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Division of labor in the honey bee (*Apis mellifera*): the role of tyramine β-hydroxylase

Herman K. Lehman[1,2,*], David J. Schulz[4], Andrew B. Barron[4], Lydia Wraight[4], Chris Hardison[1,2], Sandra Whitney[1], Hideaki Takeuchi[3], Rajib K. Paul[3] and Gene E. Robinson[4,5]

[1]*Department of Biology and* [2]*Program in Neuroscience, Hamilton College, Clinton, NY 13323, USA,* [3]*Department of Biological Sciences, University of Tokyo, Tokyo, Japan,* [4]*Department of Entomology and* [5]*Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*

*Author for correspondence at address 1 (e-mail: hlehman@hamilton.edu)

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Summary

The biogenic amine octopamine (OA) is involved in the regulation of honey bee behavioral development; brain levels are higher in foragers than bees working in the hive, especially in the antennal lobes, and treatment causes precocious foraging. We measured brain mRNA and protein activity of tyramine β-hydroxylase (Tβh), an enzyme vital for OA synthesis, in order to begin testing the hypothesis that this enzyme is responsible for the rising levels of OA during honey bee behavioral development. Brain OA levels were greater in forager bees than in bees engaged in brood care, as in previous studies, but Tβh activity was not correlated with bee behavior. Tβh mRNA levels, however, did closely track OA levels during behavioral development, and Tβh mRNA was localized to previously identified octopaminergic neurons in the bee brain. Our results show that the transcription of this neurotransmitter synthetic enzyme is associated with regulation of social behavior in honey bees, but other factors may be involved.

Key words: *Apis mellifera*, octopamine, tyramine, tyramine beta-hydroxylase.

Introduction

Age-related division of labor in honey bee colonies is based on a striking pattern of behavioral plasticity that involves lifelong behavioral development (Robinson, 1992). Worker honey bees tend the queen, rear brood, and maintain the hive for the first few weeks of adult life, and then forage outside of the hive for the final weeks of life (Winston, 1987). The transition from spending almost all of the time working in the hive to foraging for nectar and pollen outside the hive is a major change in lifestyle for the bee, and is preceded by changes in endocrine and exocrine gland secretions (Fahrbach and Robinson, 1995; Robinson and Vargo, 1997), behavioral diurnal activity rhythms (Moore et al., 1998), brain structure (Fahrbach and Robinson, 1996; Fahrbach et al., 1998), and brain gene expression (Robinson et al., 2005).

Over the past few years several studies have focused on the neural mechanisms underlying the behavioral transition from nurse to forager. Biogenic amines (dopamine, serotonin and octopamine) are likely candidates to mediate this transition because of their widespread role as modulators of behavior (Huber, 2005). Of the three biogenic amines, octopamine (OA) is the most strongly associated with the transition to foraging. OA levels are higher in the brains of foragers, regardless of age (Wagener-Hulme et al., 1999), particularly in the antennal lobes (Schulz and Robinson, 1999). OA receptors from the honey bee brain have been isolated and characterized, and are localized in antennal lobes (Grohmann et al., 2003; Farooqui et al., 2004); treatment with OA causes bees to forage precociously (Schulz and Robinson, 2001; Barron et al., 2002; Barron and Robinson, 2005). OA treatment causes bees to be more responsive to foraging-related stimuli (Barron et al., 2002; Barron and Robinson, 2005), providing a plausible mechanism to explain how increased brain levels of OA affect honey bee behavioral development.

Despite the wealth of knowledge regarding OA and its role in behavioral plasticity, little is known about the mechanisms controlling OA levels in the honey bee brain. The levels of neurotransmitters such as OA are determined by the rates of presynaptic synthesis, release, synaptic uptake and degradation, and each of these mechanisms has multiple elements. For example, OA synthesis in insects is dependent on the levels and activity of two enzymes, tyrosine decarboxylase (Tdc) and tyramine beta-hydroxylase (Tβh) (Livingstone and Temple, 1983). The activity and stability of these enzymes is regulated by transcriptional, translational and post-translational modifications. Furthermore, they require substrates, co-substrates and cofactors for activity; Tdc requires tyrosine and pyridoxal phosphate whereas Tβh

requires tyramine, ascorbate and copper, and each of these factors is dependent on specific transporters (Cole et al., 2005; Lehman et al., 2000a; Malutan et al., 2002). Thus, the regulation of neurotransmitter levels is complex and involves many processes.

We hypothesized that changes in OA synthesis, in particular those involving Tβh, are involved in the transition from working in the hive to foraging. As stated above, OA treatment caused precocious foraging, but treatment with tyramine, a neuroactive compound that is the immediate precursor in OA biosynthesis, did not (Schulz and Robinson, 2001). These results suggest tyramine does not promote precocious foraging and that the amount and/or activity of Tβh, the enzyme that converts tyramine to OA, may be an important part of the mechanism regulating honey bee behavioral development. We evaluated this hypothesis by measuring behaviorally related changes in brain *Tβh* mRNA, Tβh activity, and octopamine levels themselves. In addition, we determined whether *Tβh* mRNA was localized in neuronal populations that were previously shown to be octopaminergic (Kriessl et al., 1994; Spivak et al., 2003; Sinakevitch et al., 2005). We also compared these localization patterns in nurses and foragers to explore whether the higher OA levels seen in forager brains are related to changes in OA synthesis in existing octopaminergic neurons or due to the appearance of new octopaminergic neurons.

