Search behavior of individual foragers involves neurotransmitter systems characteristic for social scouting

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SUMMARY

Behavioral specialization in honey bees is regulated by hormones and neuromodulators that tune neuronal activity and gene expression, and can be viewed as a temporarily fixed behavioral state associated with a specific brain state. Honey bee scouts, which search for new food sources, show a higher expression of genes involved in glutamate, GABA and catecholamine signaling than recruits that remain loyal to a food source. We asked whether recruits visiting a feeder initiated a search behavior when the feeder was experimentally removed, and if similar neuromodulators might be involved in the initiation and performance of that behavior. We found that recruits perform a relatively stereotyped search behavior that shows inter-individual variation in its intensity. Quantitative single brain mass spectrometric analyses showed that glutamate and GABA titers changed during search behavior supporting the hypothesis that behavioral specialization in social insects is based on reinforcing brain molecular processes involved in solitary behavior.

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INTRODUCTION

Division of labor among the non-reproductive worker caste is a hallmark of eusocial insect societies (Michener and Michener, 1974; Wilson, 1971). In honey bees, the most prominent division of labor is the one between workers that stay in the hive nursing the brood and maintaining the nest and workers that leave the hive foraging for food and other resources. Each worker performs both tasks during her life, at a young age she performs in-hive tasks and at an older age, she becomes a forager (Lindauer, 1952; Seeley, 1982). Nurses and foragers present distinct "long-term" behavioral phenotypes differing in hormone titers, brain anatomy, neuromodulator titers, and brain gene expression patterns (Lehman et al., 2006; Robinson, 1987a; Scheiner et al., 2006; Schulz et al., 2002; Whitfield et al., 2003, 2006; Withers et al., 1993).

Among foragers, different behavioral phenotypes exist concerning the food or resource they collect (nectar, pollen, and water), or whether they search for new food sites (scouts) or get recruited to a known food site and continue to forage at that site till it is fully exploited (recruits) (Lindauer, 1952; Seeley, 1983; zu Oettingen-Spielberg, 1949). Finally, recruits foraging at the same food source show consistent differences in their dance activity, suggesting a finer grained division of labor involving different behavioral phenotypes even among foragers performing the same task (George et al., 2020; George and Brockmann, 2019; Seeley, 1994). Although foragers can switch tasks, there is evidence that most of them temporarily show behavioral consistency. Additionally, there is some evidence that there is a genetic and genomic basis for the probability to engage in a specific task (Dreller, 1998; Liang et al., 2014; Robinson and Page, 1989; Rueppell et al., 2006). Similar to the differences between nurses and foragers, these foraging related behavioral phenotypes differ in hormone and neuromodulator titers as well as brain gene expression patterns (Barron and Robinson, 2005; Božič and Woodring, 1998; Brockmann et al., 2009; Giray et al., 2007; Liang et al., 2012; Reim and Scheiner, 2014; Taylor et al., 1992).

One of the first mechanistic hypotheses regarding the regulation of division of labor in social insects was that workers differ in behavioral response thresholds which would be regulated by hormones (Beshers et al., 1999; Page and Erber, 2002; Robinson, 1987a, 1987b). Following more elaborate hypotheses proposed that behavioral specialization is based on a temporal persistence of a certain brain state associated with specific neuromodulator titers and specific expression of transmitter receptors as well as any genes involved in cellular information processing and homeostasis (Barron and Robinson, 2008; Chandrasekaran et al., 2011; Marder and Goaillard, 2006). Most recently, it was even suggested that these brain states might be associated with specific gene-regulatory networks that are synchronously expressed in all brain cells involved in regulating the respective behaviors (Sinha et al., 2020).

All these experimental data and ideas suggest that behavioral specialists might be temporarily tuned in to a specific brain and behavioral state that occurs in behavioral generalists (e.g., solitary bees) when they perform the corresponding solitary behavior. Foragers are "skinny bees" that are hungry (Toth et al., 2005), guards show a higher state of aggressiveness (Alaux et al., 2009;

Shpigler et al., 2017). Recently, a series of transcriptomic and genomic studies indicated that scouts and recruits show strong differences in the brain expression of genes involved in catecholamine, glutamate, and γ-aminobutyric acid signaling (Liang et al., 2014, 2012). Furthermore, manipulative experiments confirmed that glutamate and octopamine treatment increased, and dopamine antagonist treatment decreased the likelihood of scouting (Liang et al., 2014, 2012). With respect to the idea that behavioral specialists may present a specific brain state which increases the probability of performing the corresponding behavior, we were interested whether those neurotransmitter systems differing between scouts and recruits are also involved in search behaviors occurring during regular foraging of recruits (Chatterjee et al., 2019; Townsend-Mehler et al., 2011; Townsend-Mehler and Dyer, 2012). In particular, we used mass spectrometry measurements (Ramesh and Brockmann, 2019) to test whether the search behavior of recruits which was induced by the removal of a visited feeder is associated with short-term changes in neuromodulators involved in social scouting.

RESULTS

Absence of an expected feeder elicited a series of search flights and subsequent cessation of foraging

Honey bee foragers (n=16, 2015 and n=16, 2020) that had continuously visited a feeder for a few hours immediately initiated a search when they did not find the feeder at the expected location (Figures 1A-C). Already the mean duration of the foraging trip when they did not find the feeder (FS, combined foraging/search flight; Table 1) was significantly longer than the mean duration of the foraging trip (FS, Figures 1D and 1E). In contrast, the mean duration of the hive stay after this first unsuccessful trip (HFS) was as short as those after the previous regular foraging trips (HF; Figures 1F and 1G). One of the foragers directly stopped foraging after the FS flight (BeeID: E26; Figure 1C), whereas all the other foragers performed one to four additional search flights (Figures 1B and 1C). All foragers stopped foraging within 100 mins after the removal of the feeder. The mean duration of the consecutive search flights was relatively consistent (S: 12.37 ± 6.02 min) and lasted about 3 times longer than the mean duration of the foraging trips (F: 3.51 ± 1.02 min; Figures 1D and 1E; GLMM gamma family and generalized linear hypothesis test; see also data file S1 for details of the GLMM and GLHT results). In contrast to the search flights, the mean duration of the intermittent hive stays (HS1 - HS3) increased with the number of search trips (Figures 1F and 1G; GLMM gamma family and generalized linear hypothesis test; see also data file S1 for details of the GLMM and GLHT results). The duration of the hive stay might, therefore, correlate to some extent with the probability to stop foraging.

In an additional control experiment in which we put the feeder back after 1 hour, foragers landed on the feeder as soon as it was opened (Figure S1). This finding suggests that the search flights were more or less restricted to the close vicinity of the expected feeder location and the foragers were not searching for any other food location.

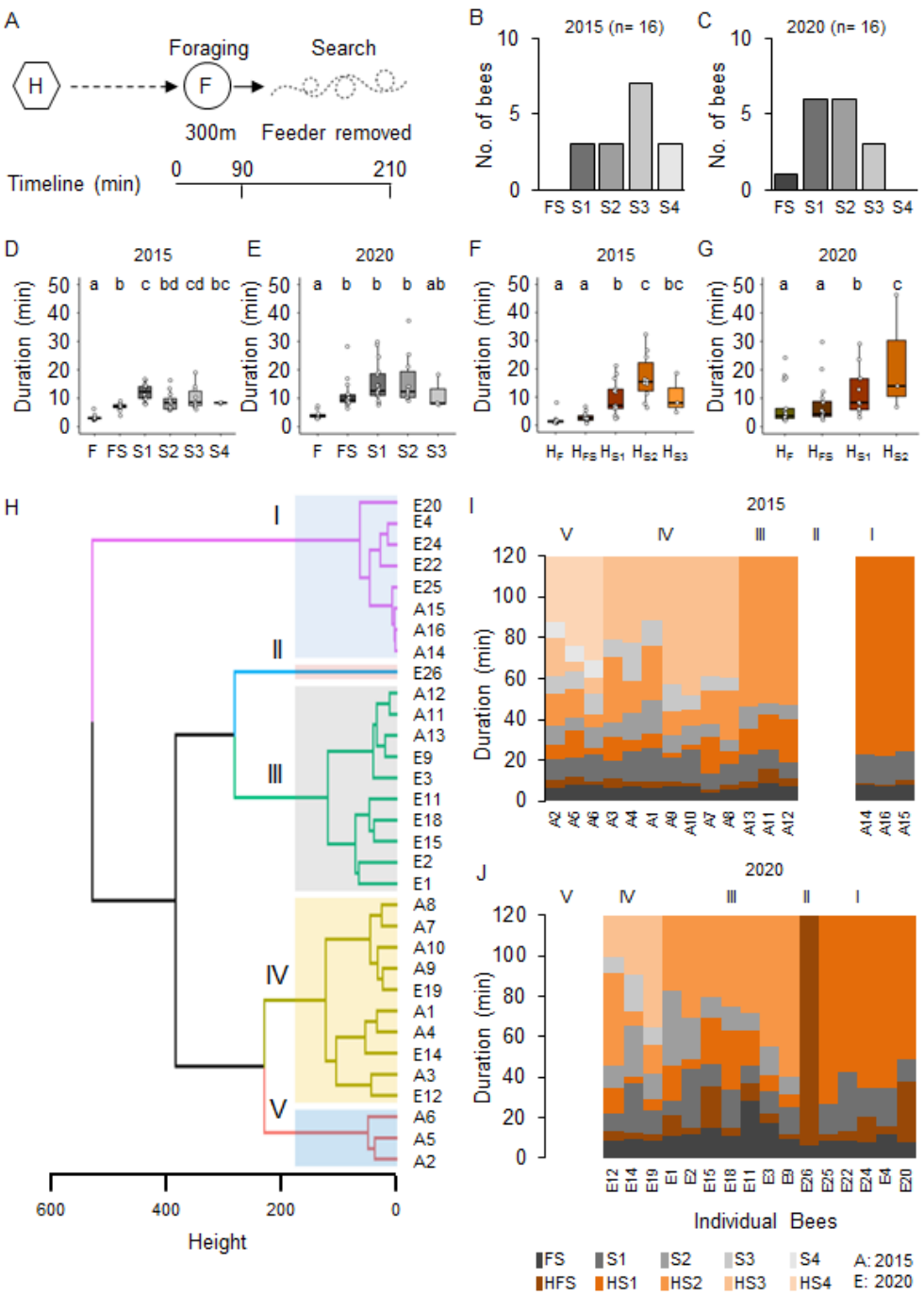

Figure 1. Removal of the feeder led foragers to perform search flights. (A) Experimental design to study the dynamics and persistence of search behavior and after foragers were confronted with the absence of a known feeder. Individually marked foragers are allowed to visit the feeder at 300m distance from the hive for an initial 1.5 hrs. The feeder is removed, and the outbound flight activity of the marked foragers is monitored at the hive entrance for following 2 hrs. (B-C) Search flights following feeder removal for two colonies. (D-E) Increase in hive-to-hive duration and (E-F) duration of hive stays before and after feeder removal. (H) Hierarchical clustering of foragers based on search behavior sequence. The maximum average silhouette width 0.58 gave a five-cluster solution with agglomerative coefficient 0.93. (I-J) Search behavior sequences for foragers along with cluster information for two colonies.

