protein as a ligand involved in specifying the termination sites of axon branches.

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Gene Expression Profiles in the Brain Predict Behavior in Individual Honey Bees

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We show that the age-related transition by adult honey bees from hive work to foraging is associated with changes in messenger RNA abundance in the brain for 39% of ~5500 genes tested. This result, discovered using a highly replicated experimental design involving 72 microarrays, demonstrates more extensive genomic plasticity in the adult brain than has yet been shown. Experimental manipulations that uncouple behavior and age revealed that messenger RNA changes were primarily associated with behavior. Individual brain messenger RNA profiles correctly predicted the behavior of 57 out of 60 bees, indicating a robust association between brain gene expression in the individual and naturally occurring behavioral plasticity.

Behavior in nature arises through the interaction of an individual with its environment. Several forms of animal behavior are known to be influenced by the activity of specific genes (*1, 2*). Microarray studies have been used to relate changes in behavior with changes in gene expression in the brain (*3–7*). These studies identified average trends for groups of animals, but did not examine gene expression in individuals. But identifying average differences between groups is not the same as identifying differences that can reliably predict behavior in individuals (*8*). This is especially the case for behavior performed outside of a laboratory setting where the experiences of each individual can vary widely. To predict behavior from gene expression profiles in a natural context would demonstrate a more robust relation between genes and behavior than is commonly thought to exist (*9*).

We used the honey bee (*Apis mellifera*) to examine the relation between gene expression profiles in individual brains and behavior. Honey bee workers are well suited for this purpose because they exhibit strong and stable differences in behavior between individuals, and they can be readily studied under naturalistic conditions. Behavioral differences arise as part of a system of age-related, socially regulated division of labor common to species of social insects (*10*). Bees perform several different tasks in the hive during the first 2 to 3 weeks of adult life, including brood care ("nursing"), and then shift to foraging for nectar and pollen outside the hive for the remainder of their 5- to 7-week life. Like other forms of behavioral plasticity, such as social dominance and sexual behavior in vertebrates (*11, 12*), the transition to foraging in the honey bee involves long-term, environmentally modulated changes in behavior that are associated with changes in brain structure, brain neurochemistry, and expression in the brain of at least a few genes (*13, 14*). However, the extent of gene expression changes associated with bee behavioral plasticity is not known (*15*).

Individual bees vary in several ways in how they perform their jobs, due to both intrinsic factors and changes in the environment that affect colony needs. For example, foragers fly to food sources located at distances from several meters to several kilometers from the hive, under variable weather conditions, and may specialize on collecting pollen or nectar, or on one or more floral species (*10*). Both nurses and foragers vary in performance tempo, with some individuals working harder than others (*16*). The timing of the transition from hive work to foraging is itself variable and may be accelerated, delayed, or reversed depending on the needs of the colony (*13*).

We measured genome-wide gene expression in individual dissected brains from nurses and foragers using a total of 72 microarrays (*17*). The microarrays were made from a collection of >20,000 cDNAs generated from a bee brain expressed sequence tag (EST) project (*18*). A total of 6878 cDNAs were analyzed in this study, estimated to represent ~5500 different genes (perhaps ~40% of genes in the honey bee genome) (*17, 18*). To minimize the effect of genetic variation, full-sister nurses and foragers were collected in equal numbers from several genetic sources [full sisters are 75% related due to haplodiploidy (*10*)]. Statistical power was maximized (*19–21*) by pairing individual nurse and forager brains and comparing them directly (fig. S1; each brain was analyzed on either two or four microarrays). Microarray hybridization data were analyzed with Bayesian statistics (*20*) and analysis of variance (ANOVA) (*19*). Our initial analyses were designed to describe group tendencies, before examining individual profiles.