Materials and methods

Bees

Bees (*Apis mellifera* L.) used in this study were reared at the Bee Research Facility at the University of Illinois at Urbana-Champaign, Urbana, IL, USA. Behavioral groups were collected according to standard methods of identification (Robinson, 1987). Nurses were identified as workers with heads in cells containing larvae, and foragers were identified as bees flying into the hive with pollen loads or abdomens distended with nectar. 'Single-cohort colonies' were formed by housing approximately 1000 one-day-old worker bees and a queen in a small hive with one frame of pollen and honey, and one empty frame in which the queen could lay eggs. Single-cohort colonies were used to dissociate worker age and behavior; the absence of older bees leads to precocious foraging in a subset of younger individuals (Robinson et al., 1989; Huang and Robinson, 1992). 5- to 8-day-old normal age nurses and precocious foragers were collected from single-cohort colonies. Within each trial, single-cohort colonies were made to be as similar as possible in terms of genotypic composition, population size, age demography, amount of comb and food, and queen age and source.

Nurses and foragers were collected by vacuuming directly into liquid nitrogen to preserve brain chemistry at the time of collection and stored at –80°C (Wagener-Hulme et al., 1999; Schulz and Robinson, 1999). Brains (brain and suboesophageal ganglion less optic lobes) were removed on dry ice so the tissue never thawed, and stored at –80°C until analysis.

Quantification of octopamine levels by HPLC

Quantification was conducted according to previously described methods (Wagener-Hulme et al., 1999; Schulz and Robinson, 1999).

Protein assay

Protein concentrations in bee brain extracts were determined prior to Tβh enzyme assays (described below) so that an equal amount of protein could be added to each enzymatic reaction. Brains were homogenized in saline and amount of soluble protein was determined from a 2 μl sample using the bicinchoninic acid method, with BSA as a protein standard (Pierce, Rockford, IL, USA).

Tβh activity assays

Tβh activity was measured using a slightly modified version of a method developed previously (Lehman et al., 2000a). The assay relies on the conversion of [^{3}H]tyramine to [^{3}H]OA and has been used previously to characterize Tβh in the developing nervous system of the hawk moth, *Manduca sexta* (Lehman et al., 2000a; Lehman et al., 2000b). Here, individually dissected bee brains were each homogenized in 10 μl bee saline and 2 μl samples were assayed for total protein concentration. The samples were then frozen overnight (–80°C) to liberate membrane and soluble forms of Tβh; on the following day samples were thawed and 30 μg samples of the brain homogenate added to a Tβh reaction buffer [final concentrations: 0.1 mol l^{-1} potassium phosphate (pH 7.0), 1.0 mg catalase, 0.05 mmol l^{-1} $CuSO_4$, 5.0 mmol l^{-1} disodium fumarate and 5.0 mmol l^{-1} ascorbic acid] containing 0.5 mmol l^{-1} (0.2 Ci mmol l^{-1}) [^{3}H]tyramine. Samples were incubated for 3 h at room temperature with mixing and the reactions stopped by adding 10 μl percholoric acid and boiling. Samples were centrifuged (20 min, 10 000 ***g***) and the reaction products separated and identified by high-performance liquid chromatography (HPLC). The HPLC apparatus consisted of an ESA Model 542 automatic injector (Chelmsford, MA, USA), a Shimadzu Model LC-10AS pump (Columbia, MD, USA), and an ESA Coulochem Model 5200 electrochemical detector. Coulophase (ESA) was used as the isocratic mobile phase, the separation column was a high efficiency reverse-phase column (ESA Catecholamine HR-80), and the electrochemical detector was set at 750 mV for OA detection. Radioactivity eluting from the column was either manually collected and counted by liquid scintillation counting (Trials 1–3) (Wallac Model 1409, PerkinElmer, Boston, MA, USA) or counted with the use of an in-line radioactive detector (Trials 4–7) (β-RAM Model 3, INUS, Tampa, FL, USA). In the latter case, the electrochemical and radioactive detectors were sequentially arranged and scintillation fluid was pumped at a 3:1 ratio. The HPLC system was connected to HP ChemStation Software (Agilent Technologies, Palo Alto, CA, USA) for analysis of peak areas. Radioactivity co-eluting with OA was identified in each sample by comparing its elution time to unlabeled OA, subtracted from boiled enzyme controls, and converted to counts min^{-1} (c.p.m.). Kinetic parameters of the crude enzyme

for tyramine were determined by plotting double-reciprocal plots of OA synthetic rates *vs* substrate concentrations. Estimates of V_{max} and K_m were obtained from the slopes and intercepts of the straight lines generated in these plots.

