Mushroom bodies of the honeybee brain show cell population-specific plasticity in expression of amine-receptor genes

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Dopamine and octopamine released in the mushroom bodies of the insect brain play a critical role in the formation of aversive and appetitive memories, respectively. As recent evidence suggests a complex relationship between the effects of these two amines on the output of mushroom body circuits, we compared the expression of dopamine- and octopamine-receptor genes in three major subpopulations of mushroom body intrinsic neurons (Kenyon cells). Using the brain of the honeybee, Apis mellifera, we found that expression of amine-receptor genes differs markedly across Kenyon cell subpopulations. We found, in addition, that levels of expression of these genes change dramatically during the lifetime of the bee and that shifts in expression are cell population-specific. Differential expression of amine-receptor genes in mushroom body neurons and the plasticity that exists at this level are features largely ignored in current models of mushroom body function. However, our results are consistent with the growing body of evidence that short- and long-term olfactory memories form in different regions of the mushroom bodies of the brain and that there is functional compartmentalization of the modulatory inputs to this multifunctional brain center.

Mushroom bodies (MBs) of the insect brain play a critical role in the formation and recall of associative olfactory memories (for reviews, see Menzel 2001; Heisenberg 2003; Davis 2005; Keene and Waddell 2007). During aversive learning, dopamine (DA) released at this level conveys the reinforcing properties of aversive stimuli (Schwärrzel et al. 2003; Riemensperger et al. 2005; Schroll et al. 2006; Vergoz et al. 2007; Claridge-Chang et al. 2009). Food rewards, on the other hand, activate modulatory neurons that release octopamine (OA), which supports the formation of appetitive memories (Hammer and Menzel 1998; Menzel et al. 1999; Schwa¨rzel et al. 2006; Vergoz et al. 2007; Claridge-Chang et al. 2009). Food rewards, on the other hand, activate modulatory neurons that release octopamine (OA), which supports the formation of appetitive memories (Hammer and Menzel 1998; Menzel et al. 1999; Schwa¨rzel et al. 2006; Vergoz et al. 2007; Claridge-Chang et al. 2009).

Interestingly, abnormal expression of the Drosophila DA receptor dDA1 not only impairs appetitive learning but also appetitive learning (Kim et al. 2007), and in bees and flies, appetitive memory recall can be suppressed by DA (Menzel and Menzel 1982; Krashes et al. 2009). Treatment with OA has recently been shown also to reduce avoidance learning behavior in the bee (Agarwal et al. 2011). These results suggest a complex interplay between the effects of DA and OA on MB circuits and ultimately on learning performance, but the nature of this interplay remains unclear. To begin to address this issue, we have examined in this study patterns of expression of DA and OA receptor genes in MBs of the honeybee, Apis mellifera.

MB intrinsic neurons (Kenyon cells) in the honeybee express three DA-receptor genes, Amdop1 (Blenau et al. 1998; Kurshan et al. 2003), Amdop2 (Humphries et al. 2003; Kurshan et al. 2003), and Amdop3 (Beggs et al. 2005). To date, only one OA receptor gene, Amoa1, has been characterized and identified in this region of the brain (Grohmann et al. 2003); however, several putative OA receptors have been identified in the honeybee genome (Hauser et al. 2006) and are currently being characterized. Amdop1, Amdop2, and Amoa1 code for G-protein-coupled receptors that increase intracellular levels of cAMP when activated (Blenau et al. 1998; Grohmann et al. 2003; Humphries et al. 2003; Mustard et al. 2003, 2005; Beggs and Mercer 2009), whereas activation of the Amdop3 receptor generally reduces intracellular levels of cAMP (Beggs et al. 2005; Clark and Baro 2007; Beggs and Mercer 2009). Increases in cAMP levels resulting from Amdop3 receptor activation can be observed also under some conditions (Clark and Baro 2007), and AmOA1 receptors and the dopamine receptor, Amdop2, signal not only via cAMP but also through changes in intracellular Ca2+ (Grohmann et al. 2003; Beggs et al. 2011).