We first measured gene expression in the brains of nurses and foragers exhibiting age-typical behavior from "typical" colonies. Nurses (5 to 9 days old) and foragers (28 to 32 days old) showed significant differences in brain gene expression for 39% (2670) of microarray cDNAs (Bayesian analysis; $P < 0.01$, $n = 18$ individuals per group) (table S1). This number is 39 times larger than the 69 false-positives expected under the null hypothesis when $P < 0.01$. Genes identified by Bayesian analysis were consistent with those identified by ANOVA (table S2). Although many genes exhibited highly significant differences in expression, the magnitude

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of these differences was typically small (Fig. 1A) (only 24 cDNAs showed average differences greater than twofold). Significant differences of small magnitude may reflect subtle modulation by widely diffusible neuroendocrine factors acting over large parts of the brain, or may reflect large differences in particular neural subpopulations.

Because foragers are older than nurses in a typical population, examination of bees from typical colonies did not reveal whether gene expression differences are associated with behavior or with age. To resolve this question, we created "single-cohort" colonies initially composed entirely of young bees (17); in the absence of old bees, some individuals initiate foraging as much as 2 weeks earlier than usual (13). Subsequently, as the entire worker population ages, the lack of young bees causes some individuals to continue working as nurses despite advancing chronological age. We obtained individual brain gene expression profiles for age-matched young nurses and young ("precocious") foragers, and age-matched old foragers and old ("overage") nurses (n = 6 individuals per group, 24 total). These four groups were analyzed in conjunction with nurses and foragers from typical colonies (above), for a total of six sample groups, from two colony types, represented by 60 individuals.

Results of two analyses revealed a strong association between brain gene expression and behavior. Hierarchical clustering (22) showed that the important distinguishing characteristic of the mean expression profiles for the six groups was behavior (nurse or forager), not age (Fig. 1B). Principal component analysis (22) revealed that one pattern of gene expression (PC1) dominated, accounting for 49% of the variance among the six groups (Fig. 1C). This dominant pattern of expression was clearly associated with behavior. Another pattern of expression, accounting for 25% of the variance among the six groups (PC2), was associated with differences in age and colony environment (typical versus single-cohort colonies).

Do extensive but mostly subtle differences in brain gene expression between nurses and foragers reflect only group tendencies, or are they representative of the brain profiles of individual bees? Visual inspection of brain gene expression profiles for the 60 bees (Fig. 2A) suggests that most individuals can be readily distinguished as nurse or forager, irrespective of age, genetic source, colony type, or (unknown) prior experience. To formally test whether gene expression profiles in individual bee brains can predict behavior, we employed two methods used to classify tumor types from microarray-generated expression profiles (22, 23).

"Leave-one-out" cross-validated class prediction accurately classified single brain expression profiles as nurse or forager for 95% of the individuals (57 out of 60) (Fig. 2B). Accurate classification did not depend on genes with large expression differences. Correct behavioral classification was high (92%; 55 out of 60) even when we excluded all genes showing greater than 1.5-fold mean expression difference between nurses and foragers (Fig. 2B, rightmost column). This result indicates that at least some expression differences of less than 1.5-fold are robustly associated with behavioral differences in individuals. Using an arbitrary fold-difference criterion (e.g., twofold) to interpret microarray data thus risks overlooking changes that might be important (21). Similar results were obtained with principal component analysis, this time applied to the 60 individual (not average group) expression profiles; individual nurses and foragers were partitioned into two separate groups with the exception of only three foragers (Fig. 2C).