Tβh mRNA quantification

Tβh mRNA levels were quantified with real-time quantitative PCR using an ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA, USA). Total brain RNA was isolated from an individual brain using a RNeasy total RNA isolation kit (Qiagen, Valencia, CA, USA). Total RNA was reverse-transcribed according to the manufacturer's instructions with 100 μg of total RNA using default parameters (TaqMan Reverse Transcription Reagent Kit, PE Applied Biosystems). Specific primers for *Tβh* were designed using PrimerExpress software (Applied Biosystems): forward primer (5′-GGCTAAAAGGTTTAGGACCACTATCA-3′), reverse primer (5′-AATTCTGTTCTAGACATACCAGCAGTTT-3′), and probe (5′-CTTTGACTGGTTTGCAAGTATCCGATG-CA-3′). Sequence information for the *Tβh* gene was obtained by locating an ortholog to the *Drosophila melanogaster Tβh* gene in the sequence of the honey bee genome (see below). Brain levels of *Tβh* mRNA were measured relative to two well-characterized control genes: *rp49* was used for Trial 1 while both *s8* and *rp49* were used for Trials 2 and 3 (both control genes resulted in very similar results for *Tβh*). Each sample was analyzed in triplicate. To quantify mRNA, we recorded the number of PCR cycles required for each reaction's fluorescence to cross a threshold value of intensity (Ct), using the the $2^{-\Delta\Delta Ct}$ technique (Livak, 1997).

Tβh mRNA in situ hybridization

Sections (10 μm thick) from two frozen nurse and two forager brains were collected on silane-coated slides (Matsunami, Japan), air-dried overnight, and stored at –20°C until use. Sections were fixed in 4% paraformaldehyde in phosphate buffer (10 mmol l^{-1} sodium phosphate buffer, pH 7.4) at room temperature for 15 min, in 10 μg ml^{-1} Proteinase K in RNase-free TE buffer (10 mmol l^{-1} Tris-HCl buffer, pH 8.0, containing 1 mmol l^{-1} EDTA) for 30 min, re-fixed in 4% paraformaldehyde in PBS for 10 min, and then treated in RNase-free 0.2 mol l^{-1} HCl for 10 min. Sections were placed in 200 ml RNase-free 0.1 mol l^{-1} triethanolamine-HCl buffer, pH 8.0, containing 0.5 ml acetic anhydride for 10 min with constant stirring and then washed with RNase-free PBS at room temperature for a few minutes. Sections were then dehydrated in RNase-free 70%, 80%, 90% and 100% ethanol. Hybridization solution [10 mmol l^{-1} Tris-HCl buffer, pH 7.6 containing 50% formamide, 200 μg ml^{-1} tRNA, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, Fraction V), 10% dextran sulfate, 600 mmol l^{-1} NaCl, 0.25% SDS, and 1 mmol l^{-1} EDTA] containing 1–2 μg ml^{-1} digoxigenin (DIG)-labeled RNA probes was preincubated at 85°C for 10 min and then placed on ice. Hybridization solution was added to the sections and they were then covered with parafilm to prevent evaporation of the probe. The DIG-labeled antisense RNA probe was prepared by *in vitro* transcription using a partial *Tbh* cDNA (5′-AAGAATGTACAGGCATTGGTCTCCC to GTGCTATTAGCAATGATGCTCTAAG-3′) as a template with a DIG RNA labeling kit (Roche, Basel, Switzerland). Hybridization was performed at 50°C overnight in a humidified chamber (50% formamide). After hybridization, the sections were washed with 2× SSC (1× SSC=150 mmol l^{-1} sodium chloride, 15 mmol l^{-1} sodium citrate) containing 50% formamide at 50°C for 1 h. Sections were then pre-treated with TNE buffer (10 mmol l^{-1} Tris-HCl buffer, pH7.6 containing 1 mmol l^{-1} EDTA, and 0.5 mol l^{-1} NaCl) at 37°C for 15 min. RNaseA (200 μl of 10 μg ml^{-1}) was added to TNE buffer with stirring, and the sections incubated for 30 min at 37°C followed by washing with TNE buffer at 37°C for 10 min, 2× SSC at 50°C for 20 min, and two times with 0.2× SSC at 50°C for 20 min. DIG-labeled RNA was detected immunocytochemically with alkaline phosphatase-conjugated anti-DIG antibody using a DIG nucleic acid detection kit (Roche Applied Sciences, Indianapolis, IN, USA). Digital images of honey bee brains were captured using a digital scanning camera (HC-2500, Fujifilm, Stamford, CT, USA) mounted on a BX-50 microscope (Olympus, Melville, NY, USA). Brightness and contrast of the image were adjusted using Photoshop 4.0 software (Adobe System Inc., San Jose, CA, USA). No staining was observed using sense strand RNA controls (data not shown).

Statistical analysis

A two-way analysis of variance (ANOVA) was performed to determine effects of colony type (typical or single-cohort) and behavior (nurse or forager) on levels of OA, Tβh activity, and mRNA levels. Results of this ANOVA together with Fisher PLSD *post-hoc* tests were used to determine differences between nurses and foragers within each colony. All statistical analyses were performed using StatView5 (Abascus Concepts, Inc., Berkeley, CA, USA). OA and Tβh mRNA measurements were made in (the same) three trials, each using one typical and one single-cohort colony (derived from the typical colony). Tβh activity was measured in seven trials. Colonies in different trials were unrelated to each other.