The diversity of receptor types mediating the effects of DA and OA in the insect brain suggests that responses to these amines will vary depending on the complement of receptors expressed by target cells. As differential expression of amine-receptor genes in MB neurons could have important functional implications, we compared the levels of DA- and OA-receptor gene expression in three major subpopulations of MB Kenyon cells and examined the extent to which shifts in the expression of amine-receptor genes in this important learning and memory center are cell population-specific.

Results

Age-related changes in amine-receptor gene expression

In whole-brain samples, significant shifts in expression levels were identified for Amdop1 (Fig. 1A), Amdop2 (Fig. 1B), Amdop3 (Fig. 1C), and Amoa1 (Fig. 1D). Data were normalized using Rpn2 and Rps8 (see Fig. 2B,C), but similar results were also obtained when 18S was used as an alternative stable reference gene (Fig. 1E–H). Data obtained for candidate reference genes tested are shown in Figure 2. Each of the amine-receptor genes examined in this study exhibited a unique expression profile. Post hoc analyses revealed that levels of Amdop1 mRNA in whole-brain samples were significantly lower in 15-d-old bees than in adult bees of all other ages examined (Fisher’s least significant difference [LSD], P < 0.05)

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Amdop2 transcript levels, on the other hand, were remarkably stable over the first 15 d of adult life but were significantly higher in whole-brain samples from pollen foragers than in all of the other groups examined (Fisher’s LSD, \( P < 0.05 \)) (Fig. 1B). Levels of expression of Amdop3 in the brain increased significantly during the first 2 d of adult life (linear mixed-effects model [LMM], \( P < 0.001 \)) and were higher in pollen forager bees when compared to 2-d-olds (\( P < 0.05 \)) but did not show an obvious change correlated with foraging (Fig. 1C). Overall, the expression profile of Amoa1 was most similar to that of Amdop2 (cf. Fig. 1, B and D); levels of expression of Amoa1 in the brains of 4-, 6- and 15-d-olds were significantly lower than the level detected in the brains of pollen foragers (Fisher’s LSD, \( P < 0.05 \)).

Similar changes in receptor-gene expression were apparent also in the brains of bees collected and analyzed the following summer (Fig. 3A–D). Consistent with the data set collected during the first summer, we found no significant differences in levels of Amdop1 expression between newly emerged (NE) bees, 6-d-olds, and pollen foragers (Fig. 3A). Based on the first data set, we predicted also that levels of expression of Amdop2 and Amoa1 would be higher in the brains of pollen foragers than in 6-d-old bees. While this prediction held true for Amoa1 (Fisher’s LSD, \( P < 0.05 \)) (Fig. 3D), it did not hold true for Amdop2. In the second data set, the level of expression of Amdop2 in pollen foragers was similar to that detected in 6-d-olds (Fig. 3B). In brain samples collected during the second summer, as in the first, expression of Amdop3 was significantly higher in the brains of 6-d-olds than in NE bees (Fisher’s LSD, \( P < 0.05 \)) (Fig. 3C). The level of Amoa1 expression detected in the brains of 6-d-olds was significantly lower than the level detected in NE bees (Fisher’s LSD, \( P < 0.05 \)) (Fig. 3D), a result that was also consistent with trends apparent in the first data set.

### Amine-receptor gene expression in MB Kenyon cells

The analyses outlined above revealed significant changes in amine-receptor gene expression in the brain, not only early in adult life but also correlated with a transition to duties outside the colony (pollen foraging). For this reason, we conducted two independent experiments to look for changes in levels of receptor-gene expression in subpopulations of MB neurons. To reveal potential shifts in gene expression occurring early in adult life, we compared levels of amine-receptor gene expression in NE bees and 15-d-olds (Fig. 4). To examine changes in gene expression that may be associated with the transition from duties within (Fig. 1A). Amdop2 transcript levels, on the other hand, were remarkably stable over the first 15 d of adult life but were significantly higher in whole-brain samples from pollen foragers than in all of the other groups examined (Fisher’s LSD \( P < 0.05 \)) (Fig. 1B). Levels of expression of Amdop3 in the brain increased significantly during the first 2 d of adult life (linear mixed-effects model [LMM], \( P < 0.001 \)) and were higher in pollen forager bees when compared to 2-d-olds (\( P < 0.05 \)) but did not show an obvious change correlated with foraging (Fig. 1C). Overall, the expression profile of Amoa1 was most similar to that of Amdop2 (cf. Fig. 1, B and D); levels of expression of Amoa1 in the brains of 4-, 6- and 15-d-olds were significantly lower than the level detected in the brains of pollen foragers (Fisher’s LSD, \( P < 0.05 \)).

