in the three observation colonies. The distribution of the number of trips performed per forager was highly right-skewed (Figure S1). This means that most bees performed only a small number of trips by the age of 21 days, while a few did many trips. The foraging trip duration increased with the age of the bees (LME, *t* = 8.53, *P* < .001; Figure 3). While the mean trip duration of 10-day old bees was 13.9 ± 14.5 minutes, it increased to 44.7 ± 39.8 minutes for the 22-day old bees. However, we found no significant relationships between the gene expression of biogenic amine receptors and our measures of cumulative foraging activity (Table 4).

3.3 | Biogenic amine receptor gene expression and foraging state

We tested if biogenic amine receptor gene expression was linked to foraging state and training group in time-trained foragers. The expression of *Amdop1* was influenced by foraging state: *Amdop1* showed significantly higher mRNA levels during foraging than when bees were inactive (LME, foraging state, *LRT* = 20.76, *P* < .0001; training group, *LRT* = 0.74, *P* = .39; Figure 4A).

TABLE 3 *P*- and *z*-values determined by linear mixed models for the pairwise comparison tests

Pairwise comparison	LME	*Amdop1*	*Amdop3*	*AmoctαR1*	*Amtar1*
~9:00 vs ~ 12:00	*p*	<0.0001	0.0001	0.0094	<0.0001
	z	5.45	4.2	−3.04	−4.84
~9:00 vs ~ 16:00	*p*	<0.0001	<0.0001	<0.0001	<0.0001
	z	4.43	5.99	−5.34	−4.29
~9:00 vs ~ 22:00	*p*	0.22	<0.001	<0.0001	0.0001
	z	1.59	4.4	−6.01	−4.18
~12:00 vs ~ 16:00	*p*	0.59	0.19	0.34	1.00
	z	−0.53	1.87	−1.36	0.31
~12:00 vs ~ 22:00	*p*	0.0055	0.76	0.086	1.00
	z	−3.2	0.3	−2.19	0.40
~16:00 vs ~ 22:00	*p*	0.037	0.25	0.34	1.00
	z	−2.5	−1.53	−0.99	0.09

Abbreviation: LME, linear mixed-effects.