Individual foragers showed different search phenotypes

Cluster analysis based on the number and temporal dynamics of the search flights and hive stays identified five different search phenotypes independent of the behavioral experiment (I-V; Figures 1H-J; see Figure S2A for optimum number of clusters). Cluster 1 includes bees that stopped foraging after the first search flight (S1; n=3 2015; n=5 2020) and cluster 2 includes the single forager that already stopped foraging after FS (BeeID: E26, 2020). Foragers in Cluster III (n=3, 2015 and n=7, 2020) made 2 search flights and Cluster IV (n=4, 2015 and n=3, 2020) made 3 search flights within the observation period. Cluster III and IV formed the largest groups each with 10 bees. Cluster V comprised 3 foragers (all in 2015) which performed 4 search trips (see supplementary data file S1 for details). As the number of search flights is the parameter with the strongest impact, the different clusters present behavioral phenotypes that vary in their motivation to search and their persistence to continue foraging.

Search behavior led to a robust reduction of glutamate and GABA titers in the central brain

Neurotransmitter analysis of the brain samples from different foragers was done in multiple batches, each containing approximately equal numbers of representatives of behavioral groups and brain regions. The batch identity was added as a random factor in the statistical model. Comparing neurotransmitter titers in the central brain (CB) between foragers caught during foraging, searching for the feeder, or revisiting the feeder (Figure 2A), we found robust differences for glutamate and GABA (Figures 2B and 2C). Foragers that had experienced the absence of the feeder for the first time (FS) and were caught as they were leaving for their first search trip already showed significantly lower GABA titers in the CB (Figure 2C; decrease by 29.7 +/- 10.3 ng, p=0.025) than successful foragers. In contrast, glutamate titers in the CB declined after a first search flight (Figure 2B; decrease by 242.2 +/- 80.8 ng, p=0.018). Further, glutamate levels continued to linearly decrease with the number of search trips (Figure 2D; decrease by 111.7 +/- 34.8 ng with every search flight, p=0.001). Similarly, GABA levels also showed a significant linear decrease, however the largest reduction occurred during the FS trip and the following hive stay (Figure 2E; decrease after first experience by 29.5 +/- 10.6 ng, p=0.032).

In addition to the changes in GABA and glutamate titers, we also found differences in the histamine and aspartate levels in the central brain samples (Figures 2F and 2G). Foragers with 2 search flights had significantly higher histamine levels than those that were foraging, and the histamine levels showed a significant linear increase with number of search flights (0.21 +/- 0.096 ng per search flight, p=0.025). In contrast, aspartate levels showed a significant linear decrease with number of search flights (decrease by 86.8 +/- 40.28 ng per search flight, p=0.034).

Restarting foraging led to an increase of glutamate and GABA titers in the optic lobes

In contrast to the CB, we did not detect any changes in neuromodulator titers in the optic lobes (OL) samples during the search flights (Figures 3A and 3B). However, when we reinstalled the feeder, the foragers, that had restarted foraging, showed significantly higher levels of glutamate

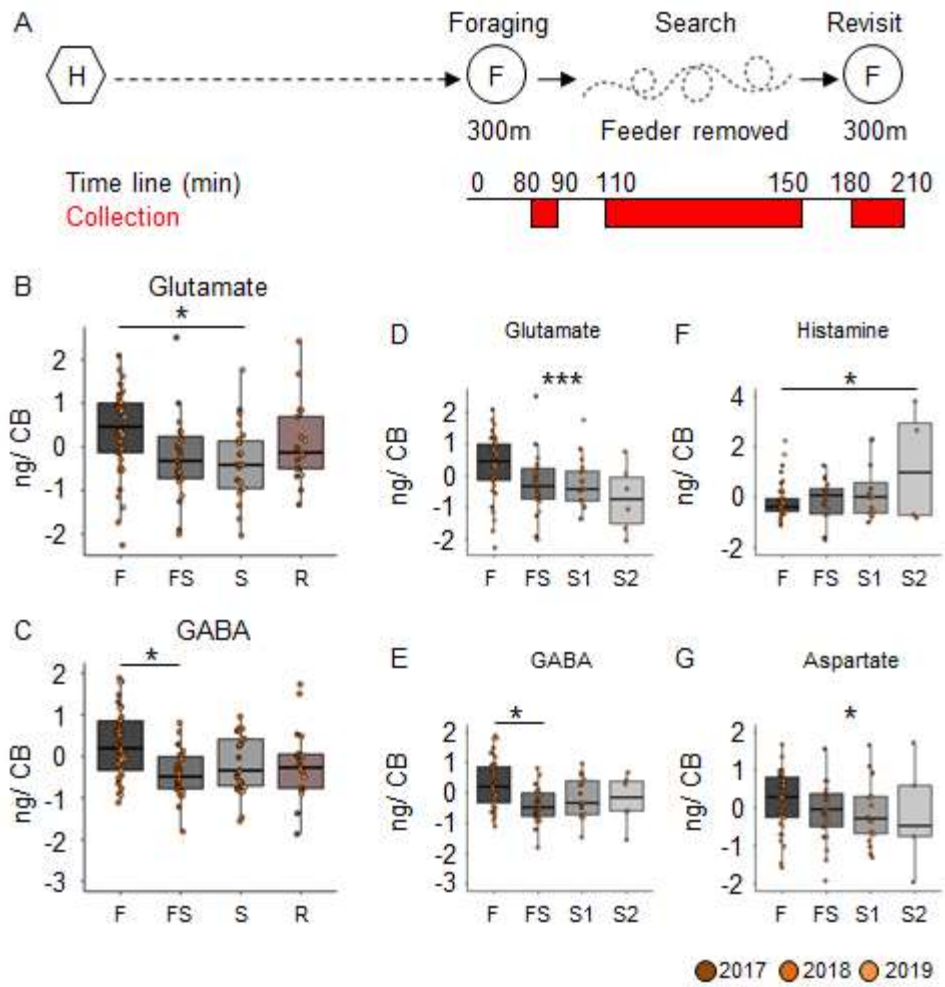

Figure 2. Search behavior is correlated with reduced GABA and glutamate titers in the central brain. (A) Experimental design to collect foragers for mass spectrometric analysis of brain neurotransmitter titers. Individually marked foragers are allowed to visit the feeder at 300m distance from the hive for an initial 1.5 hrs (foraging phase). The feeder is removed for 1 hr (search phase) and reinstalled for 1 hr (revisit phase). Foragers were captured at hive entrance during each behavior phase (marked red on experiment timeline). (B-C) Glutamate and GABA levels decrease in the central brain (CB) after bees experience a loss of their expected feeder. (D-E) A detailed look at the dynamics of change indicates that glutamate levels gradually but linearly decrease over increasing search trips (decrease by 112 ng per search flight, p-value = 0.001), but GABA levels only decrease post the first experience and stay that way. (F) Histamine (G) Aspartate (decrease by 87 ng per search flight, p-value = 0.031). The neurotransmitter values are scaled by the MS batch. *p<0.05; **p<0.01; ***p<0.001.

and GABA than any other behavioral group (Figures 3A and 3B). Furthermore, we also found changes in the titers of other neurotransmitters and their precursors in the optic lobes after the bees restarted foraging (Figures 3E-3O). Tyrosine and L-DOPA, but not dopamine, were significantly higher in foragers that had restarted foraging compared to the foragers before we removed the feeder (Figures 3F and 3G). Tryptophan, aspartate, histamine, and serine were higher in the foragers that had re-started foraging compared to those that had performed search flights and those