Similar changes in receptor-gene expression were apparent also in the brains of bees collected and analyzed the following summer (Fig. 3A–D). Consistent with the data set collected during

![Figure 1](image1.png)

**Figure 1.** Amine-receptor gene expression levels in the adult bee brain from emergence to foraging age. (A–D) Expression levels normalized using the geometric mean of Rpn2 and Rps8. Statistics (LMM): Amdop1, age \( P < 0.0001 \); Amdop2, age \( P < 0.0001 \); Amdop3, age \( P < 0.0001 \); Amoa1, age \( P < 0.0001 \). (E–H) Data normalized using 18S. Statistics (LMM): Amdop1, age \( P < 0.0001 \); Amdop2, age \( P < 0.0001 \); Amdop3, age \( P < 0.0001 \); Amoa1, age \( P < 0.0001 \). Bars that do not share a letter differ significantly (Fisher’s LSD, \( P \leq 0.05 \)). Data are presented as mean ± standard error. Numbers in parentheses indicate sample size. (NE) Newly emerged bees, (PF) pollen foragers.

![Figure 2](image2.png)

**Figure 2.** Levels of expression in the brain of candidate reference genes. Bars indicate means ± standard error. Numbers in parentheses indicate sample size. Asterisks indicate the most stable pair of genes as determined using Normfinder. Rpn2 and Rps8 were used to normalize gene expression levels in this study. (NE) Newly emerged bees, (PF) pollen foragers.
Amdop1 Analysis of ICC, NCC, and OCC in the MBs of NE adults and Shifts in gene expression early in adult life

compact cells (ICC), and noncompact cells (NCC) (see Fig. 6Ai). Kenyon cell populations as outer compact cells (OCC), inner-

the terminology introduced by Farris et al. (2004), we refer to these of Kenyon cells in the honeybee (Fig. 6A–D), we compared (where

zation analysis (Fig. 6) suggested to us that the genes of interest with levels detected in pollen foragers (Fig. 5). As in situ hybridi-

the hive to foraging, we compared expression levels in 6-d-olds (LMM): (Fig. 4B) early in adult life. Levels of Amdop1 expression in young bees did not vary significantly across the three subpopu-

lations of MB neurons (LMM, P = 0.0022) (Fig. 4B) early in adult life. Levels of Amdop1 expression in young bees did not vary significantly across the three subpopulations of Kenyon cells in the honeybee (Fig. 6A–D), we compared (where possible) levels of expression in all three cell types. Consistent with the terminology introduced by Farris et al. (2004), we refer to these Kenyon cell populations as outer compact cells (OCC), inner-compact cells (ICC), and noncompact cells (NCC) (see Fig. 6Ai).

Shifts in gene expression early in adult life

Analysis of ICC, NCC, and OCC in the MBs of NE adults and 15-d-old bees revealed significant changes in expression of Amdop1 (LMM, P < 0.0001) (Fig. 4A) and Amdop2 (LMM, P = 0.0022) (Fig. 4B) early in adult life. Levels of Amdop1 expression in young bees did not vary significantly across the three subpopulations of MB neurons (LMM, P = 0.3093), but in all three cell groups Amdop1 expression levels were lower in 15-d-olds than in NE bees (Fisher's LSD, P < 0.05). No significant interaction was identified in this data set between cell type and age (LMM, P = 0.141). Amdop2 expression levels (Fig. 4B), like those of Amdop1 (Fig. 4A), were significantly higher in the ICC of NE adults than in the ICC of 15-d-olds (Fisher's LSD, P < 0.05). In contrast to Amdop1, however, levels of expression of Amdop2 tended to be lower in OCC and NCC of NE bees than in 15-d-olds. While differences in Amdop2 expression levels across the three subpopulations of Kenyon cells were not statistically significant (LMM, P = 0.1439), our analysis identified a significant interaction between cell type and age in levels of expression of Amdop2 in the MBs of young (NE and 15-d-old) bees (LMM, P < 0.0001).