Results

Behaviorally related differences in brain octopamine levels

In this set of experiments, we measured OA levels in nurse and forager bees from typical and single cohort colonies to confirm previous studies. In this study, OA brain levels differed significantly with bee behavior. In 3 out of 3 trials there were significantly higher OA levels in foragers as compared to nurses, in both typical colonies and single-cohort colonies, in which (precocious) foragers were the same age as nurses (Fig. 1). Foragers from typical colonies had higher levels of octopamine compared to precocious foragers and both groups of nurses, as revealed by both colony and

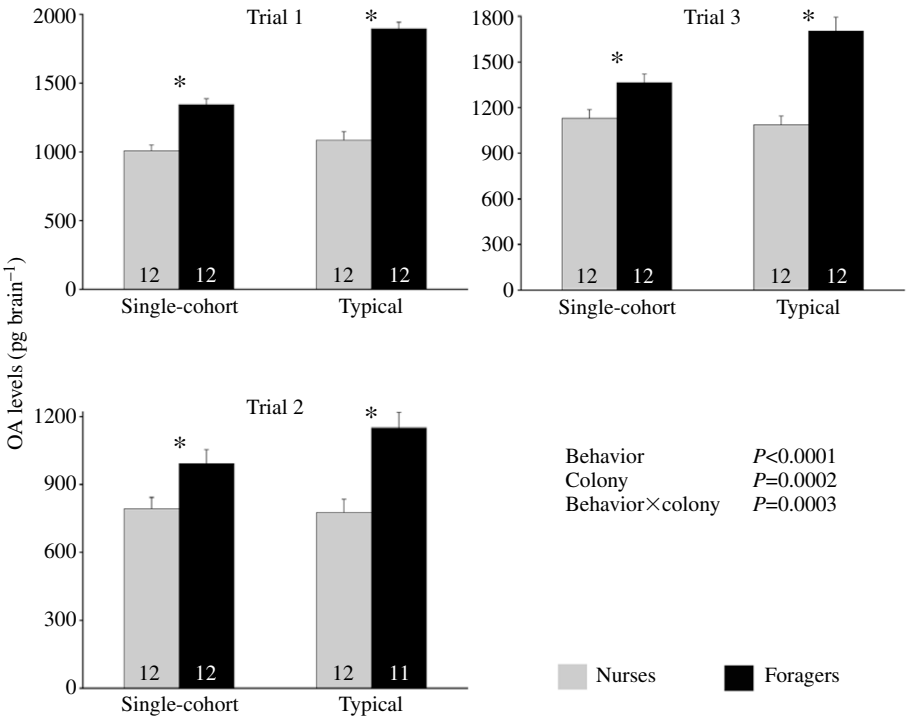

Fig. 1. Octopamine (OA) levels in honey bee brains. OA levels for bees from single-cohort colonies (normal-aged nurses and precocious foragers) and typical colonies (typical foragers and typical nurses) are shown. Values are mean ± s.e.m.; sample size is indicated in each bar. *Significant differences between nurses and foragers (ANOVA and Fisher PLSD *post-hoc* tests). The results of two-way ANOVA for OA levels as a function of behaviour and colony type are also shown. Three trials were performed; the single-cohort colony was derived from the typical colony in each trial, and colonies in different trials were unrelated to each other.

colony×behavior interaction effects. It is not possible to ascribe this difference to either age or foraging experience, since foragers from typical colonies are both older and more experienced than precocious foragers (Farris et al., 2001), and effects of foraging experience on brain OA levels have not been detected (Schulz et al., 2003).

Tβh assay

In this series of experiments we adapted a previously developed Tβh assay to characterize and measure Tβh activity in protein extracts from single honey bee brains. Incubation of substrate ([ring-^{3}H]tyramine hydrochloride) with crude bee brain extracts resulted in the formation of a single enzymatic product that was distinguished by reverse-phase HPLC (Fig. 2). The total amount of radioactivity collected from this peak was typically <5% of radioactivity added as substrate. [^{3}H]OA eluted at ca. 5.0 min and its identity was confirmed by comparing elution times of the radiolabeled product to unlabeled OA detected with electrochemical detection.

The formation of [^{3}H]OA by bee brain homogenates was linear for at least 6 h, and a linear rate of [^{3}H]OA synthesis was observed with protein levels between 10 μg and 50 μg (data not shown). The rate of [^{3}H]OA synthesis was a function of the concentration of tyramine in the incubation mixture; the apparent K_m values for tyramine ($K_{m,tyramine}$), calculated from forager and nurse brain extracts, were similar (0.25 mmol l^{-1} and 0.32 mmol l^{-1}, respectively). Our estimation of the apparent $K_{m,tyramine}$ from the honey bee is similar to the $K_{m,tyramine}$ of Tβh from *Manduca sexta* (0.22±0.047 mmol l^{-1}), *Homarus americanus* (0.15±0.015 mmol l^{-1}) and mammalian Dβh (0.55 to 2.8 mmol l^{-1}) (Lehman et al., 2000a; Wallace, 1976; Stewart and Klinman, 1991).