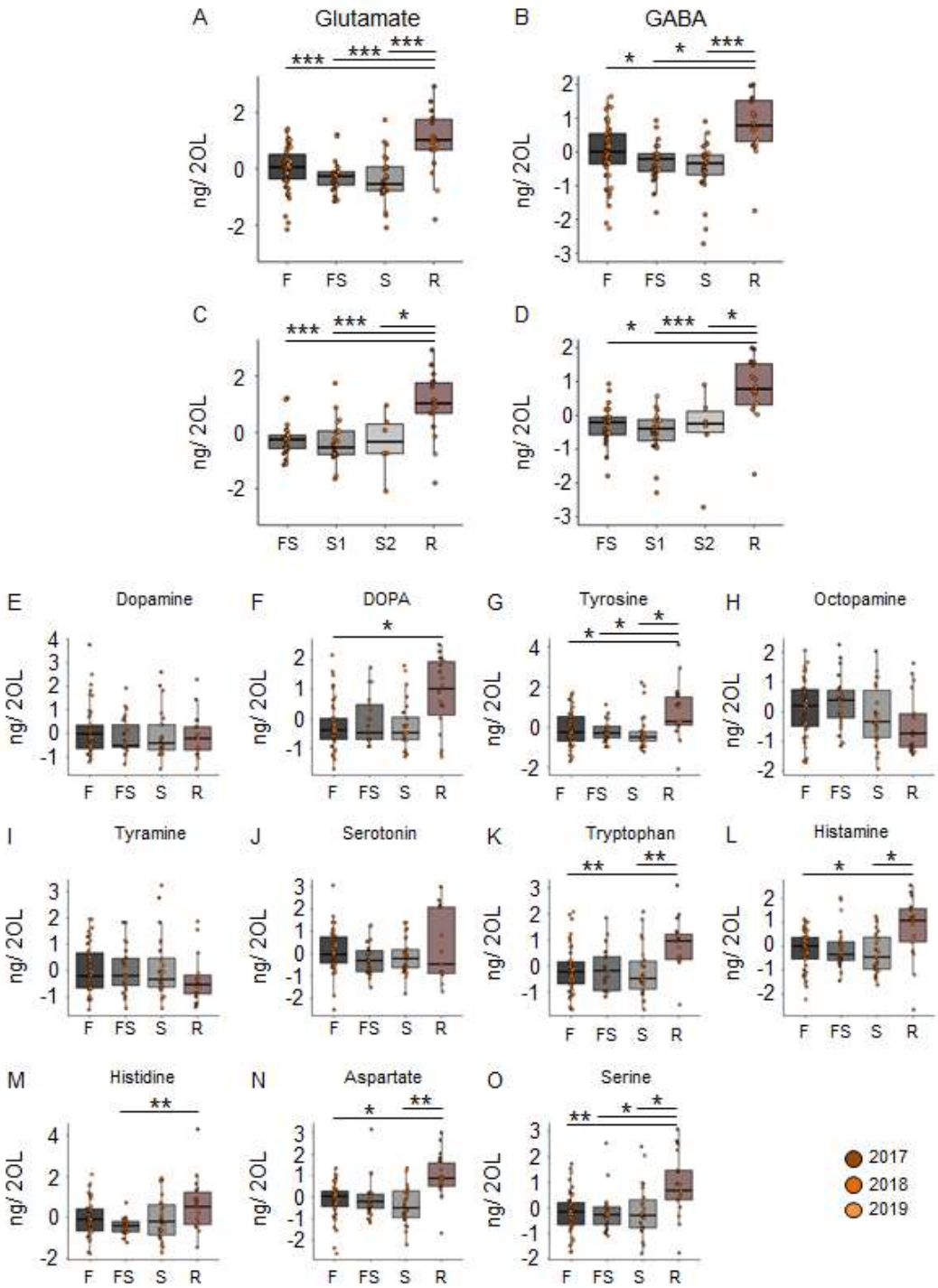

Figure 3. Reinitiation of foraging increases levels of Glutamate and GABA in the optic lobes. (A-B) Glutamate and GABA levels increase after bees start revisiting the feeder. There are no changes due to the experience of feeder loss. (C-D) A detailed look at the dynamics show that the number of search trips do not affect the modulator levels, but only the experience of the feeder does. (E-O) Replacement of the feeder causes abrupt and global changes in multiple modulators in the OL. The neurotransmitter values are scaled by the MS batch. *p<0.05; **p<0.01; ***p<0.001.

that foraged before the removal of the feeder (Figures 3K, 3L, 3N, 3O; supplementary data file S1). All neuromodulators, for which we detected a change, showed a significant increase in their titers due to revisiting the feeder. These dramatic changes were independent of the number of search flights (Figure S3).

Forager search phenotypes show differences in the titers of HA, Octopamine and L-Dopa

Based on our findings that foragers differ in their motivation to search and their persistence to forage we performed a cluster analysis on the individual temporal search dynamics of the collected foragers. Of course, the behavioral data of the collected foragers do not allow a clear identification of the search phenotype for all collected foragers because we collected them during their search behavior instead of after they had stopped leaving the hive. However, we were able to identify a group of foragers that performed several search flights with short intermittent hive stays (Cluster IIIe, Figures 4A and 4B) and foragers that already showed a long hive stay after the foraging/search flight (FS, Cluster I, Figures 4A and 4B) before they were collected. With respect to our behavioral analyses, these two search phenotypes strongly differ in their motivation to search. Only for the collection experiment performed in 2018 we found a sufficient number of foragers with different search phenotypes for a comparison of the neurotransmitter levels (Figures 4A and 4B).

Foragers of the cluster IIIc that had performed 2 search flights with short intermittent hive stays showed significantly higher DOPA and HA levels and significantly lower octopamine levels in the central brain samples compared to one or more groups of foragers with fewer search flights (Figures 5A-C). Foragers of the cluster IIIc also showed a lower level of aspartate in the optic lobes as compared to foragers of the other three clusters (Figure 5F). This difference was also observed between similar phenotypes in the 2017 collection (Figure 5E).

Colonies vary significantly in their neurochemical signatures

Neurochemical content from the CB and OL were quantified from foragers from three different colonies and used for analyzing differences in behavior. In addition to finding changes related to search and restarting of foraging, we found significant differences across colonies as well. A PCA analysis of the CB and OL titers showed that the colonies clustered separately (Figures 6A and 6B), and that more than 50% of the variance in transmitter content is explained by the colony differences alone. Individually as well, transmitters showed significant differences between colonies (Figures 6C-6N and Figure S4). Specifically, the colony used for CE 1 had lower amounts of transmitters in general, in comparison to the other colonies. In the OL, 12 out of 14 transmitters in CE 1 were significantly lower than CEs 2 and 3, while in the CB, 10 transmitters were significantly lower than at least one of the other colonies (Figure S4). In spite of the large differences in transmitter titers due to the colony identity, we were still able to detect the changes in neurochemicals due to the behavioral state

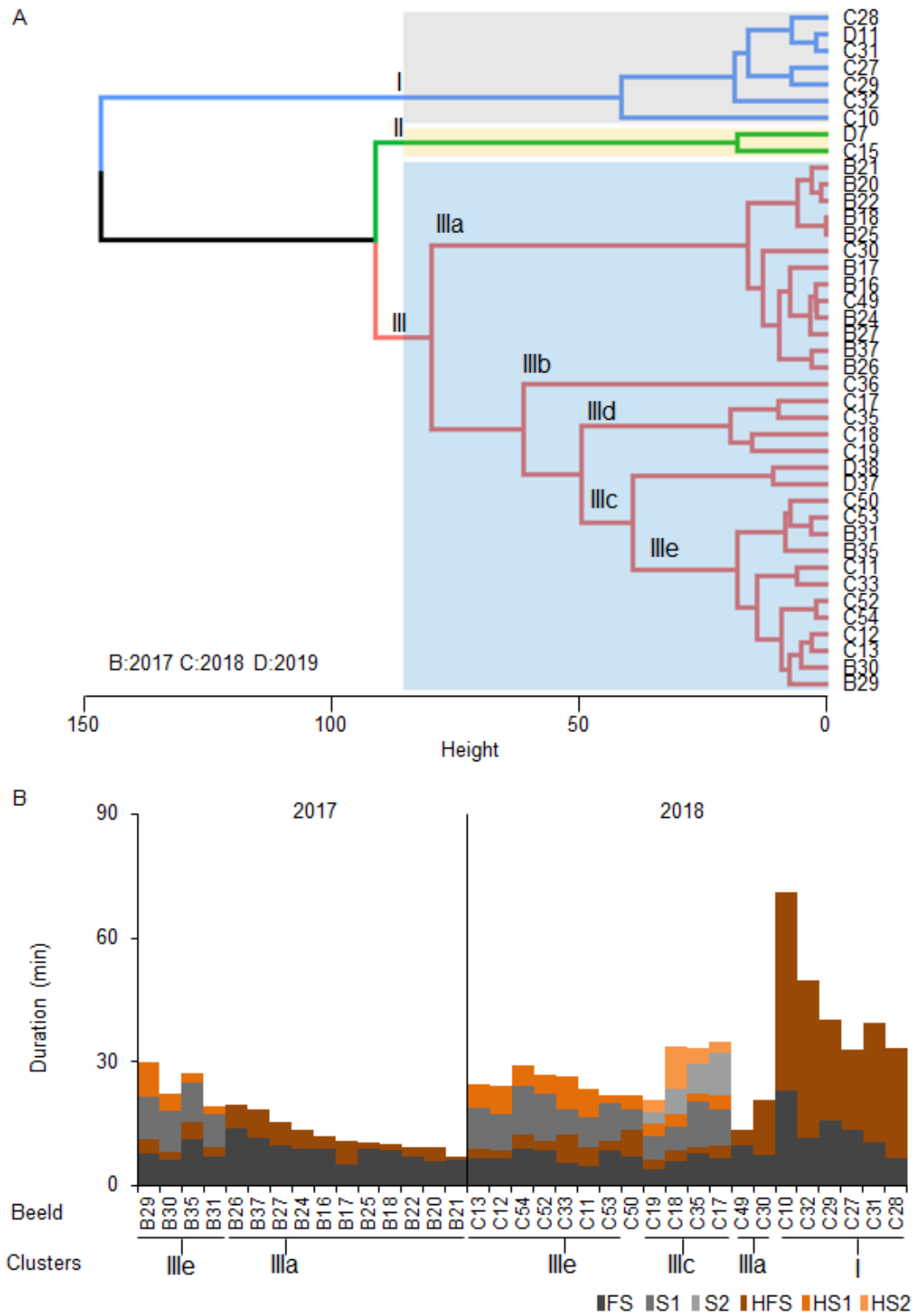

Figure 4 Phenotypes of the collected foragers. (A) Hierarchical clustering of foragers collected during search phase based on search behavior sequences. The maximum average silhouette width 0.52 gave a three-cluster solution with agglomerative coefficient 0.94 (B) Search behavior sequences for the foragers from the collection experiments along with cluster information. Only bees used for cluster analysis of neurotransmitter titers are shown.

DISCUSSION

The principal result of our study is that glutamate and GABA titers in the central brain region (comprising mushroom bodies, central complex and adjacent protocerebral areas) decreased during continuous search behavior for a previously visited but absent feeder. This finding corresponds with the fact that the brains of scouts show a higher expression of genes involved in glutamate and GABA signaling. This correlation suggests that, at least to some degree, the behavioral specialization of scouts is based on genomic mechanisms that modulate signaling mechanisms used in regular search behavior.