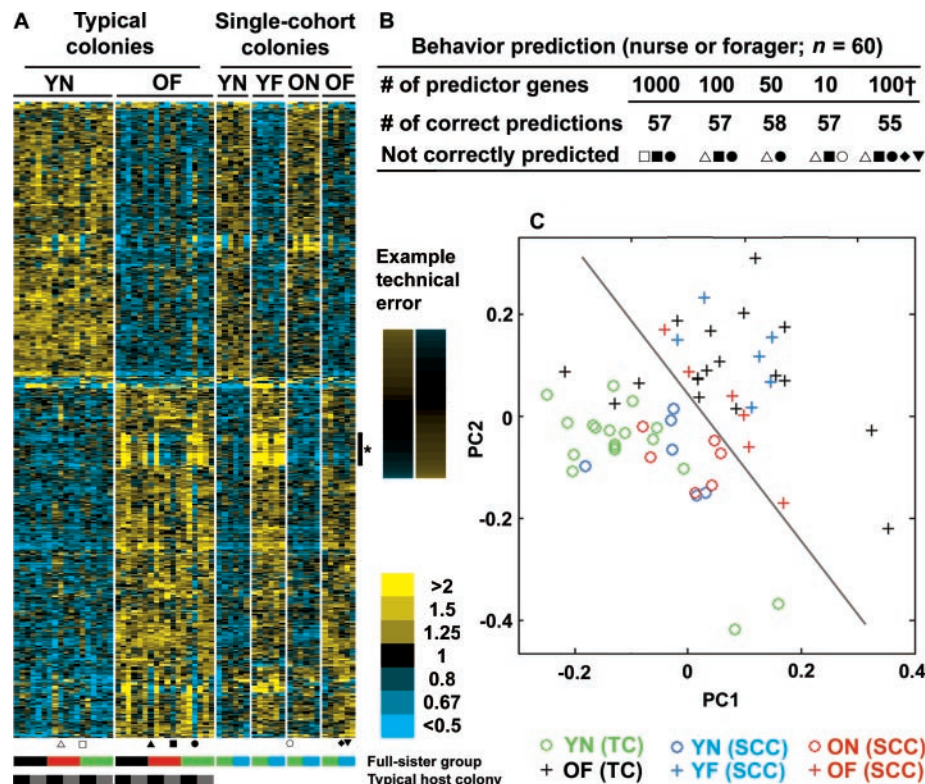In addition to their intrinsic value in cancer studies for prognostication and treatment, class prediction analyses have increased our understanding of how distinct classes of tumors can arise despite essentially infinite variation in tumor genotype and environment (23). Of the thousands of genes differentially expressed between tumors, only relatively small numbers of genes (~6 to 70) are necessary to successfully discriminate between classes of tumors, and identification of these "predictor" genes has led to important insights into the cellular mechanisms that underlie particular types of cancer (23). Like tumors, behavior is a product of genotype and environment and is unique for each individual. Despite the individuality of behavior, distinct classes of behavior (both normal and pathological) can be recognized in humans and animals. Class prediction analysis—applied here to a microarray study of behavior—readily identified a set of predictor genes expressed in the brain that could discriminate between two classes of behavior in the honey bee. Genes that predict behavior in this way might provide new insights into neural mechanisms in the brain that underlie behavioral plasticity.
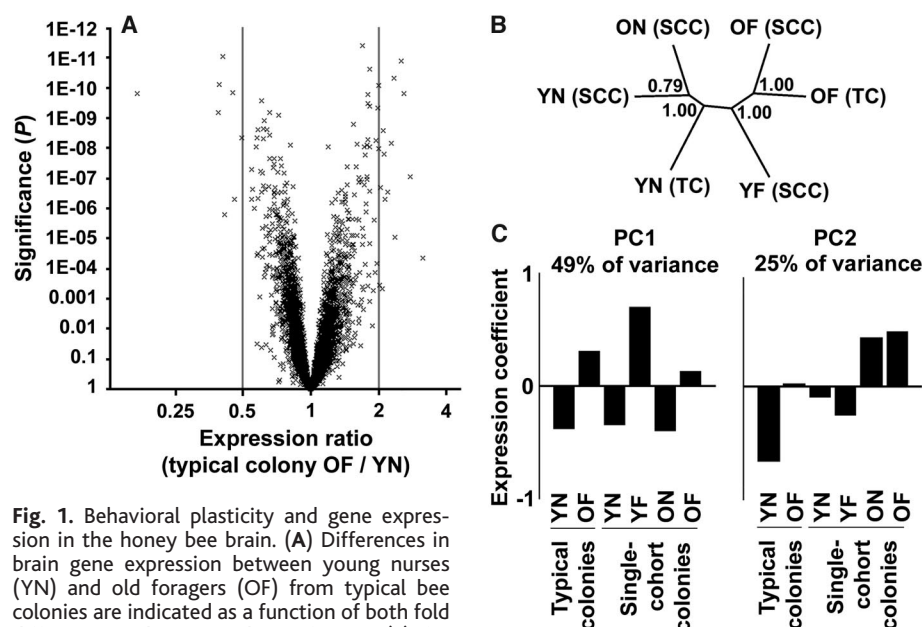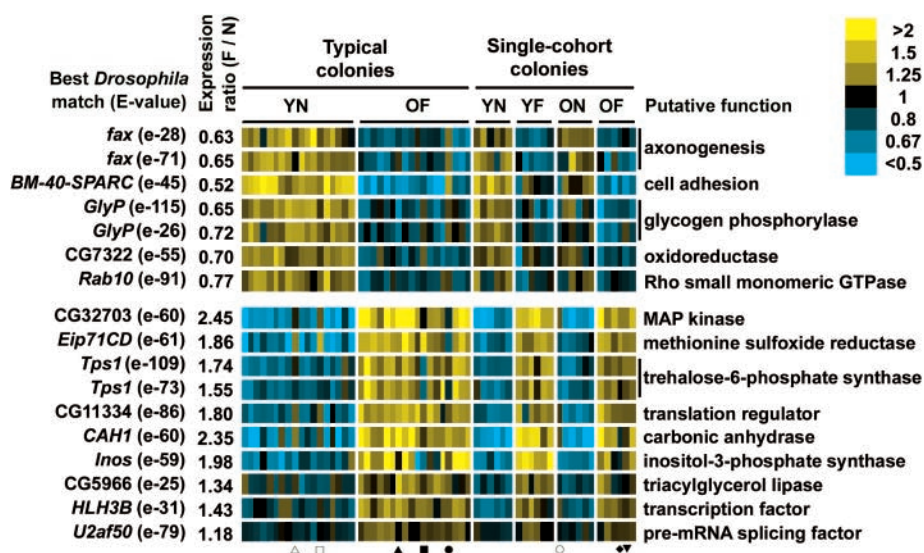
Fig. 1. Behavioral plasticity and gene expression in the honey bee brain. (**A**) Differences in brain gene expression between young nurses (YN) and old foragers (OF) from typical bee colonies are indicated as a function of both fold difference and statistical significance (P) for each of the 6878 cDNAs on the microarray (tabulated P-value from ANOVA; n = 18 individuals per group; similar plot for Bayesian analysis is not shown because this method estimates P-values only to the second decimal place). Vertical lines indicate twofold expression difference in either direction. (**B** and **C**) Single-cohort colonies were used to dissociate behavior and age (see text). Statistical analyses were performed on mean expression levels of all 6878 cDNAs in each of six experimental groups: young nurses and old foragers from typical colonies (TC; analyzed in Fig. 1A), and young nurses, young foragers (YF), old nurses (ON), and old foragers from single-cohort colonies (SCC; n = 6 individuals per group). (B) Hierarchical clustering indicated that behavioral phenotype was more important than age in separating expression profiles in the six groups. Numbers indicate bootstrap support (from 100 iterations) for each node, and branch lengths indicate Euclidian distances (UPGMA). (C) Principal component analysis indicated that the most important pattern of brain gene expression (PC1, accounting for 49% of variance in the data) was associated primarily with differences in behavior. A second pattern (PC2; 25% of variance) was associated with differences in both age and colony type. Bar graphs depict PCs in a sign-independent manner; i.e., PC1 reflects both genes up-regulated in foragers and genes up-regulated in nurses (across age groups and colony types). Methods are given in (17).