Behaviorally related differences in brain Tβh activity

We used the assay developed above to measure Tβh activity in nurse and forager bee brains isolated from typical and single cohort colonies to determine if OA levels were correlated with Tβh activity. Behaviorally related differences in brain Tβh activity were variable (Fig. 3). In two trials (1 and 7) there were significantly higher levels of Tβh activity in foragers compared to nurses from typical colonies, in two trials (4 and 6) the opposite result was obtained, and in three other trials no differences were observed. Overall, the results of a two-way ANOVA revealed that Tβh activity differed significantly with behavior in three trials (1, 4, 6). In addition, differences in brain Tβh activity between precocious foragers and normal age nurses from single-cohort colonies also varied from trial to trial. In trials 4 and 6, Tβh activity was greater in normal-aged nurses than precocious foragers, while in the other five trials there were no differences. There were significant differences between colony types (single-cohort *vs* typical) in 3 out of 7 trials.

Tβh sequence

We identified the Tβh gene from *Apis mellifera* to explore aspects of its regulation. Sequence information for the *Tβh* gene was obtained by locating an ortholog to the *Drosophila melanogaster Tβh* gene in the sequence of the honey bee genome. We identified a *tβh* gene in the honey bee genome and based on the evidence presented here we have named this gene *Apis mellifera Tβh* (*AmTβh*). Nucleotide sequence data reported are available in the Third Party Annotation section of the DDBJ/EMBL/GenBank databases under the accession number TPA: BK005823. The deduced amino acid sequence for *AmTβh* indicated an ORF of 613 amino acids constituting a protein with a molecular mass of 70.09 kDa (Fig. 4). The

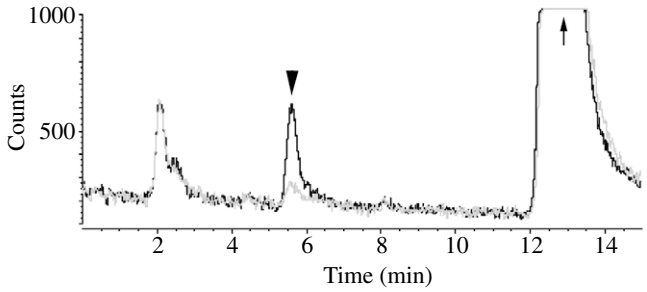

Fig. 2. Reverse-phase HPLC radiochromatograms of the enzymatic products from incubation of honey bee brain extracts with [ring-^{3}H]tyramine. Large arrowhead indicates the elution of synthetic OA and the small arrow indicates the elution of synthetic tyramine as detected by electrochemical detection. Dark trace: radiochromatogram of the enzymatic products from untreated brain extracts. Light trace: compounds recovered from boiled enzyme reaction.

deduced amino acid sequence of *AmTβH* shares up to 44% identity with other insect Tβh proteins and up to 42% identity with mammalian dopamine beta-hydroxylase proteins. Analysis of the AmTβH amino acid sequence revealed several structural and functional motifs that are consistent with hydroxylation activity. Domain analysis using ScanProsite (Swiss Institute of Bioinformatics, Basel, Switzerland) and NCBI Entrez (National Center for Biotechnology Information, Bethesda, MD, USA) showed that this protein contains a catecholamine-binding domain (Ponting, 2001), a DOMON domain (Aravind, 2001), and both a copper type II, ascorbate-dependent monooxygenase signature 1 domain and a copper type II ascorbate-dependent monooxygenase signature 2 domain (Southan and Kruse, 1989). In addition, 14 cysteine residues are responsible for intra- and intermolecular disulfide linkages in bovine Dβh (Robertson et al., 1994), and 12 of these cysteine residues are located in similar positions in the AmTβH protein. Based on this analysis, we are confident we identified a *bona fide* ortholog of *Tβh* in the honey bee.

Behaviorally related differences in brain AmTβh mRNA levels

In this series of experiments we measured *AmTβh* mRNA levels in nurse and forager bees from typical and single cohort colonies to determine if *AmTβh* was correlated with Tβh activity. *AmTβh* mRNA brain levels differed significantly with behavior. In 3 out of 3 trials there were significantly higher *AmTβh* mRNA levels in forager brains compared to nurses. This result was seen in both typical colonies and in single-cohort colonies, in which (precocious) foragers were the same age as nurses (Fig. 5).

AmTβh mRNA localization

We determined the cellular location of *AmTβh* to confirm the identity of *AmTβh* and to identify neurons that might mediate changes in bee social behavior. *In situ* hybridization revealed four clusters of *AmTβh* expressing neurons in the honey bee brain, in both nurses and foragers. There were no obvious differences in the distribution of *AmTβh* expressing neurons in nurses and foragers (data for foragers not shown). The four clusters are described in the following paragraphs.

A frontal, anterior, view of the cerebral and subesophageal ganglia showing the antennal lobes, mushroom bodies and optic lobes contained a group of *AmTβh* expressing neurons medial to the antennal lobes (Fig 6A–C). A higher magnification image of this region revealed a compact cluster of *AmTβh* expressing neurons medial to the antennal lobe and adjacent to the esophageal foramen (Fig. 6C). This group closely resembles the location of a cluster of 8–9 OA-immunoreactive cells observed by Spivak et al. (Spivak et al., 2003), the group of 6–7 OA-immunoreactive cells medial to each antennal lobe observed by Kreissl et al. (Kreissl et al., 1994), and the 7–9 OA-immunoreactive somata medial to the antennal lobe observed by Sinakevitch et al. (Sinakevitch et al., 2005). All these neurons appear to belong to octopaminergic-immunoreactive cell group 3 as named by Kreissl et al. (Kreissl et al., 1994) and later subdivided into two groups (G3a and G3b) by Sinakevitch et al. (Sinakevitch et al., 2005). In our study, the intensity of staining was greatest in this cell cluster, as in Spivak et al. (Spivak et al., 2003) and Kreissl et al. (Kreissl et al., 1994). It is thus likely that this cluster of neurons contains more OA than other OA-immunoreactive and *AmTβh* expressing cells.