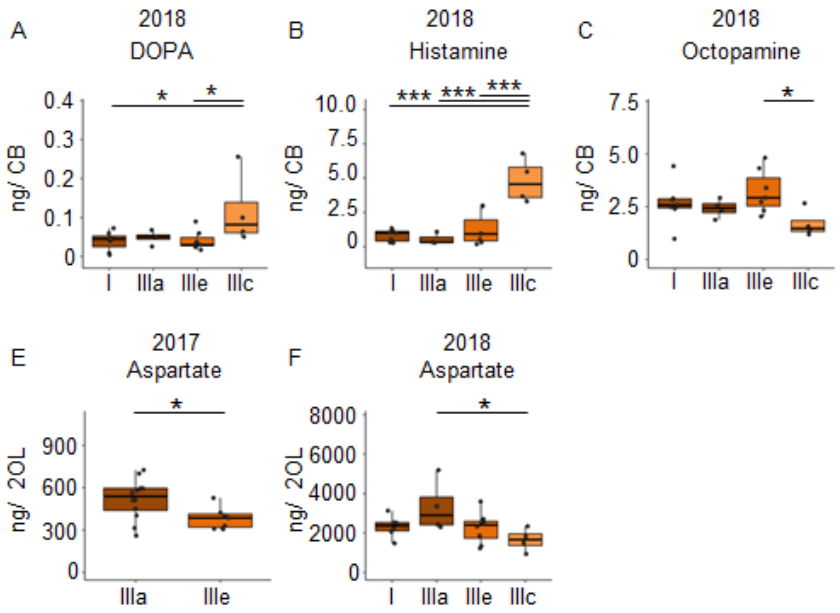

Figure 5. Search intensity negatively correlates with OA and positively correlates with DOPA and HA titers in the CB. (A-B) DOPA and Histamine levels show an increase with increased search trips. (C) Octopamine levels decrease with increased search trips. (E-F) Foragers most motivated to continue search flights showed decreased levels of aspartate in the optic lobes. In addition, bees that performed the FS trip but are phenotypically different in their hive stay times show differences in aspartate levels, though non-significant. Bees without detailed behavior data were added to the phenotype analysis by comparing available relevant behaviors. *p<0.05; **p<0.01; ***p<0.001

Search behavior of foragers that do not find a known feeder at the expected location

Our analyses of the temporal dynamics of flight and hive stay durations of foragers that did not find a known feeder at the expected location suggest that the initiated search behavior consists of a few to several search flights of relatively similar duration (range 1-4 search flights; mean 12.37 ± 6.02 min; range = 5.79 - 37.3 min, Figures 2C-D) and intermittent hive stays that get longer with the increasing number of search trips (13.43 ± 9.37 min, range= 2.33 - 46.4 min; Figures 2E-F). The duration of the hive stay appears to be a good predictor of the probability to stop the search (and foraging) for a period of time. In the additional control experiment, in which we reinstalled the feeder after 1 hour (BE2), foragers almost immediately started landing on the feeder, indicating that the bees' search flights were more or less restricted to the vicinity of the feeder location.

These observations nicely correspond to findings of other studies in which similar experiments were performed. Radar tracking experiments showed that foragers that did not find the expected feeder started flying in loops around the expected location of the feeder and after some time returned to the hive (mean duration 4.49 ± 2.44 min, Reynolds et al., 2007). Furthermore, the search flights were mostly oriented in the hive to feeder direction (Reynolds et al., 2007). Al Toufailia et al., (2013) reported that foragers trained for a few days revisited an empty feeder at an average of 4.29 ± 4.47 trips (range: 0–25) over a 6-hour recording period. The persistence to revisit the temporarily unrewarded feeder, measured as number of trips and total duration of trips, correlated with previous foraging experiences, e.g., duration of feeder availability and profitability, as well as season, which affects colony food stores (Al Toufailia et al., 2013; Townsend-Mehler et al., 2011; Townsend-Mehler and Dyer, 2012). Furthermore, trained foragers were found to continue to visit an emptied feeder for a few days. (1.89 ± 1.56 days, range 0–7 days, Al Toufailia et al., 2013; see table S3).

Together all these studies indicate that the search behavior elicited by the absence of a known feeder induces a search behavior for this feeder. These foragers are not searching for a new food source or food location as scouts do. Only after repeated unsuccessful visits over a few days, is it reported that the majority of foragers might start searching for a new food source, and that too, most likely only after following dances (Biesmeijer and de Vries, 2001; Seeley, 1983). None of the searching bees were found to follow any unmarked dancer during their hive stay within the observation period.

Individual variation and search phenotypes

In addition to the description of the general temporal dynamics of this search behavior, cluster analysis showed that the individual foragers visiting the same feeder varied in the intensity of search behavior or persistence in revisiting the feeder location. The strongest search response is characterized by fast repetition of search flights which includes short intermittent hive stays. The weakest response was characterized by 1-2 search flights with long intermittent hive stays. These differences are likely based on variations in the behavioral state depending on previous experience and genotype.

In an earlier study, our lab reported that there are consistent long-term individual differences in the dance activity among foragers visiting the same feeder. Interestingly, these differences were, at least to some degree, dependent on the composition of the group (George et al., 2020). Furthermore, the individual variation in dance activity correlated with expression differences in the foraging gene (*Amfor*). Similarly, linear discriminant analysis of the brain gene expression pattern of individual scouts and recruits showed a separate but overlapping distribution suggesting a more quantitative than qualitative difference between these phenotypes (Biesmeijer and de Vries, 2001; Liang et al., 2012). For the future, it will be interesting to explore whether differences in foraging, dance and search activity among foragers visiting the same feeder are correlated and based on the same physiological processes or not. In addition, it would be interesting to see whether scouts resemble one of these forager phenotypes or represent a separate one.

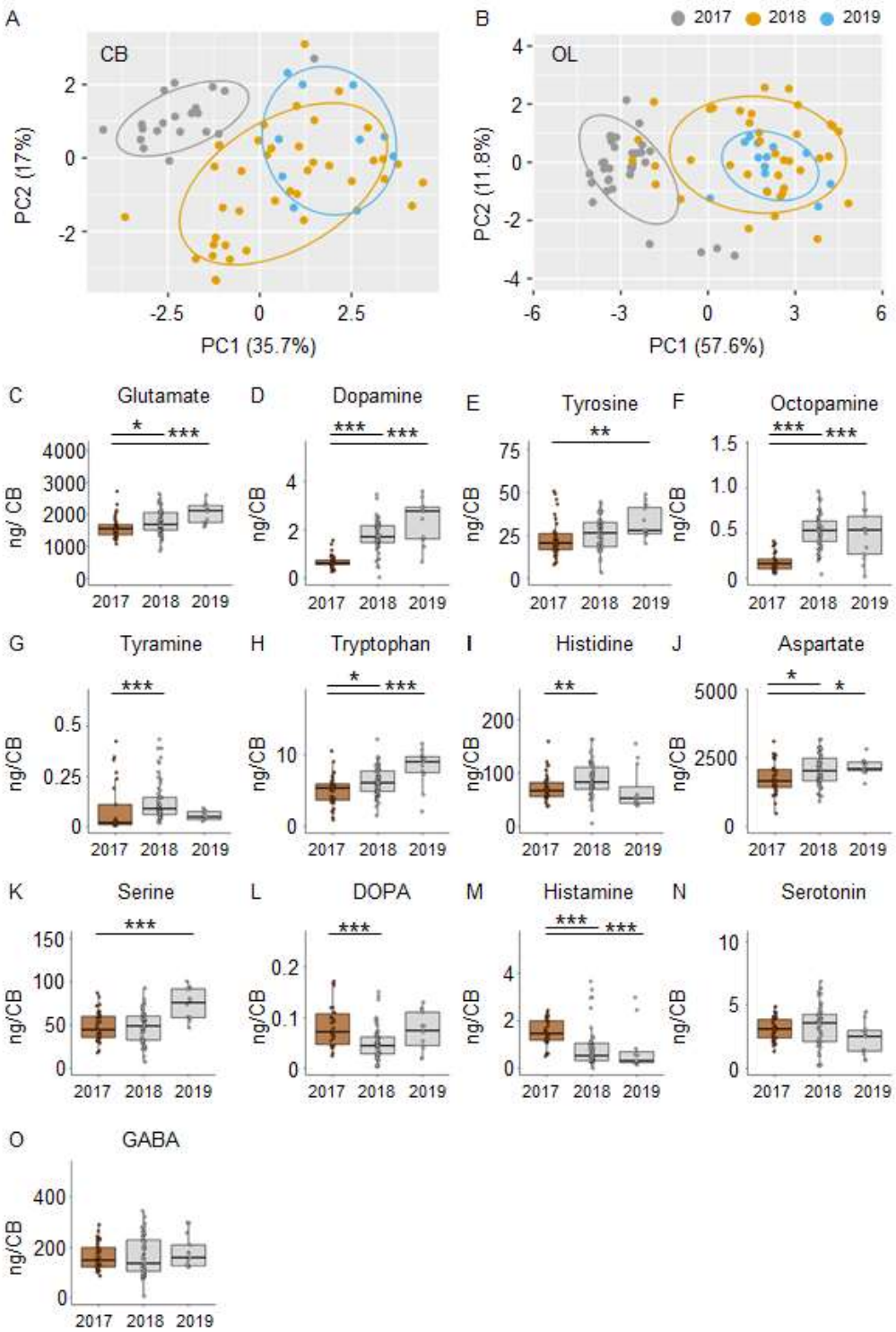

Figure 6. Neuromodulators vary significantly between different colonies. PCA of modulator titers in the CB (A) and OL (B) show >50% variance explained by colony membership. (C-K) Individual modulators vary significantly between the different colonies. In general, the 2017 colony shows a lower amount of most transmitters than the 2018 and 2019 colonies. Only the differences between the 2017 and the other two years are shown. *p<0.05; **p<0.01; ***p<0.001.

GABA and glutamatergic systems are involved both in scout- and recruit-mediated food search

The comparison of foragers with different numbers of search flights suggest that glutamate and GABA titers continuously decrease with the number of search trips in the central brain (i.e., mushroom bodies, central complex and surrounding protocerebral brain areas). In contrast, glutamate and GABA titers in the optic lobes did not change during search but showed an abrupt increase when the foragers had started revisiting the feeder again. This kind of rebound increase in titers was not observed for the central brain region; moreover, the titers were still lower compared to the foraging group at the beginning of the experiment.

Liang et al., (2012) reported a higher expression of several glutamate and GABA receptor and transporter genes in the brain of scouts compared to recruits. In addition, treatment experiments with monosodium glutamate (MSG) increased scouting behavior. Although we do not know the exact function of glutamate and GABA in search behavior (Carr-Markell and Robinson, 2014; Cook et al., 2019; Filla and Menzel, 2015; Locatelli et al., 2005), the comparison of scouts and recruits and our studies on search behavior in foragers (i.e., recruits) strongly suggest that these neuromodulators have an important function in search behavior in general. Changes in the glutamate and GABA signaling appear to be major physiological underpinnings of the behavioral specialization of scout bees. Furthermore, this molecular mechanism might not be unique to honey bees, as it was found that glutamate receptors are also upregulated in scouts of *Temnothorax* ants (Alleman et al., 2019).