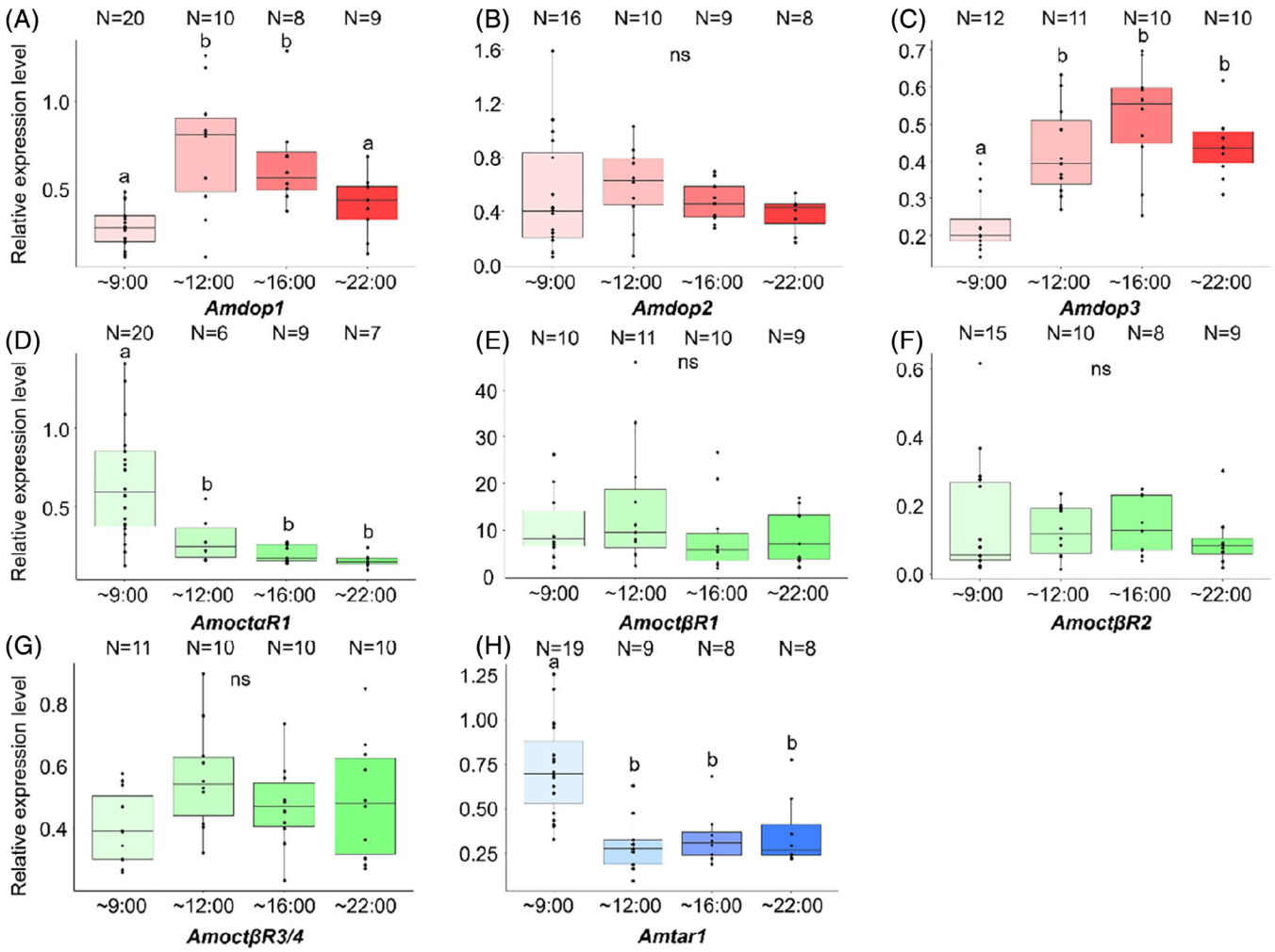

FIGURE 2 Biogenic amine receptor gene expression (A-E) in the mushroom bodies depends on time of day. Boxplots show the medians, the 25%- and 75% quartiles of expression levels relative to the two reference genes (*GAPDH* and *eiF3-S8*) ± SE. Numbers above bars indicate sample size. Letters indicate significant differences between groups. Bars not sharing a letter differ significantly ($P < .05$). Bars without letters mean no significant

For *AmoctαR1* and *AmoctβR3/4*, the gene expression differed among bees with different foraging state, whereas the training group showed no effect. The expression of *AmoctαR1* and *AmoctβR3/4* was higher in bees engaged in foraging compared with inactive bees (LME, *AmoctαR1*, foraging state, *LRT* = 4.37, *P* = .037; training group, *LRT* = 0.013, *P* = .91; *AmoctβR3/4*, foraging state, *LRT* = 8.60, *P* = .0034; training group, *LRT* = 0.66, *P* = .42; Figure 4D,G). The expression of *AmoctβR3/4* was higher in bees engaged in foraging compared with inactive bees (LME, foraging vs inactive, *z* = −2.96, *P* = .0093). The expression of *Amdop2*, *Amdop3*, *AmoctαR1*, *AmoctβR1*, *AmoctβR2*, and *Amtar1* did not depend on either of the investigated factors (LME, *Amdop2*, foraging state, *LRT* = 2.74, *P* = .098; training group, *LRT* = 0.73, *P* = .39; *Amdop3*, foraging state, *LRT* = 3.22, *P* = .073; training group, *LRT* = 1.02, *P* = .31; *AmoctβR1*, foraging state, *LRT* = 0.11, *P* = .74; training group, *LRT* = 0.16, *P* = .69; *AmoctβR2*, foraging state, *LRT* = 0.083, *P* = .77; training group, *LRT* = 0.30, *P* = .58; *Amtar1*, foraging state, *LRT* = 3.71, *P* = .054; training group, *LRT* = 0.032, *P* = .86; Figure 4B,C,E,F,H).

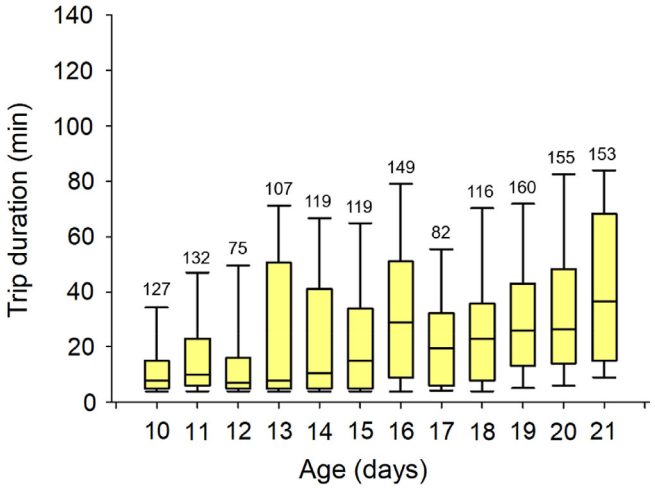

FIGURE 3 The effect of age in days on the trip duration in minutes. Boxplots show the medians, the 25%- and 75% quartiles. Numbers above bars indicate sample size

4 | DISCUSSION

Our findings suggest that forager age and foraging state are linked to biogenic amine signaling in the mushroom bodies. In addition, the expression of some biogenic amine receptor genes depended on the time of day. Our measures of cumulative foraging activity, on the other hand, did not correlate with the expression of the receptors included in this study.