Frontal, median sections of the honey bee brain contained a small cluster of *AmTβh* expressing cells proximal to the optic lobes and lateral and posterior to the antennal lobes (Fig. 6D). The location of this neuronal cluster is similar to the 5–6 neurons of OA-immunoreactive group 5 (Kreissl et al., 1994) and the group of ca. 13 OA-immunoreactive neurons in group 5b identified by Sinakevitch et al. (Sinakevitch et al., 2005).

In the same plane as in Fig. 6D, one *AmTβh* expressing cell body was observed in a region lateral and posterior to the antennal lobe (Fig. 6E). The staining appears to be due to a single cell on each side of the ganglion and resembles the location of two neurons in cell group 6 observed by others (Kreissl et al., 1994; Spivak et al., 2003) and cell groups G6a and G6b consisting of two distinct groups of 3 and 5 OA-immunoreactive neurons observed by Sinakevitch et al. (Sinakevitch et al., 2005).

Other *AmTβh* expressing neurons were observed in a more posterior frontal section of the brain and subesophageal ganglion (Fig. 6F,G). This group of cells resembles the ventral median neuron located in the medial region of the subesophageal ganglion (Hammer, 1993; Bicker, 1999). Kreissl et al. observed three groups of OA-immunoreactive cell bodies containing 6–10 somata, each clustered in the ventral median portion of the subesosphageal ganglion (Kreissl et al., 1994), and a group of six cells are shown in a similar location in Spivak et al. (Spivak et al., 2003); Sinakevitch et al. reported five large cells in the same location (Sinakevitch et al., 2005). These Tβh expressing and OA-ir neurons appear to belong to cell group 7 (Kreissl et al., 1994) or the ventral unpaired median neurons and flanking neurons as identified by Sinakevitch et al. (Sinakevitch et al., 2005).

Discussion

We have observed that elevated levels of octopamine in the brains of forager honey bees are correlated with an increase in the expression of the gene encoding tyramine beta-hydroxylase (Tβh), an enzyme vital for OA synthesis. Our results provide a link between transcriptional activity and levels of neurotransmitter in the brain, and the regulation of social behavior in honey bees.

Changes in the expression of many genes are associated with the shift from working in the hive to foraging (Whitfield et al.,