Finally, one of the most original recent molecular studies in honey bees showed an increase in activity in GABA-ergic neurons of the optic lobes during re-orientation flights in which the foragers learn the hive entrance and hive location (Degen et al., 2018; Kiya and Kubo, 2010). In our behavioral experiments the foragers that found the feeder again also performed learning flights involving circling over the feeder (Figure S5; Lehrer, 1991). Thus, the abrupt increase in GABA titers in the optic lobes in the revisiting foragers might be related to the phenomenon described by Kiya and Kubo (2010). GABA-ergic neurons in the optic lobes of *Drosophila*, for example, are involved in tuning the sensitivity and selectivity of different visual channels (Keleş et al., 2020; Keleş and Frye, 2017). Similarly, glutamate signaling in the optic lobes is involved in shaping object recognition and directional motion vision (Bicker et al., 1988; Rossi et al., 2020; Sinakevitch and Strausfeld, 2004).

In addition to the differences in the glutamate and GABA titers, we found changes in histamine and aspartate. Histamine levels in the CB increased with the intensity of search (Figures 2F and 5B), and in the OL, they increased due to the re-initiation of foraging (Figure 3L). Aspartate was found to decrease linearly with increasing search trips in the central brain (Figure 2G) as well as in the optic lobes (Figures 5E and 5F). Later, during the re-initiation of foraging, aspartate levels in the OL increased (Figure 3N). There is growing evidence that neuromodulation in the optic lobes plays a significant role in selecting and adjusting visual processing according to the behavioral context (Cheng and Frye 2020). Our results suggest that HA and aspartate, which showed significantly higher titers in the optic lobes of revisiting foragers, might play an important

role in modulating visual processing (Hamanaka et al., 2012; Sinakevitch and Strausfeld, 2004; Thamm et al., 2017; Ziegler et al., 2013). The changes in the central brain are more difficult to interpret. Previously, we reported that aspartate levels increased globally during anticipation of food (Ramesh and Brockmann, 2019). It is therefore likely that aspartate and histamine play a role in regulating foraging and motivation (see Torrealba et al., 2012), although this remains to be investigated.

Forager search phenotypes show differences in the titers of HA, Octopamine and L-Dopa

Comparison of search phenotypes revealed that foragers with a high intensity of search behavior (several successive search flights with short intermittent hive stays, Cluster IIIe) had higher DOPA and HA levels and lower OA levels in the central brain region than foragers with less intense search behavior. These differences in the levels of neurotransmitters among search phenotypes could be a result of a higher degree of neural signaling activity or differences in the baseline levels of neurotransmitter levels among search phenotypes.

Liang et al., (2012) reported that octopamine treatment resulted in a weak but significant increase in scouting behavior and that scouts showed a higher expression of the OctƷ2R receptor. Interestingly, they also found that a dopamine antagonist treatment inhibiting dopamine signaling caused a significant decrease in scouting, but their molecular data indicated that dopamine signaling might be downregulated in scouts. The two dopamine receptors, AmDopR1 and AmDopR2 showed a lower whole brain expression in scouts compared to non-scouts (Liang et al., 2012). More recently, Linn et al., (2020) showed that foragers treated with octopamine revisited an emptied feeder more often than a sham-treated control group. More importantly, they preferred the known but emptied feeder instead of searching for a new feeder indicated by other nestmates, suggesting that octopamine might increase foraging activity in the sense of persistence (or probability of leaving the hive) but not in a specific sense of searching (A. Barron et al., 2002; Barron and Robinson, 2005; Wagener-Hulme et al., 1999). Recently, Cook et al., (2019) reported that the brains of scouts showed higher tyramine levels than those of recruits. However, similar to octopamine, the function of tyramine might not be directly involved in search behavior but foraging and flight activity, as suggested by QTL studies on honey bee foraging behavior (Hunt et al., 2007).

Colonies differ in neuromodulator levels as well as in search phenotypes

In our study, we found that greater than 50% of the variance in neurotransmitter titers were due to the identity of the colony from which the foragers were caught (Figures 6A and 6B). An interesting observation was that the foragers of colony CE 1 showed lower titers compared to foragers of CEs 2 and 3 for almost all neuromodulators tested (Figures 6C-N and Figure S4). These were bees that were housed in an observation hive. Many previous studies on honey bees reported variation in brain neurotransmitter and neuromodulator titers with colony state and season (Božič and Woodring, 1998; Harris and Woodring, 1992). The colony hosted in the observation hive likely differed from the others in the density and crowding of bees, as well as in the available food stores (impacting the hunger state), both of which are known to affect the neurochemical composition of

the brain (Hewlett et al., 2018; Mayack and Naug, 2015). In spite of the large differences in transmitter titers due to the colony identity, we were still able to detect the changes in neurochemicals due to the behavioral state.

Colonies also differed in the composition of search phenotypes. In the behavior experiment (Figures 1I and 1J) as well as in the collection experiment (Figure 4B), performed over 5 years, we found that colonies differed in the amount and even the presence of phenotypes. These differences are likely due to colony conditions and forage availability modulating the foraging force.

Novelty seeking, glutamate, GABA and the honey bee brain

Liang et al., (2012) suggested that the brain expression differences between scouts and recruits have something to do with novelty seeking as scouts are obviously searching for new food sites, and studies in vertebrates indicated that the identified neuromodulator systems (glutamate, GABA, and catecholamine) are involved in novelty seeking. Novelty seeking is certainly a complex behavior composed of different behavioral routines or modules, e.g., a specific flight pattern and increased visual and olfactory attention when searching for flowers. The differences in the changes in glutamate and GABA titers in the central brain region and optic lobes in our study might correspond to these different behavioral modules. Our finding that GABA titers increase with relearning the re-established feeder corresponds with the finding that GABA neurons in the optic lobes showed an increased activity during relearning the nest entrance and its surrounding after the hive had been experimentally relocated (Kiya and Kubo, 2010). Another question is whether glutamate and GABA initiate or modulate (enhance) search and scouting behavior (Palmer and Kristan Jr, 2011). A recent study in *Drosophila*, for example, showed that the majority of octopaminergic neurons are also glutamatergic and that both transmitters in these neurons affect the same and different behaviors, and thus might be involved in selection of behavioral modules (Sherer et al., 2020). In honey bees, glutamate and GABA have mainly been studied in the context of learning and memory (Gauthier and Grünewald, 2012; Leboulle, 2012; Locatelli et al., 2005; Shyu et al., 2017; Xia et al., 2005; Zwaka et al., 2018). A study in ants aimed at identifying negative effects of increased monosodium glutamate consumption over several days showed a decrease in “precision of reaction”, a decrease in “the response to pheromones”, a decreased “impacted cognitive ability”, and “largely reduced learning and memory” (Cammaerts Tricot and Cammaerts, 2016). Thus, the most plausible assumption at the moment might be that glutamate and GABA are involved in modulating brain circuits involved in search behavior and thus changing probabilities to perform behavioral routines involved in search behavior. The insect central brain region including mushroom bodies, central complex and adjacent protocerebral areas are involved initiating and selecting behaviors (Huber, 1955; Hulse et al., 2020; Tsao et al., 2018; Varga et al., 2017), and the mushroom bodies are important for learned visual navigation (Buehlmann et al., 2020; Kamhi et al., 2020). For the future it would be interesting to identify neuron populations that are involved in search and scouting. One approach would be to perform double in-situ staining for neuronal activity-regulated genes and genes involved in glutamate and GABA signaling in brains

of recruits and scouts caught during search behavior as it was done for re-orienting foragers by (Kiya and Kubo, 2010; Sommerlandt et al., 2019). Having identified neurons involved in searching as well as scouting, one could compare their expression pattern to identify the molecular changes underlying behavioral specialization at the cellular level. Regarding the neuronal mechanisms that determine scouts, it might be promising to first compare how scouts differ from recruits in the search behavior. For example, do scouts perform longer and more extended search flights? Subsequently, one could study whether the gene expression differences between scouts and recruits are based on changes in gene expression in the same cells or an extension of gene expression in different cells and identify in which brain areas these differences occur.

Honey bee foraging at an artificial feeder is one the most fruitful and successful experimental assays in the study of animal behavior. Most, if not all, of the fascinating behavioral and cognitive capabilities of honey bees have been identified using the feeder training assay (Giurfa, 2015; Seeley, 1995; Von Frisch, 1965). As a behavior, foraging can be nicely dissected into different behavioral routines which can be studied separately, e.g., anticipating foraging in the hive (Ramesh and Brockmann, 2019; Reinhard et al., 2004; Shah et al., 2018), flying towards the feeder, food collection at the feeder (Brockmann et al., 2009; Singh et al., 2018), flying back to the hive, and recruiting nestmates with the dance (Barron et al., 2007; Chatterjee et al., 2019). In addition, there is increasing evidence that foragers vary in the intensity of each behavioral module (Cook et al., 2019; George et al., 2020; George and Brockmann, 2019; Jeanson and Weidenmüller, 2014). We suggest that detailed behavioral experiments combined with sophisticated molecular techniques will help to identify candidate neuronal mechanisms involved in elaborated behavioral and cognitive capabilities (Kiya and Kubo, 2011; Shah et al., 2018; Sommerlandt et al., 2019). Of course, decisive causal mechanistic studies will only be possible if genetically engineered honey bees are widely available (Değirmenci et al., 2020; Kohno et al., 2016; Roth et al., 2019), or we might use Drosophila for comparative studies to identify neural underpinnings of some of the behavioral modules (Brockmann et al., 2018; Kamhi et al., 2017; Murata et al., 2017; Reaume and Sokolowski, 2011).

METHODS

Materials availability

Apis mellifera colonies were procured from HoneyDay Bee Farms Pvt. Ltd., Bangalore. All standards, formic acid (FA), hydrochloric acid (HCl), boric acid and ascorbic acid as well as reagents required for 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) synthesis were obtained from Sigma-Aldrich (Bangalore, In- dia). Acetone was obtained from Fisher Scientific. Solid phase extraction cartridges (Strata-X, 8B-S100-TAK) were obtained from Phenomenex, Inc. (Hyderabad, India). High purity MS grade solvents (methanol, acetonitrile, and water) were obtained from Merck Millipore (Merck Millipore India Pvt. Ltd., Bangalore). Deuterated internal standards were supplied by CDN isotope (Quebec, Canada). Glass beads and the bead beater were purchased from BioSpec.