Fig. 2. Individual brain gene expression profiles predict behavioral phenotype. (**A**) Expression levels are indicated by color scale for each of the 60 individual bee brains. Sample group abbreviations are as in Fig. 1. Only the 548 cDNAs exhibiting >1.25-fold mean difference between nurses and foragers are shown (arbitrary fold criterion for graphic representation only). cDNAs are arranged on the *y* axis by hierarchical clustering (tree not shown). Asterisk (*) indicates a set of coordinately expressed chaperones up-regulated in some, but not all, foragers (listed in table S4). Groupings by genetic source (full-sisters) and host colony are indicated. Some individuals are identified (△□▲■●○◆▼) for classification analyses in (B) and (C), below. "Example technical error" is indicated for one representative experiment that measured relative expression in two biologically identical samples (derived from a homogenate of bee brains; see fig. S2). For analysis of technical error, cDNAs are ordered on the *y* axis by magnitude of error (i.e., measured fold difference between the two identical samples). (**B**) Cross-validated class prediction classified 95% of bees (*n* = 60) to the correct behavioral category (nurse or forager) on the basis of individual brain gene expression profiles. For each brain tested, "predictor genes" were selected with expression data from the other 59 brains (i.e., independently of the single "test" brain being classified). Predictor genes were selected from all 6878 cDNAs, except as indicated in the rightmost column (†), where all cDNAs that exhibited >1.5-fold mean expression difference between nurses and foragers in either direction were excluded. Individual nurses (△□○) and foragers (■●◆▼) not correctly classified are indicated for each analysis. Correct behavior prediction was not a function of age; bees with an experimentally induced age-behavior "mismatch" (YF and ON; *n* = 12) were correctly classified (with the exception of only one bee and just when only 10 predictor genes were used). (**C**) Principal component analysis was performed with expression levels for all 6878 cDNAs in all 60 individual brains. The 60 brains are plotted as a function of PC1 and PC2, which together accounted for 26% of variance in individual expression data. Individual nurses and foragers were partitioned into two separate groups (arbitrary line) with the exception of three foragers (▲■●). In contrast, clear partitioning of individuals by age group was not possible. Methods are given in (*17*).