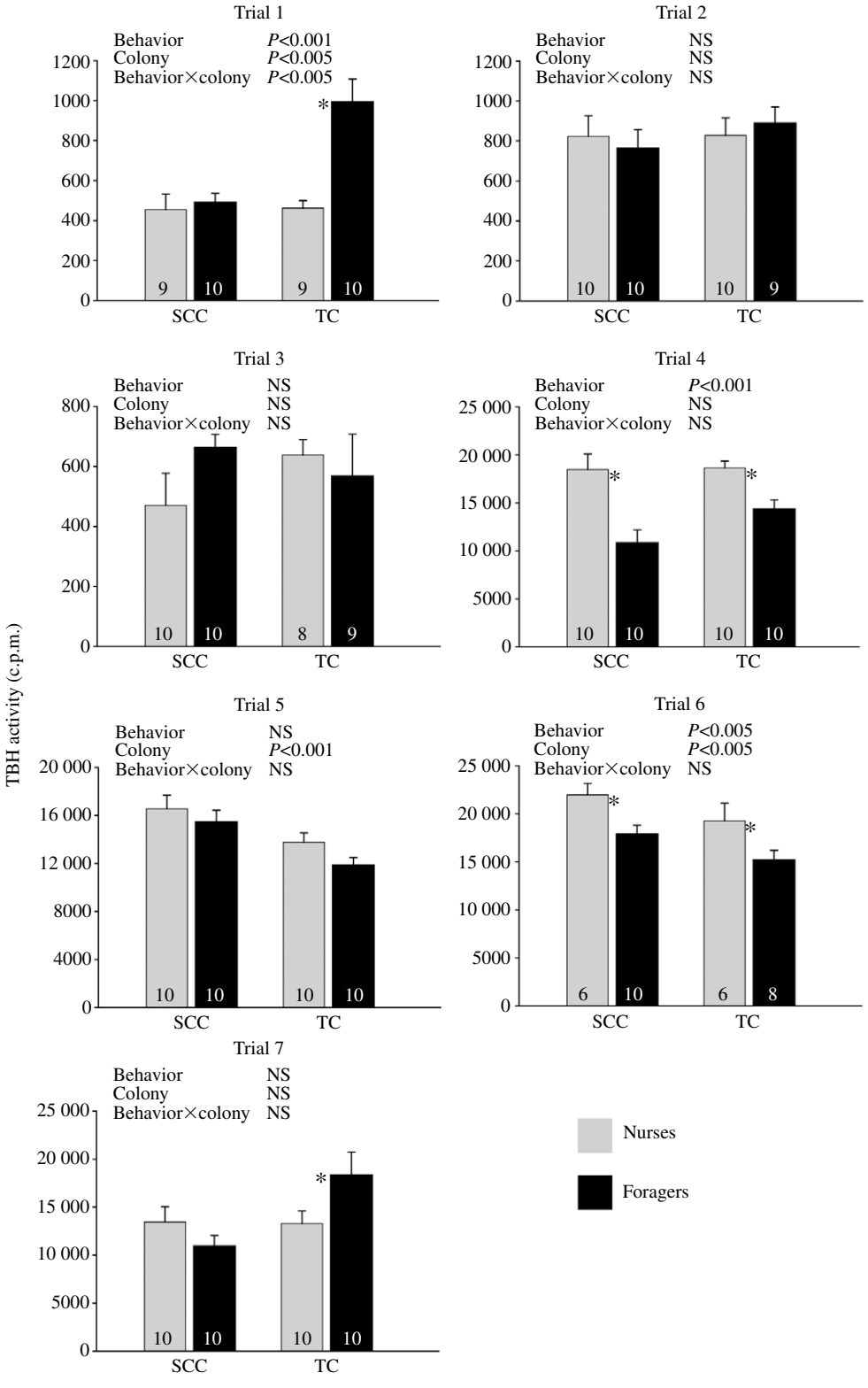

Fig. 3. Tyramine β-hydroxylase (Tβh) activity in honey bee brains. Tβh activity from bees from single-cohort colonies (SCC, normal-aged nurses and precocious foragers) and typical colonies (TC, typical foragers and typical nurses) are shown. Values are mean ± s.e.m.; sample size is indicated in each bar. *Significant differences between nurses and foragers (ANOVA and Fisher PLSD *post-hoc* tests). The results of two-way ANOVAs for Tβh activity as a function of behavior and colony type are also shown. Seven trials were performed; see Fig. 1 legend.