Animals and feeder training

Honey bee colonies (*Apis mellifera*, n = 6) were located inside the NCBS campus, Bangalore, India. Foragers were trained from the hive to an unscented sugar water feeder (concentration 1.75 M). The feeder distance was gradually increased to 300 m from the hive over two days and foragers were trained along a road surrounded by dense vegetation in the neighboring UAS-GKVK Campus, Bangalore, India (Chatterjee et al., 2019).

Behavior Experiments

Two kinds of behavior experiments were performed. In the first behavior experiment (BE 1), foragers (n = 10-12) were color-marked individually prior to the day of the experiment (Chatterjee et al., 2019). On the day of the experiment, marked foragers were allowed to visit the feeder at 300 m for 1.5 h (foraging phase). The feeder was then removed and kept hidden away from the reach of the foragers for another 1.5 h (search phase; Figure 1A). In the second behavior experiment (BE 2), the feeder was presented initially for 1.5 h (foraging phase) for marked foragers (n = 50-60) to visit. The feeder was then removed for 1 h (search phase) and reinstalled at 300 m for another 1.5 h (revisit phase).

Collection Experiments

Three collection experiments (CE 1-3) were performed. During each experiment, color-marked foragers (n = 50-60) initially visited the feeder (concentration 2 M) for 1.5 h (foraging phase). At the end of the foraging phase (last 10 min), color-marked outbound foragers (n = 7-14) from the hive opening were captured in plastic tubes and flash-frozen in liquid nitrogen. The feeder was then removed and kept hidden during the search phase (1-1.5 h). Foragers flying out did not find the feeder at 300m and came back to the hive. Marked Individuals were caught while flying out after 10-90 mins of feeder removal. The feeder was reintroduced at 300m (revisit phase) and foragers readily continued foraging at the feeder for another hour. Revisiting individuals were captured at the hive entrance making outbound flights, provided each made 3-4 trips to the feeder (Figure 2A).

Colonies used for BE 1 (n = 2, 2015 and 2020) and CE 1 (n = 1, 2017) were housed in a glass observation hive located within a wooden hut devoid of any external illumination (Chatterjee et.al., 2019). In case of BE 2 (n = 1, 2019) and CE 2-3 (n = 2, 2018 and 2019), the colonies were housed in a Langstroth hive box and placed under the shade of a tree next to the site of the hut. Experiments were performed in the summers between the months of May – July each year.

Monitoring search flights

For BE 1 and CE 1-3, a video camera (Sony HDR-CX220V Tokyo, Japan) was mounted at the hive entrance. Experimenters recorded the number and duration of search flights made by individuals from the time of exit and entry of the foragers in and out of the hive from video

playback. Video recordings were done at the feeder for BE 2 during foraging and revisit phase to monitor the rate of bees arriving at the feeder.

Brain dissections

The foragers collected in liquid nitrogen were transferred to a -80 ℃ freezer. Individual bee brains were dissected on dry ice into two different parts, the optic lobe pair (OL) and the region of the brain containing the mushroom bodies and the central complex (CB). The antennal lobe pair (AL) and the subesophageal zone (SEZ) were discarded.

Mass spectrometry of neurotransmitters

For mass spectrometric measurements, brain samples were prepared as in Ramesh and Brockmann, 2019. Briefly, to the vial containing the individual bee brain part, 100 µL of 0.5 mm glass beads, 190 µL of acetone containing 0.1% formic acid, 10 µL of 10 mM freshly prepared ascorbic acid, and 10 µL of 0.5 µg/mL internal standard mixture was added. A standard addition mixture of serotonin and tryptamine was added to each sample so that the spiked amount on column was 0.5 ng. A bead beater was used to homogenize the samples, and the supernatant was collected in a new vial and lyophilized. For the derivatization procedure, 80 µL of 200 mM borate buffer and 10 µL of 10 mM ascorbic acid was added to the lyophilized extract, mixed well and 10 µL of a freshly prepared solution of 10 mg/mL AQC was added. The reaction was incubated at 55 °C for 10 min and stopped by the addition of 3 µL of 100% formic acid.

MS grade water (500 µL) was then added to the samples, and samples were loaded onto activated and equilibrated RP-SPE columns and washed twice with 1 mL of water containing 0.1% formic acid. Elution was done with 1 mL of ACN−MeOH (4:1) containing 0.1% formic acid and lyophilized and stored at -20 °C until injection into the instrument. Samples were reconstituted in 50 µL of 2% ACN containing 0.5% formic acid. The calibration curve ranges for each compound were made according to the abundance in the biological matrix and were the same as in Ramesh and Brockmann, 2019.

A Thermo Scientific TSQVantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), connected to an Agilent 1290 infinity series UHPLC system (Agilent Technologies India Pvt. Ltd., India) was used for the neurochemical quantification. The column oven was set at 40°C, and the autosampler tray at 4°C. The mobile phase solvent A was 10 mM ammonium acetate containing 0.1% formic acid, and solvent B was ACN containing 0.1% formic acid. A C-18 column (2.1 mm × 100 mm, 1.8 µm, Agilent RRHD ZORBAX) fitted with a guard column (2.1 mm × 5 mm, 1.8 µm Agilent ZORBAX SB- C18) was used. The LC gradient was as follows: (2% B at 0 min, 2% B at 3 min, 20% B at 20 min, 35% B at 25 min, 80% B at 25–27 min, 2% B at 27–35 min) at a flow rate of 0.2 mL/min. The MS operating conditions were as follows: 3700 V spray voltage (positive ion mode); 270 °C capillary temperature; 20 (arbitrary units) sheath gas pressure; 10 (arbitrary units) auxiliary gas; argon collision gas. The S lens voltage and collision energy were as given in Ramesh and Brockmann, 2019. Quantification was done using the Xcalibur software version 2.2.

Statistical Analysis

(i) Classification of outbound trips

Trips of the foragers were classified based on the foragers' knowledge whether the feeder was present or not into F, FS and S (Table S1). In both F and FS, a forager flying out of the hive had the information that the feeder was present whereas in S(s) the forager had the information that the feeder is no more present.

(ii) Changes in hive-to-hive duration and hive stays

For behavior experiment BE 1, we wanted to know if the removal of the feeder led to changes in (a) hive-to-hive duration for an outbound flight and (b) duration of the hive stays between two outbound trips. We used generalized linear mixed-effects model (GLMM) with a Gamma error distribution, considering the individual identity of the bee as a random factor (individual effect) and compared (a) hive-to-hive duration (F, FS and S1-4) and (b) duration of hive stay following outbound flights (HF, HFS, HS1-3) using a generalized linear hypothesis test (GLHT). P-values were corrected for multiple testing using single step adjustment. Distribution structures of the data were determined prior to model building by comparing AIC values as a goodness of fit criteria. Models were used separately for the two colonies.

(iii) Search behavior sequence and cluster analysis

A combination of sequence and cluster analysis (Lowe et.al., 2020) was used to identify common search behavior patterns among individual foragers following feeder removal in the behavior experiment BE1. First, consecutive search flights and in-between hive stays for a forager were arranged as a search behavior profile. Each profile started with the exit of the bee for FS (t=0) and was terminated at 120 min. When a bee came back from a search flight and did not appear outside until the end of the observation period, the time the forager spent inside the hive was counted as her final hive stay. For example, if a bee made 4 search flights, the search behavior profile would include the search behavior states: "FS - HFS - S1 - HS1 - S2 - HS2 - S3 - HS3 - S4 - HS4" where HS4 is the final hive stay (i.e., final state in the sequence). Next, the duration a forager spent in a given state was rounded to its nearest whole number (minimum duration of stay in a given state is 1 min). Then, each search behavior profile (SPELL format) was converted into a search behavior sequence (STS format). The whole observation period of 120 min was divided into bins of 1 min and a forager occupied a given state for 1 min and moved into a new one or continued to be in the same state in the next 1 min depending on the search behavior profile. The sequences of search behavior states occupied by foragers were stored chronologically in a matrix (rows for every forager and the columns for a given state, see also additional data file S1 for details).

Finally, we calculated a distance matrix, i.e., the distances between all pairs of search behavior sequences using the optimal matching distance metric. The metric used an insertion/deletion cost of 1 and substitution cost using transition rates (min = 0 for identical substitution and max = 2 for a transition not observed) between observed states in the search behavior sequence. Individuals with common search behavior sequences were grouped by hierarchical agglomerative clustering using Ward's D2 clustering criterion based on the distance matrix computed earlier. The optimal

number of clusters was determined by selecting the maximum average silhouette width (Figure S2A; Levshina, 2015).

For collection experiments (CE 1-3), the search behavior profile for an individual making 2 search flights before being captured included the search behavior states: "FS - HFS - S1 - HS1 - S2 - HS2" and HS2 was the final state in the sequence. A combination of sequence and cluster analysis was further done similar to behavioral experiments as mentioned before to identify common search behavior patterns among individual foragers following feeder removal (see also additional data file S1 for details). Foragers in CE 1-3 (n=41) were grouped into three clusters (I-III; Figure 4A) based on the similarity in duration of stay in given states in their search behavior profile (Figure 4B; see also Figure S2B for optimum number of clusters). Cluster I consisted of 7 FS bees (n=6, 2018 and n=1, 2019) which had their final stay in the hive (HFS) more than 20 mins. (Figure 4A-B). Cluster II had 2 S1 bees (n=1, 2018 and n=1, 2019) which stayed in the hive (HS1) for more than 35 mins before being captured. The biggest group, Cluster III consisted of S1, S2 and S3 bees (n=15, 2017, n=15, 2018 and n=2, 2019) which all had their final stay in the hive (HS1, HS2 and HS3 respectively) for less than 20 mins.