In contrast to Amdop1 and Amdop2, levels of expression of Amdop3 in MB Kenyon cells early in adult life were clearly influ-

enced by cell type (LMM, P < 0.0001), and a strong interaction was identified between cell type and age (LMM, P < 0.0001). In NCC, levels of Amdop3 expression were significantly higher in 15-d-olds than in NE bees (Fisher's LSD, P < 0.05), whereas in the OCC and ICC of NE bees and 15-d-olds, Amdop3 expression levels were remarkably similar (Fig. 4C). A strong overall trend suggesting that levels of Amoa1 expression in the MBs may be influenced by age and/or cell type encouraged us to undertake post hoc analyses also on data obtained for Amoa1 (Fig. 4D). While Amoa1 expression levels in the OCC of 15-d-olds were lower than those detected in the OCC of NE bees (Fisher's LSD, P < 0.05), no age-related differences in the levels of expression of this gene were detected in NCC or ICC. Analysis of the data obtained for Amoa1 confirmed a significant overall effect of cell type on levels of expression of Amoa1 (LMM, P < 0.0001) and, in addition, a strong interaction between cell type and age (LMM, P = 0.0045).

Amine-receptor gene expression and the shift to foraging

Comparisons were made also between levels of amine-receptor gene expression in the ICC and NCC of 6-d-old bees and pollen foragers (Fig. 5). While the results revealed few age-related differences in this data set (Fig. 5A–D), clear differences were apparent between the levels of gene expression detected in the two subpopulations of MB neurons. In 6-d-olds (Fisher's LSD, P < 0.05) and in pollen foragers (Fisher's LSD, P < 0.05), levels of expression of Amdop3 were significantly higher in NCC than in ICC, whereas levels of expression of Amoa1 were higher in ICC than in NCC, both in 6-d-olds (Fisher's LSD, P < 0.05) and in pollen foragers (Fisher's LSD, P < 0.05). In contrast to Amdop3 and Amoa1, levels of Amdop2 expression in ICC and NCC were remarkably similar in 6-d-old bees and in pollen foragers (Fig. 5A). The same was true of levels of Amdop2 expression in ICC and NCC of 6-d-old bees (Fisher's LSD, P < 0.05). In pollen foragers, Amdop2 expression was higher in ICC than in NCC (Fisher's LSD, P < 0.05). Arguably the most complex changes in gene expression at the level of the MB were those detected for Amdop2 (cf. Fig. 4 and Fig. 5).

Discussion

Our data show that each of the genes investigated in this study exhibits a unique pattern of expression in the MBs, a property that generates striking differences in the expression of amine-receptor genes across subpopulations of MB neurons. In light of this

www.learnmem.org 153 Learning & Memory
heterogeneity, it is, perhaps, unsurprising that shifts in gene expression in whole-brain samples proved to be a poor indicator of changes occurring in the MB of the brain. Differential expression of amine-receptor genes at the level of the MBs is, nonetheless, consistent with a growing body of evidence that subpopulations of Kenyon cell subpopulations exhibit distinct DA and OA receptor expression profiles and that there is significant spatial and temporal plasticity in the levels of expression of amine-receptor genes in the MBs of the bee. Analysis of predictable shifts in receptor expression promises insights into the functional significance of subpopulations of MB neurons and the role of the amine receptors in this important learning center of the brain.

Materials and Methods
Selection of age groups
To determine which age groups to examine in detail, we began by examining age-related changes in levels of expression of amine-receptor genes in the whole brain of worker bees. Three honeybee colonies permanently housed at the University of Otago Department of Zoology were used to source brood frames with emerging adult worker bees. Brood frames were held overnight in a humidified incubator at 34°C. The following morning, bees that had emerged overnight were collected (typically 100–200 bees), cold-anesthetized, and marked on the thorax with nontoxic acrylic paint. Once the marked bees had recovered from chilling, they were returned to their parent colony for 2, 4, 6, or 15 d. Newly emerged bees (NE) were obtained from frames cleared at 8:30 a.m. and then left for 1.5–2 h to accumulate new bees. Pollen foragers were captured at the hive entrance and identified by the presence of full pollen baskets on the hind legs. Whole-brain samples were collected from NE, 2-d-old, 4-d-old, 6-d-old, and pollen forager bees (10 bees/sample). To determine how consistent changes in expression were from year to year and
whether changes in expression could be detected using individual brains rather than pooled tissue, a second set of samples was collected and analyzed the following summer. During the second summer, whole brains were collected from NE bees, 6-d-olds, and pollen foragers, and the brain of each bee was analyzed separately.