Fig. 2 panels A, B, and C.

B. Behavior prediction (nurse or forager; *n* = 60)

# of predictor genes	1000	100	50	10	100†
# of correct predictions	57	57	58	57	55
Not correctly predicted	□■●	△■●	△●	△■○	△■●◆▼

Color scale: >2, 1.5, 1.25, 1, 0.8, 0.67, <0.5

YN (TC) ○, OF (TC) +, YN (SCC) ○, YF (SCC) +, ON (SCC) ○, OF (SCC) +

Fig. 3. Genes predictive for behavior in individuals. The 50 brain cDNAs most predictive for behavior (table S3) were determined with the same class prediction method used in Fig. 2B (using all 60 brains). These 50 cDNAs included 17 with strong sequence similarity (BLASTX; E-value <10^{-20}) to functionally annotated *Drosophila* genes (*34*). Expression of these 17 cDNAs in the 60 individual bee brains is indicated (color scale and individual identification as in Fig. 2A). Sample group abbreviations are in Fig. 1. Expression ratio (F/N) is average for all foragers and nurses (*n* = 30 per group). See fig. S4 for independent confirmation of selected gene expression results with quantitative RT-PCR. Expression changes in the gene similar to *BM-40-SPARC* were consistent with results of a previous study (*14*).

Best *Drosophila* match (E-value)	Expression ratio (F/N)	Putative function
fax (e-28)	0.63	axonogenesis
fax (e-71)	0.65	
BM-40-SPARC (e-45)	0.52	cell adhesion
GlyP (e-115)	0.65	glycogen phosphorylase
GlyP (e-26)	0.72	
CG7322 (e-55)	0.70	oxidoreductase
Rab10 (e-91)	0.77	Rho small monomeric GTPase
CG32703 (e-60)	2.45	MAP kinase
Eip71CD (e-61)	1.86	methionine sulfoxide reductase
Tps1 (e-109)	1.74	trehalose-6-phosphate synthase
Tps1 (e-73)	1.55	
CG11334 (e-86)	1.80	translation regulator
CAH1 (e-60)	2.35	carbonic anhydrase
Inos (e-59)	1.98	inositol-3-phosphate synthase
CG5966 (e-25)	1.34	triacylglycerol lipase
HLH3B (e-31)	1.43	transcription factor
U2af50 (e-79)	1.18	pre-mRNA splicing factor