Cluster III was further subdivided into 5 subgroups post-hoc (IIIa-IIIe; Figures 4A-B). Subcluster IIIa comprises 13 FS bees which had their final stay in the hive (HFS) no longer than 14 mins. Subcluster IIIc (n=4) had all S2 bees but one individual (BeeID: C36) forming an outlier in subcluster IIIb with longer FS and search flights (S1 and S2 more than 18mins). Subclusters IIId (n=2) housed S1 bees which stayed in the hive (HS1) longer than 15mins (but less than 35 mins) before being captured. Finally, subcluster IIIe (n=12) housed the rest of the S1 bees which spent time in the hive (HS1) less than 9 mins before they were captured (see also additional data file S1 for details). This clustering further led to comparing neurotransmitters among individuals.

The sequence analysis was performed using the R package "TraMineR" and agglomerative clustering was done by using the "agnes" function from R package "cluster".

To help with the neurochemical data analysis, the FS and S bees without full behavioral data (7 bees) were manually classified into the identified clusters. For this purpose, the number of search flights and the total amount of time a bee experienced a loss of feeder before being caught were used in addition to incomplete flight duration and hive stay data. The details of the clustering criteria are given in the supplementary data file S1.

(iv) Quantification of foragers dynamics at the feeder

For BE 2, the total number of marked foragers at the feeder was counted every 2 minutes (Figure S1). We asked if the rate of foraging was different during the foraging and revisiting phases. We fit non-linear growth curves to the number of bees at the feeder every 2 min to evaluate and compare the rate of foraging:

Gompertz curve: $y = a * exp(-b * exp(-c * t)$……..…..……
[1]

where a = carrying capacity (maximum number of bees), b = sets the displacement along the time-axis, c = growth rate, t = time in minutes and the inflection point (I) is given as equation 2 (Jukić et al., 2004).

Inflection Point: $I = \{(lnb)/c, exp(a - 1)\}$ ……...…….…….[2]

(v) Analysis of the mass spectrometry results

Neurochemical analysis was done using linear mixed effects models from the "lme4" package. Satterthwaite's t- tests from the "lmerTest" package were used to estimate significance values from the models. Analysis was done with the amount of neurochemical as the response variable, and the appropriate behavioral response/behavioral group as the fixed variable. The MS batch was used as the random effect. Post-hoc tests were done using the emmeans function from the "emmeans" package. Plots were drawn using the "ggplot2" package. For some plots, for visual purposes, the neurochemical values were scaled with respect to the MS batch and the experimental repeat.
PCA analysis was done using the "prcomps" function in the "stats" package of R. The package "ggbiplot" was used, with a minor adjustment for visual purposes, to plot the prcomp results.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one data file with 11 sheets showing raw data, and results of statistical analyses. Supplementary figures and tables referred to in the main text are at the end of this document.

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AUTHOR CONTRIBUTIONS

Conceptualization A.C., D.R. and A.B.; Field experiments and observations, A.C. and D.B.; Brain Dissections, D.B.; Behavior analysis, A.C.; Mass spectrometric measurements and analysis, D. R.; Writing, A.C. and D.R and A.B.; Supervision, A.B.

DECLARATION OF INTERESTS

The authors state no competing interests.

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Supplemental Information

Search behavior of individual foragers involves neurotransmitter systems characteristic for social scouting

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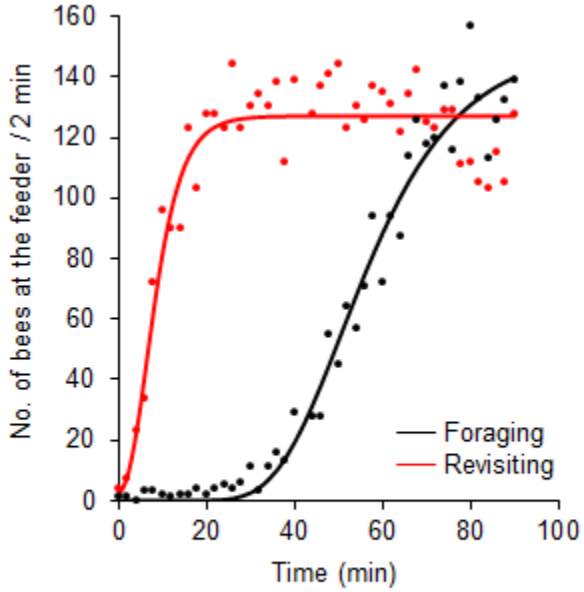
Supplemental Information

1. **Figure S1: Behavior Experiment BE2**
2. **Figure S2: Silhouette width for determining number of clusters**
3. **Figure S3: Changes in neurotransmitter titers in OL for revisiting foragers**
4. **FigureS4: Difference in neurotransmitter titers in OL among different years**
5. **Figure S5: Learning flights**
6. **Table S1: Classification of outbound and inbound flights**
7. **Table S2: Summary of collection experiments**
8. **Table S3: Comparative time scale associated with search behavior**
9. **Supplemental References**
10. **Data file S1 (uploaded separately)**
    - **(A) Sheet 1: Search behavior profile of foragers in BE1**
    - **(B) Sheet2: GLMM and GLHT for flight duration in BE1**
    - **(C) Sheet 3: GLMM and GLHT for hive stay in BE1**
    - **(D) Sheet 4: Search behavior of foragers in BE1 SPELL format**
    - **(E) Sheet 5: Search behavior of foragers in BE1 STS format**
    - **(F) Sheet 6: Clustering of foragers in BE1**
    - **(G) Sheet 7: Neurochemical values from mass spectrometric quantification for collected foragers along with batch, colony id and experiment id**
    - **(H) Sheet 8: LMM results with post-hoc comparisons for CB and OL**
    - **(I) Sheet 9: Search behavior of collected foragers SPELL format**
    - **(J) Sheet 10: Search behavior of collected foragers STS format**
    - **(K) Sheet 11: Search behavior profile of collected foragers and cluster identity**

Supplementary Figures



Curve fitted -
Foraging: y=149.799*exp(-31.061*exp.(0.067x));c=0.067, b=31.061
Revisiting: y=127.143*exp(-4.296*exp.(-0.229x)); c=0.229, b=4.296

Figure S1. Dynamics of foragers visiting the food source before removal (black) and after re-establishing the feeder (red). Foragers searching for the missing feeder started visiting at a faster rate when the feeder was re-established compared to when the feeder was opened for the first time on that day.

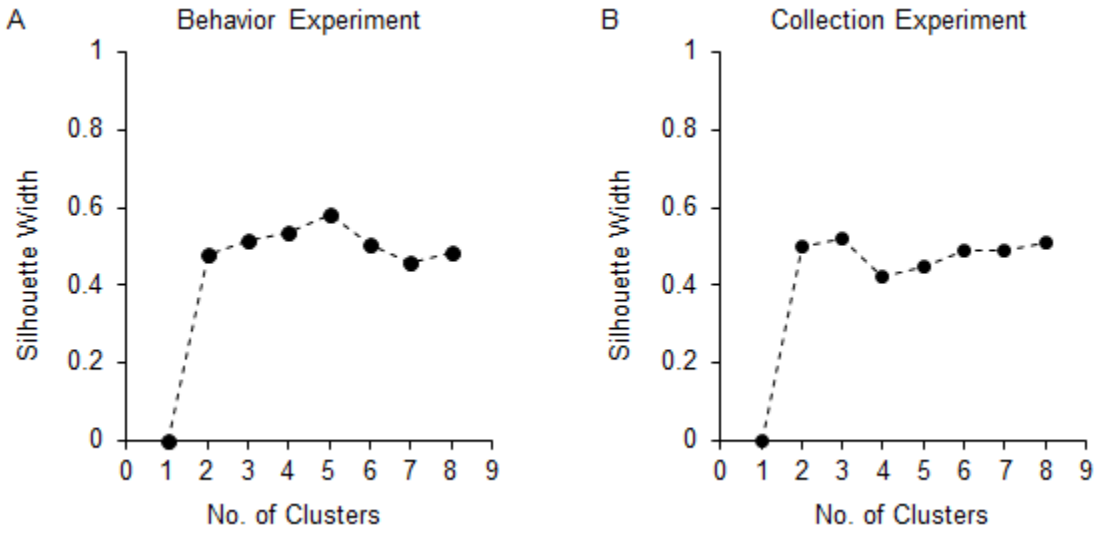


Figure S2. Silhouette width for determining number of clusters (A) Behavior Experiment (B) Collection Experiment. The maximum silhouette width determined the number of clusters.