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was used to investigate age-related changes in levels of expression of amine-receptor genes in whole-brain samples, as well as in subpopulations of MB neurons. Whole brains were dissected as quickly as possible, taking care to remove eye pigment and glandular tissue surrounding the brain. The brains were rinsed in bee saline (154 mM NaCl, 2 mM NaH2PO4, 5.5 mM Na2HPO4, pH 7.2), frozen on dry ice, and stored at −80°C until further processing. Total RNA was extracted by homogenization of brain tissue in Trizol and isolated using Micro-Midi purification columns (Invitrogen). RNA concentrations were measured using a NanoDrop ND-100 spectrophotometer (Invitrogen). qPCR analysis was performed as previously described (Vergoz et al. 2009). Briefly, 100 ng of RNA from individual brains or 200 ng of RNA from samples of pooled brains was reverse-transcribed using VILO SuperScript (Invitrogen). Gene-specific amplification products were generated using ExpressSYBR GreenER qPCR SuperMix (Invitrogen) and gene-specific primer pairs. Assay efficiencies were derived from standard curves generated using cDNA reverse-transcribed from a pool of the experimental RNA, or from a random whole-brain sample if cDNA was limited. Primer pairs, assay efficiencies, and melting product temperatures are provided in Table 1. Receptor transcript abundances were determined using the ΔΔCt method with assay efficiencies incorporated in the following formula: Normalized = (1 + Efficiency target, −ΔCt target) / (1 + Efficiency reference, −ΔCt reference). For whole-brain analysis, transcript levels were normalized using the geometric mean of two reference genes, ribophorin II (Rpn2) and ribosomal protein s8 (Rps8). Using the software packages geNorm (Vandesompele et al. 2002), BestKeeper (version 1; Pfaffl et al. 2004), and NormFinder (version 0.953; Andersen et al. 2004), these reference genes were found to be the most stable combination based on a selection of six candidates, which also included 18S, ribosomal protein 49 (rp49), elongation factor-1 alpha (ef1α), and hydroxymethylbilane synthase (hmbs) (see Table 2). Generally triplicates, but occasionally duplicates, were run of each sample.

In situ hybridization

In situ hybridization was used to examine the spatial pattern of expression of DA receptor genes across subpopulations of MB.
neurons and to determine how best to ensure that equivalent populations of cells were being examined in each age group (Fig. 6). Whole brains were dissected and fixed in 4% buffered paraformaldehyde at room temperature for 2 h and then at 4°C for an additional 20 h. Brains were then dehydrated through a graded series of ethanol and xylene, then embedded in paraffin. Sections of brain tissue (8 mm thick) were mounted on polysine slides (BDH Laboratory Supplies), and in situ hybridization was performed using ribo-probes at a concentration of 1 mg/μl. Hybridization was performed using digoxigenin (DIG)-labeled ribo-probes according to a protocol similar to that described by Braissant and Wahl (1998). Probes for Anndop1, Anndop2, and Anndop3 were synthesized as described previously (Beggs et al. 2005). Hybridization probes were detected immunologically using anti-DIG antibody. The antibody was diluted 1:5000 and applied to sections for 2 h. The sections were then washed in 450 mM NaCl in standard saline citrate (SSC) buffer, 95% ethanol (30 sec), 100% ethanol (1 min), followed by Xylene (5 min). The slides were then air dried for 5 min and used immediately for laser capture. During all steps, RNase-free solutions and handling techniques were used. Using the Leica AS LMD, a laser beam was used to isolate samples from each subpopulation of MB neurons, as shown in Figure 6E. Each sample included cells captured from between eight and 16 sections of the lateral and medial mushroom body calyces of one bee. Samples of ICC, NCC, and OCC were isolated from 3–5 bees per experimental group. Isolated cells were immediately frozen on dry ice, and RNA was isolated from the cells as soon as possible using an RNeasy Micro Kit (Qiagen). RNA quality and quantity were assessed using the 2100 expert bioanalyzer, a pico assay chip, and reagents (Agilent) according to the manufacturer’s protocols. As reported by Winnebeck et al. (2010), denatured honeybee RNA does not have a visible 28S rRNA peak due to the presence of a hidden band that leads to fragmentation of the 28S rRNA. To account for this, the 18S:28S threshold ratio anomaly setting was manually overridden to enable integrity values to be obtained. These were typically ≥ 6 for laser-captured RNA samples. Using 2 ng RNA from each sample, levels of expression of amine-receptor genes were analyzed using qPCR. Triplicates (or occasionally duplicates) were run of each sample, and transcript levels for laser capture microdissection experiments were normalized using eF1a which was found to be among the most stable in these samples (see Table 3).