Of the 50 cDNAs most predictive for behavior in class prediction analysis of individual bee brains (table S3), 17 had strong sequence matches to functionally annotated *Drosophila melanogaster* genes (Fig. 3) and belong to functional categories that can be plausibly related to neural and behavioral plasticity. For example, nurses had higher brain expression of genes that may act in axonogenesis and cell adhesion (similar to *fax* and *BM-40-SPARC*, respectively), which could be involved in changes in brain structure that precede the shift to foraging activity (*13*). Several genes elevated in either foragers or nurses are likely to act in intracellular signaling, including a putative mitogen-activated protein kinase, a gene involved in inositol-3-phosphate synthesis, a transcription factor, and a RAS-related gene (similar to CG32703, *Inos*, *HLH3B*, and *Rab10*, respectively). Expression of another intracellular signaling gene, *foraging* (encoding a cyclic guanosine monophosphate–dependent protein kinase), was previously shown to be elevated in forager brains (*24*, *25*). Pharmacological activation of the *foraging* gene product causes precocious foraging (*24*),

suggesting that changes in the expression of at least some intracellular signaling genes may play causal roles in the transition to foraging behavior. Other changes may be associated with the more cognitively demanding tasks performed by foragers (*13*). Foragers showed elevated expression of a carbonic anhydrase gene (similar to *CAH1*), which plays important roles in synaptic plasticity and cognition in mammals, including spatial learning and memory (*26*). Changes in cognitive function also may be indicated by changes in brain metabolism (*27*), suggested by changes in expression of genes similar to *Tps1*, *GlyP*, and *Eip71CD*. Of the rest of the 50 cDNAs most predictive for behavior, 5 had strong matches to *Drosophila* genes that have not been functionally annotated, and 28 had only weak matches or no matches to *Drosophila* genes (table S3), likely due to limited sequence available for honey bee cDNAs (primarily 5' ESTs) (*18*). Further speculation on the functional role of expression changes will improve after completion of the honey bee genome sequence (*28*), when full-length gene sequences corresponding to ESTs become available.

In contrast to genes discussed above, some genes were relatively poor predictors of behavior in individuals despite showing expression differences of large average magnitude between behavioral groups. For example, a set of tightly coregulated chaperones, including *HSP90*, were among genes that showed the highest average expression in the forager group (Fig. 2A, asterisk, and table S4). However, these genes were not among the 50 cDNAs most predictive for behavior because they exhibited strong up-regulation in only a subset (roughly half) of the 30 individual foragers (compare expression of genes in Fig. 2A, asterisk, and Fig. 3). Although all young foragers ($n = 6$) showed up-regulation of these genes, this apparent trend was not consistent with results from analyses of additional young foragers (*29*). Thus, differences of large magnitude inferred from group analyses do not necessarily reflect consistent or highly predictive differences in individuals. These results suggest that analyses of individual brains can provide information of potential behavioral relevance that is not apparent from average group trends.

A focus on the individual has provided key insights into behavior in both ethology (*30*) and brain imaging studies (*31*). Here we demonstrate a molecular "signature" in the individual bee brain that is robustly associated with behavior. Further experiments should help to determine how the changes in gene expression are coordinately regulated, which genes are responding to environmental cues known to be important regulators of behavior, and which genes and pathways cause changes in behavior.

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29. In addition to differences between nurses and foragers, young "precocious" foragers and old foragers appeared to be different in both principal component analysis (Fig. 1C, PC1) and in their expression of a set of coregulated chaperones including *HSP90* (Fig. 2A), asterisk). We found that 6 out of 6 young foragers showed strong up-regulation of *HSP90* whereas only 11 out of 24 old foragers showed similar up-regulation. This apparent difference between young and old foragers, although of large average magnitude, was not significant in Bayesian analysis due to large variation in *HSP90* expression between individuals. In addition, analysis of *HSP90* in six other young foragers by real-time quantitative reverse transcription (RT)–PCR (*17*) indicated strong up-regulation in just four out of six individuals (*33*), indicating that *HSP90* up-regulation does not occur consistently in all young foragers. Further analysis (fig. S3) suggested that differences between young foragers and old foragers for PC1 in Fig. 1C were associated with differences in average *HSP90* expression. In contrast, differences between nurses and old foragers for PC1 were not associated with differences in *HSP90* expression (fig. S3).
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Supporting Online Material
www.sciencemag.org/cgi/content/full/302/5643/296/DC1
Materials and Methods
Figs. S1 to S4
Tables S1 to S7
References

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