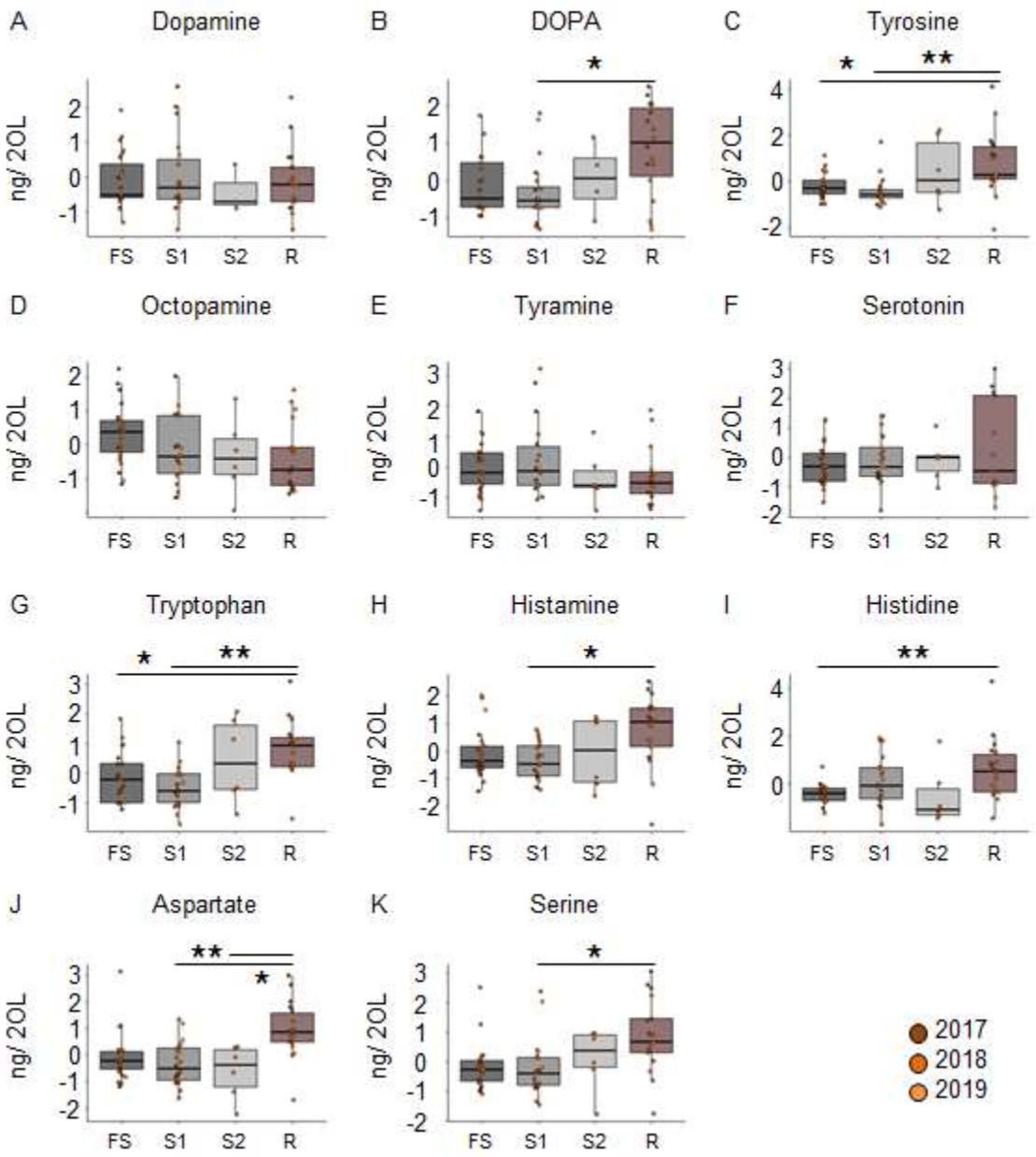


Figure S3A-K. Foragers that initiated foraging have the highest neurotransmitter titers in the OL. The abrupt increase in transmitter content seen in the OL of foragers that initiated foraging are independent of the number of search flights performed by the bees.

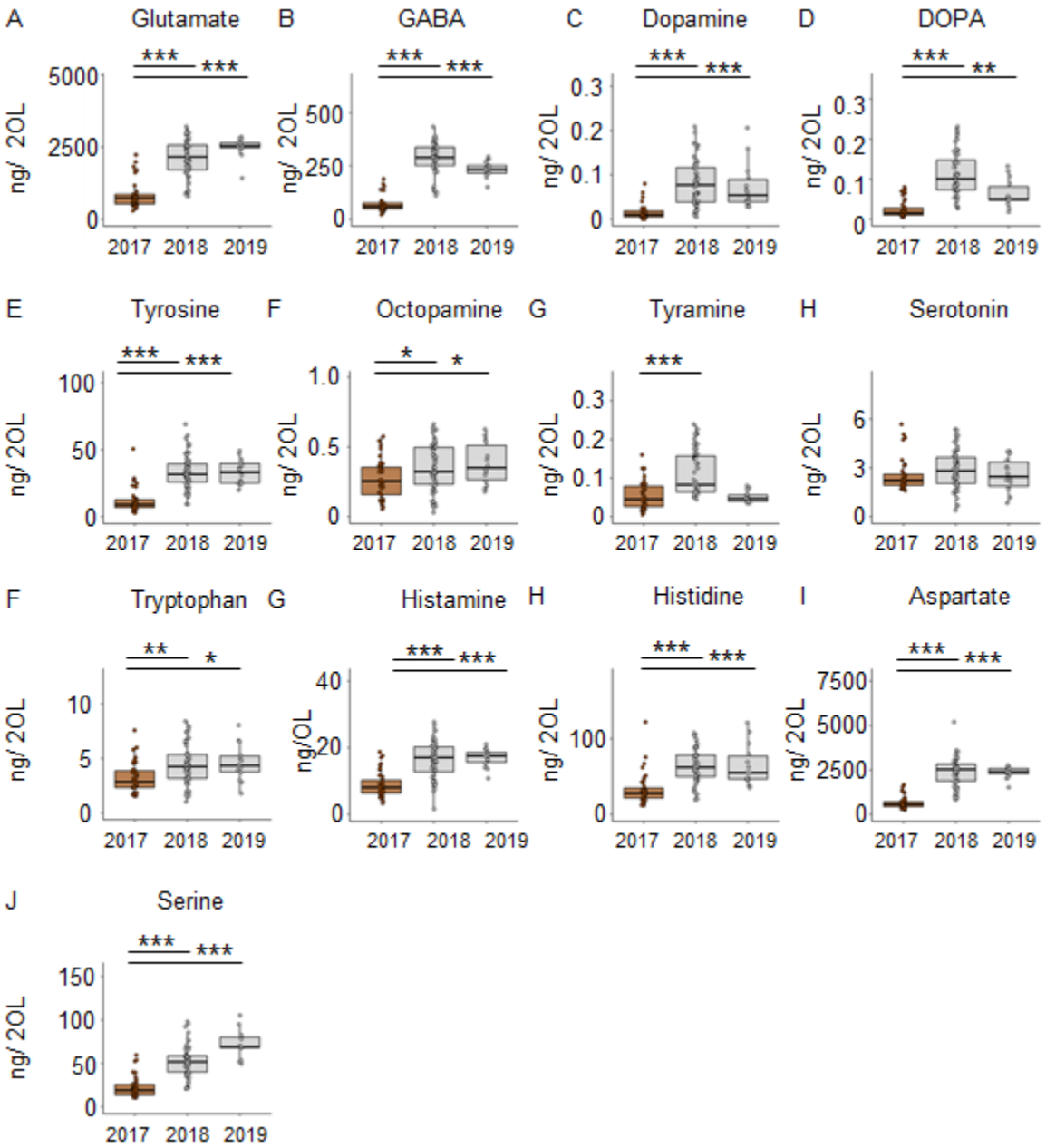


Figure S4. Colony dependent variation in neurotransmitter titers in OL. Except for serotonin (S4K), all other transmitters showed significant differences due to the colony identity. In addition, foragers from the CE 1 contained significantly lower amounts of all changed transmitters. Only the differences between the 2017 and the other two years are shown.

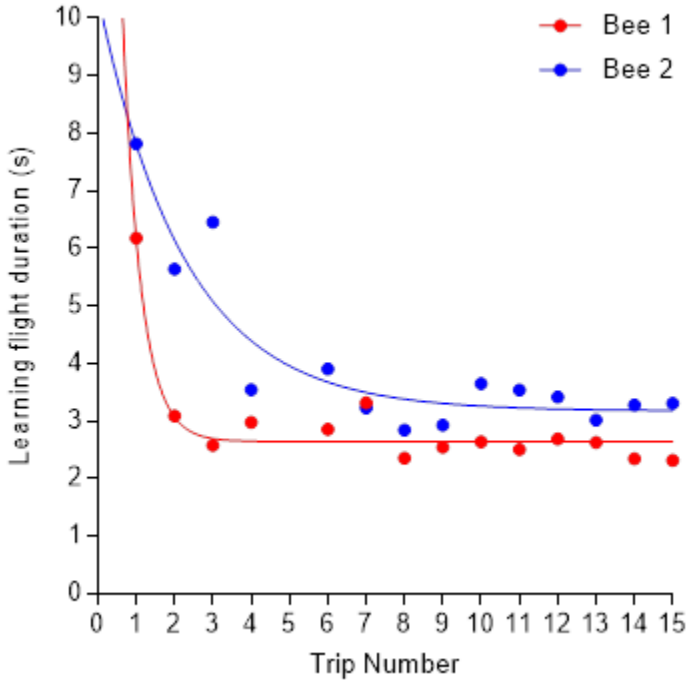


Figure S5. Learning flights. A learning flight is a specific flight pattern the forager performs after leaving the feeder prior to the vector flight to the hive. It is described as a "turn back and look" event during which the forager departing from the feeder makes consecutive circles in air with increasing radius and height for ~ 8-10 sec to examine the location to acquire the visual and landmark information to guide her return (Lehrer, 1991; Wei et al., 2002; Wei and Dyer, 2009). This behavior is an example of "backwards conditioning" where learning is followed after the reward is received and is essential since bees could not find their way back to hive without a learning flight (Lehrer, 1993). In a single shift experiment (200m-300m), the durations of the learning flights were analyzed for two foragers (red and blue). Learning flight duration decreased with successive trips to the new feeder location (unpublished data). We also found foragers making learning flights when they departed from a re-established feeder at the same location (personal observation) which might indicate re-learning the feeder location with respect to the environment around it.

Supplementary Tables

Table S1. Classification of outbound and inbound flights

Classification	What do the foragers know?	
	Outbound flight	**Inbound flight**
Foraging (F)	Present	Present
Foraging + Search (FS)	Present	Absent
Search Flight (SF)	Absent	Absent

Table S2. Summary of collection experiments

Colony	Year (Experiment)	Number of bees analysed (CB/OL)			
		F	FS	S1-S2	R
1	2017 (CE 1)	11/12	11/12	6/7	8/8
2	2018 (CE 2)	23/23	11/10	15/16	8/8
3	2019 (CE 3)	7/8	1/1	3/3	2/2

Table S3. Comparative time scale associated with search behavior

Comparative time scale associated with search behavior	Reference
Hive to hive trip to shut feeder (60 m*): < 5 min Dance guided search trip: 17 ± 11 min (mean ± sd)	Seeley, 1983
Homing flight duration of foragers captured at a feeder (10 m) and displaced to a release site (200 - 250 m): 61.78 ± 84.36 min (mean ± sd), range: 2.13 – 121.43 min	Reynolds et.al., 2007a
Search flight duration of foragers for a missing feeder (210 m): 4.49 ± 2.44 min (mean ± sd)	Reynolds et.al., 2007b
First return trip of foragers to the hive following downshift of reward (within a flight cage 35 m long): < 25 min Time spent within hive: 25.6 ± 2.8 (mean ± se)	Townsend-Mehler and Dyer, 2012
Visit-persistence to an emptied feeder (20m, 450m): 4.29 ± 4.47 trips (mean ± sd), range: 0 – 25 over a 6 hour /day	Al Toufailia et al., 2013
Hive to hive duration for a missing feeder (300 m): Foraging + Combined foraging/search flight (FS): 9.2 ± 4.5 min Search flights (S): 8.31 ± 3.5 min (mean ± sd)	Chatterjee et.al., 2019

*The distance of the feeder is in meters from the hive.

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