### Statistical methods

All statistical analyses were conducted in the R environment (version 2.12.2; R Development Core Team 2011). LMM performed using the R function lmer in the package lme4 (R package version 0.999375-42, http://CRAN.R-project.org/package=lme4) was used to examine differences in levels of receptor-gene expression between age groups and between subpopulations of Kenyon cells (fixed factors); bee identities were included as a random factor. The use of LMM enabled us to increase statistical power by incorporating variation in replicates of the same sample; in other words, LMM used each replicate as a data point while at the same time accounting for nonindependence of related replicates (Nakagawa and Huaber 2011). It is noted that the calculation of the degrees of freedom (df) required for obtaining a P value in LMM is not straightforward (Bolker et al. 2009). Therefore, we provide alternative P values estimated by Markov Chain Monte Carlo (MCMC) sampling, implemented in the R function pvals.fnc in the package languageR for all LMM analyses (Baeyen et al. 2008).

### Table 1. Real-time qPCR primer designs and assay parameters

| Gene   | Primer designs | RE (%) | MP (°C) |
|--------|----------------|--------|---------|
| Anndop1| S′-TGACCACTGCTTCGAGTAT (forward) | 90 | 84 |
|        | S′-ACACACGGCACGTTCTGAG (reverse) | | |
|        | S′-CCGATGGCATTTTACCCGTGTTT (reverse) | | |
| Anndop2| S′-CCACCTTGAACGCTTCTCATC (forward) | 97 | 86 |
|        | S′-GGCCAGGACCCGATTGAGG (reverse) | | |
| Anndop3| S′-AGAACCCACAGCCTGCTCAAC (forward) | 94 | 80 |
|        | S′-GCAAAGCAGCCGTAAGG (reverse) | | |
| Amoal  | S′-GAGGCTGACGATACTACGTCTC (forward) | 100 | 84.5 |
|        | S′-GCCATGCTGCTGCTGCTGC (reverse) | | |
| 18S    | S′-GGCGCTTGAATAGGAACTACTTTG (reverse) | 90 | 80 |
|        | S′-GATTCGCTTGAATAGGAACTACTTTG (reverse) | | |
| Rpn2   | S′-CCGCTGATGAAAGGAAATAGGAA (forward) | 97 | 79 |
|        | S′-GCAAGGGTTGCTGCGTCTG (reverse) | | |
| Rpo8   | S′-CCACGTCGAGAATTCGACTGA (forward) | 97 | 80 |
|        | S′-GGACCTGCTGCTGCTGCTG (reverse) | | |
| ef1a   | S′-AAGAGCATCAAAGCGGGAGA (forward) | 91 | 87.2 |
|        | S′-CCTCTTTAAGACGCGCCACA (reverse) | | |
| rp49   | S′-GCGAACATCGGATTGCGCAAT (forward) | 96 | 76.5 |
|        | S′-CATGAGCAATTCGACCAACA (reverse) | | |
| Hmbs   | S′-CCTGATGATGAAAGGAACTTT (forward) | 91 | 78.5 |
|        | S′-GGATGCTGAGAATTCGACTGA (reverse) | | |

(Re) Reaction efficiency, (MP) product melting point.