4.1 | Forager age, but not cumulative foraging activity, predicts biogenic amine signaling

Foraging is a complex behavior associated with physiological changes in the mushroom bodies that correlate with age and/or foraging activity.[40,44,80] However, whether both of these factors affect gene expression after workers switched to foraging is not well known. We found that OA and DA receptor genes are more expressed in the mushroom bodies of older foragers. In line with our results, previous research has shown that expression levels of *Amdop2* in the Kenyon cells of the mushroom body calyces is higher in foragers than in newly emerged bees.[46] Similar increases in the levels of expression of *Amdop1*, *Amdop2*, *Amdop3*, *AmoctαR1* were found when comparing the antennae of pollen foragers to that of younger in-hive bees (<15 day old).[47] However, in the latter two studies, foragers were compared with non-foragers. Our results show that age continues to

TABLE 4 *P*- and *t*-values determined by linear mixed models for the relationships between the foraging parameters and the relative expression values of the biogenic amine receptor genes

Parameters	LME	*Amdop1*	*Amdop2*	*Amdop3*	*AmoctαR1*	*AmoctβR1*	*AmoctβR2*	*AmoctβR3/4*	*Amtar1*
Total number of foraging trips	*p*	0.57	0.2	0.19	0.71	0.82	0.17	0.099	0.21
	t	0.57	1.31	1.32	0.38	0.24	1.39	−1.69	1.27
	N	45	40	45	33	44	45	45	45
Total foraging time	*p*	0.42	0.51	0.29	0.57	0.84	0.22	0.14	0.31
	t	0.82	0.66	1.08	0.58	−0.2	1.23	−1.5	1.02
	N	45	40	45	33	44	45	45	45
Average trip duration	*p*	0.39	0.99	0.38	0.6	0.63	0.27	0.25	0.54
	t	0.87	−0.012	0.87	0.54	−0.48	1.11	−1.18	0.62
	N	45	40	45	33	44	45	45	45
Total foraging days	*p*	0.93	0.33	0.35	0.83	0.78	0.97	0.6	0.3
	t	0.09	0.99	0.94	0.21	−0.29	−0.041	−0.53	1.06
	N	45	40	45	33	44	45	45	45

Note: N indicate the sample size.
Abbreviation: LME, linear mixed-effects.