### Table 2. Rankings of reference gene stability in the aging honeybee brain according to GeNorm, NormFinder, and BestKeeper analysis

| Stability | GeNorm* | NormFinder | BestKeeper |
|-----------|---------|------------|------------|
| Adult age series with reduced time points (n = 30) | | | |
| Highest | Rpn2 (0.295) | Hmbs (0.138) | 18S | Rpn2 (0.268) | Rpn2 (0.098) | 18S |
|         | Rpo8 (0.246) | Rpn2 (0.170) | Rpn2 | Rpn2 (0.286) | Rpn2 (0.115) | Rpn2 |
|         | 18S (0.300) | Rpo8 (0.236) | Rps8 | Rps8 (0.331) | Rps8 (0.147) | Rps8 |
|         | rp49 (0.313) | 18S (0.276) | rp49 | rp49 (0.301) | rp49 (0.140) | rp49 |
|         | ef1a (0.397) | rp49 (0.365) | hmbg | ef1a (0.301) | ef1a (0.147) | ef1a |
|         | lag (0.379) | hmbg (0.379) | hmbg | hmbg (0.306) | hmbg | hmbg |

Stability values and M values are shown in parentheses.
*M values shown indicate values calculated for the final top four genes only.
**Best combination of two genes indicated by NormFinder.

of MB intrinsic neurons were most clearly apparent (Fig. 6). The sections were briefly thaw-mounted onto 2-μm polyethylene naphthalate membrane slides (Leica), immediately reoriented on dry ice, and stored at −80°C until further processing. Prior to the capture of cell populations, the sections were thawed, fixed in 75% ethanol (30 sec), and briefly washed in MilliQ water and then in PBS (10 sec). The sections were then stained in 0.5% cresyl violet acetate in 0.1M Naacetate buffer (30 sec) and briefly rinsed twice in MilliQ water before being dehydrated in 75% ethanol (30 sec), 95% ethanol (30 sec), and 100% ethanol (1 min), followed by Xylene (5 min). The slides were then air dried for 5 min and used immediately for laser capture. During all steps, RNase-free solutions and handling techniques were used. Using the Leica AS LMD, a laser beam was used to isolate samples from each subpopulation of MB neurons, as shown in Figure 6E. Each sample included cells captured from between eight and 16 sections of the lateral and medial mushroom body calyces of one bee. Samples of ICC, NCC, and OCC were isolated from 3–5 bees per experimental group. Isolated cells were immediately frozen on dry ice, and RNA was isolated from the cells as soon as possible using an RNeasy Micro Kit (Qiagen). RNA quality and quantity were assessed using the 2100 expert bioanalyzer, a pico assay chip, and reagents (Agilent) according to the manufacturer’s protocols. As reported by Winnebeck et al. (2010), denatured honeybee RNA does not have a visible 28S rRNA peak due to the presence of a hidden band that leads to fragmentation of the 28S rRNA. To account for this, the 18S:28S threshold ratio anomaly setting was manually overridden to enable integrity values to be obtained. These were typically ≥ 6 for laser-captured RNA samples. Using 2 ng RNA from each sample, levels of expression of amine-receptor genes were analyzed using qPCR. Triplicates (or occasionally duplicates) were run of each sample, and transcript levels for laser capture microdissection experiments were normalized using eF1a which was found to be among the most stable in these samples (see Table 3).

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Table 3. Rankings of reference gene stability in laser-capture dissected Kenyon cells of newly emerged (NE) and pollen forager (PF) bees

| Stability | GeNorm* | Normfinder | BestKeeper |
|----------|---------|------------|------------|
| Highest  | Rpn2 (0.161) | Rpn2 (0.076) | Rps8       |
|          | ef1α (0.161) | ef1α (0.078) | ef1α       |
| Lowest   | Rps8     | Rps8 (0.143) | ef1α       |

Stability values and M values are shown in parentheses. *M values shown indicate values calculated for the final top two genes only. **Best combination of two genes indicated by Normfinder.

For post-hoc analyses of pairwise differences, we used Fisher’s least significant difference implemented by the R function, glht in the package, multcomp (Bretz et al. 2011).

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