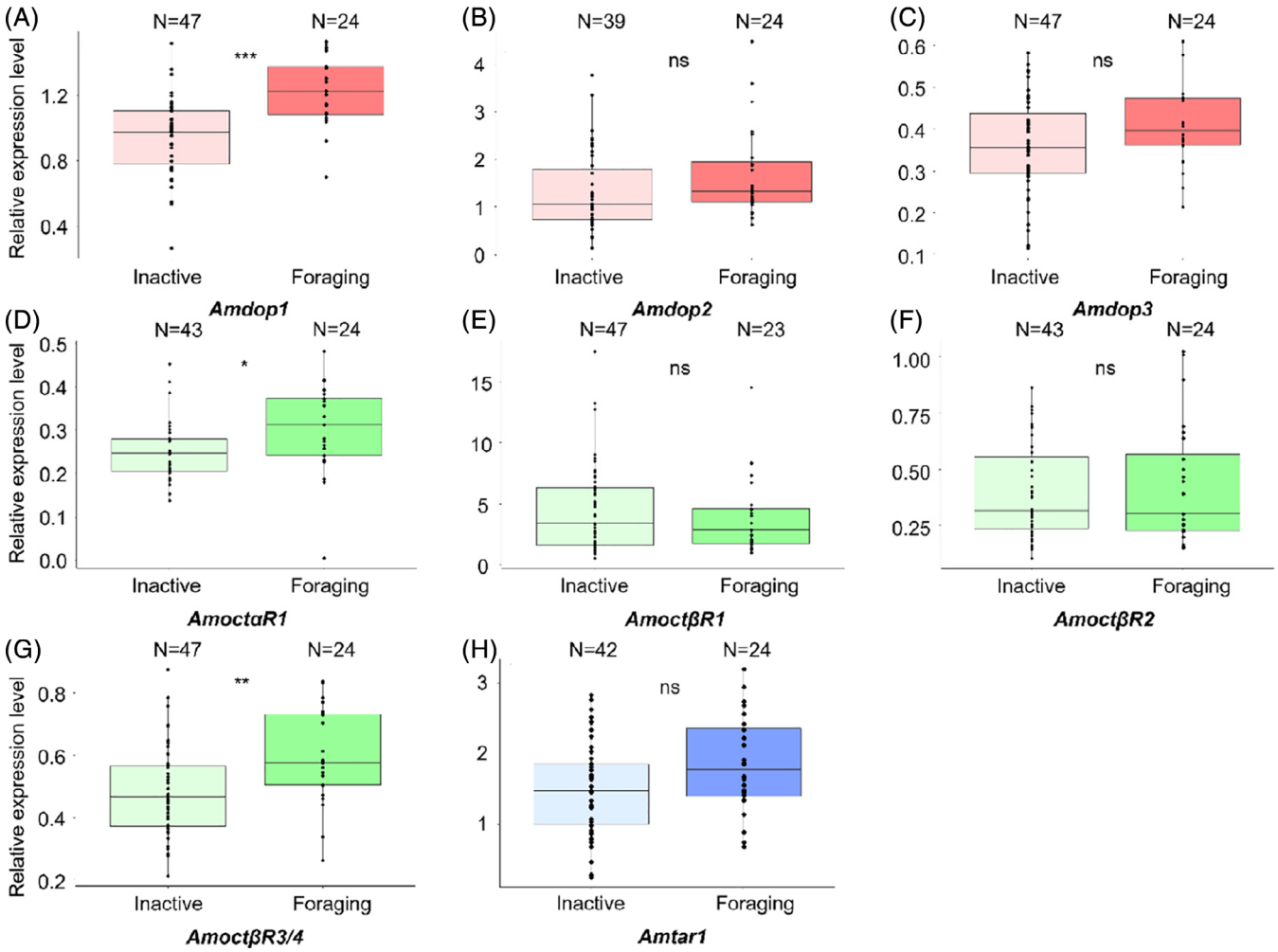

FIGURE 4 Biogenic amine receptor gene expression in relation to foraging state (A-E) in the mushroom bodies. Boxplots show the medians, the 25%- and 75% quartiles of expression levels relative to the two reference genes (*GAPDH* and *eiF3-S8*) ± SE. Numbers above bars indicate sample size. Asterisks indicate significant differences between inactive and foraging foragers (*$P \leq .05$, **$P \leq .001$, ***$P \leq .0001$)

be associated with gene expression after workers have switched to foraging.

Since octopaminergic and dopaminergic signaling are important in aversive and appetitive learning in honeybees,[26,27] forager age could correlate positively with learning performance. Ruepell et al., for example, found that older forager-aged bees tended to have a better learning performance than younger workers.[81,82] In *Drosophila*, the orthologues of AmDOP1 and AmDOP2 have been shown to play a role in aversive learning.[83,84] This raises the possibility that *Amdop1*, *Amdop2*, *AmoctαR1*, and *AmoctβR1* might mediate age-dependent learning performance in honeybees after the transition to foraging. We found that the young foragers had a higher expression of TYR receptor *Amtar1* compared with the older foragers. This effect could mediate changes in the gustatory responsiveness of bees, which often correlates positively with learning performance.[21,85] On the other hand, Scheiner et al showed that whole-brain expression of *Amtar1* was not higher in nurse bees than in foragers.[21] This suggests that age-related changes in *Amtar1* expression are tissue specific.

Bees usually start foraging after about two to three weeks after emergence,[3,4] but earlier foraging can often be observed.[2] In our study, hive excursions were observed in relatively young bees and they increased in duration with increasing age (Figure 3 and Figure S1). Some of the very early and short excursions may have been orientation flights.[86,87] An increase in foraging trip duration could indicate that bees gained experience about where to find the most profitable food sources. It could also be that the foraging skills improved with age[11] and an increase in foraging performance occurs as a result of learning.[88] On the other hand, extensive foraging activity (many days of foraging or long foraging trips) has been found to correlate negatively with associative learning performance, regardless of age.[55,89] Thus, we might have expected that the expression of receptor genes varied with cumulative foraging activity. Surprisingly, we found no relationship between biogenic amine receptor gene expression and different measures of cumulative foraging activity. It is possible that the foraging activity of our foragers (4-12 days of foraging) did not vary as much as in other studies that found effects of foraging activity, for example, on learning performance.[90]

Taken together, our results suggest that the changes in biogenic amine receptor gene expression in the mushroom bodies of foragers are explained by age, rather than cumulative foraging activity. Future research should explore whether different measures of foraging activity are linked to receptor gene expression in other regions of the brain.

4.2 | Foraging state influences biogenic amine receptor gene expression

We found that *Amdop1* expression was higher when bees were foraging than when inactive. A previous study of whole-brain gene expression discovered that *Amdop1* had a higher expression in foragers than in nurses in age-controlled colonies,[91] indicating that *Amdop1* may play important roles in honeybee foraging. In addition, in our study *AmoctαR1* and *AmoctβR3/4* expression was higher in foraging bees compared with inactive bees. These findings indicate a potential association between biogenic amine receptor gene expression and behavioral plasticity in foragers.

In contrast, the expression of *Amdop2*, *Amdop3*, *AmoctβR1*, *AmoctβR2*, and *Amtar1* were independent of foraging state or time of training. Thus, these genes might be important for functions other than the regulation of foraging activity. For instance, knockdown of *Amdop2* mRNA expression has been found to affect the time honeybees spend grooming or walking.[30] It should be noted again that our study only used the mushroom bodies and these receptor genes might have different expression profiles in different neuroanatomical areas. Future studies could use RNA interference targeting specific brain areas to better understand the roles of biogenic amine receptor genes in regulating forager behavior.

4.3 | Daytime and biogenic amine signaling

Daytime is an important extrinsic factor in the life of a honeybee forager because it determines whether certain flower species produce pollen and nectar.[16,62] Honeybees keep track of the daytime by using a time-compensated sun compass, which allows them to visit food sources at the right time of day.[9,15] So far, little is known about whether the time of day also affects biogenic amine receptor gene expression. In Experiment 1, we found that *Amdop1* showed significant down-regulation in the morning (~9:00) and at night (~22:00. Likewise, *Amdop3* showed significantly lower expression in the morning (~9:00), whereas *AmoctαR1* and *Amtar1* showed significant up-regulation in the morning. The expression of the other genes did not change during the day (Figure 2). Knockdown of *AmoctαR1* can cause impaired olfactory acquisition and recall.[92,93] Thus, expression changes during the day could affect olfactory learning performance. Indeed, honeybees exhibit better olfactory learning performance in the morning compared to the afternoon.[62] Also, our finding that *Amdop3* is more expressed in the morning could indicate that it might improve the retrieval of appetitive memory in the morning, but this requires further testing. It has been proposed that AmTAR1 might have a dual OA/TYR receptor function.[21,71,94] Thus, our finding that *Amtar1* is more expressed in the morning raises the possibility that it might support the function of *AmoctαR1* in modulating behavioral responses, such as the arousal of food seeking behavior. RNA interference[92,93,95] or null mutation of receptor genes[96] could be used to explore if receptor gene expression in the mushroom bodies mediates olfactory learning performance.

Alternatively, daytime effects could be related to the sleep-wake cycles in foragers. Unlike nurses, foragers show rhythmicity by being active during the day and showing sleep-like behavior at night.[56,97] In *Drosophila*, activating OA signaling has been suggested to cause a decrease in sleep[98] and D1 dopamine receptor (DA1) mediates the arousal effect of DA in *Drosophila*[99-101]. Therefore, our findings raise the possibility that the differential expression of *AmoctαR1* and *Amdop1* in the morning is related to the transition from sleep-like states to a more active state. Queen mandibular pheromone (QMP) has been shown to affect the olfactory system of bees and influence brain gene expression.[74,102] For example, bees have lower *Amdop1* transcript levels and lower activity levels following QMP-treatment compared to controls.[103] The finding that *Amdop1* was down-regulated at night could be explained by foragers being more exposed to queen pheromone than in the morning and during the day. It should be noted that we only tested bees at a few time points and we might have missed interesting time points, such as in the early morning. In summary, our results show complex links between forager state and biogenic amine signaling in the mushroom bodies. They also highlight that more research is needed to understand if and how biogenic amine receptor expression is linked to extrinsic and intrinsic factors.

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CONFLICT OF INTEREST

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

Tianfei Peng and Christoph Grüter conceived and designed the study, Tianfei Peng, Dennis Derstroff, Lea Maus, and Timo Bauer carried out the experiments and analyzed the data, Tianfei Peng wrote the original draft, Christoph Grüter reviewed and edited the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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