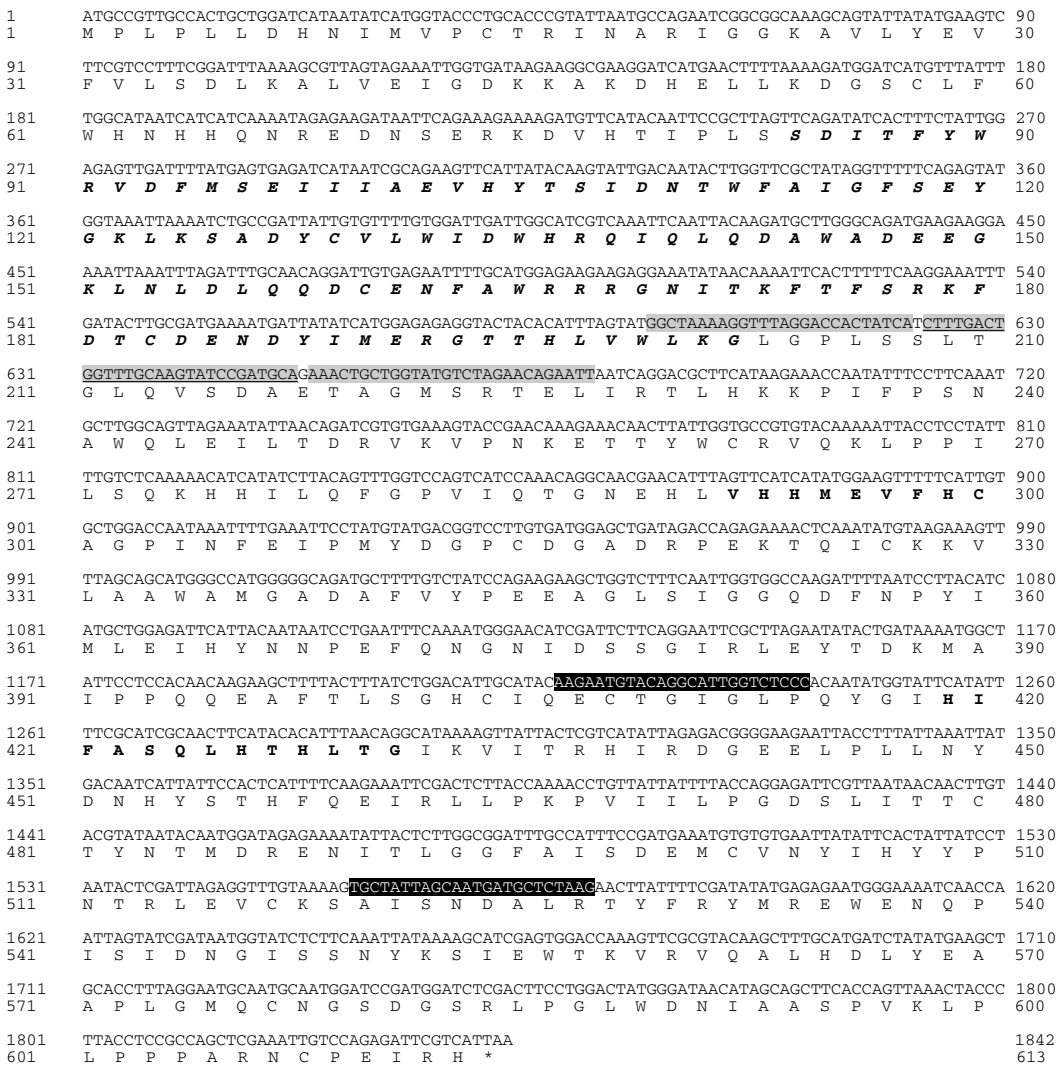

Fig. 4. Nucleotide and deduced amino acid sequence of Apis Tβh. The first nucleotide and amino acid residue of the translational start site are designated as position 1. The amino acid positions of DOMON and Cu^{2+} type II ascorbate-dependent monoxygenase domains predicted by Scansite 2.0 (Obenauer et al., 2003) are indicated by italic bold and bold text, respectively. The nucleic acid sequences in white text on a black background are the regions used to synthesize the DIG-labeled antisense RNA probe for *aTβh* in situ hybridization procedures. The nucleic acid sequences used as primers for quantitative real-time PCR analysis are shown in black text on a grey background and the sequence used as a probe is shown in underlined black text on a grey background.

2003); our results indicate that the transcriptional regulation of an enzyme controlling neurotransmitter synthesis may also be involved in these behavioral changes. This notion is supported by other studies on the molecular basis of social behavior. For example, disregulation of serotonin is an important factor in many psychiatric disorders, but tryptophan hydroxylase (Tph1), long thought to be the only rate-limiting enzyme necessary for the synthesis of serotonin, has not been linked to any specific disorder. Recently, however, a second isoform of human tryptophan hydroxylase (Tph2) was identified (Walther et al., 2003), and a single nucleotide polymorphism results in a significant decrease in serotonin production (Zhang et al., 2004), thus implicating this mutation as an important risk factor for unipolar major depression (Zhang et al., 2005). The *Dβh* gene is also polymorphic, and one allele is associated with differences in plasma and cerebrospinal fluid levels of Dβh (Kobayashi et al., 1989; Zabetian et al., 2001; Cubells et al., 1998). Several studies have now implicated low Dβh activity as a risk factor for psychotic depression (Meltzer et al., 1976; Meyers et al., 1999; Mod et al., 1986). In sum, it is clear that changes in the expression of neurotransmitter synthetic enzymes can have dramatic effects on neurotransmitter level, and behavior. It will be interesting to determine if genetic variation in behavior among bees (Robinson et al., 2005) is influenced by genetic polymorphisms in genes encoding neurotransmitter synthetic enzymes.

We did not detect a correlation between Tβh enzyme activity and octopamine levels in the honey bee brain. These

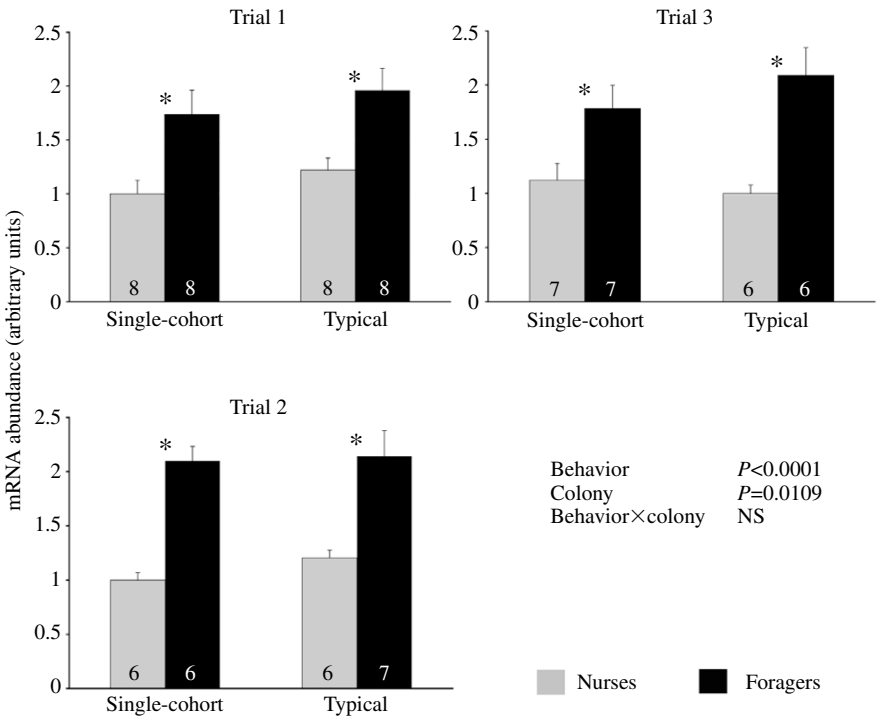

Fig. 5. *Tβh* gene expression in honey bee brains. Cycle threshold (Ct) was determined using second derivative analysis and the difference in Ct between *AmTβh* and a control gene was used to determine relative expression levels. *aTβh* mRNA levels from single bees collected from single-cohort colonies (normal-aged nurses and precocious foragers) and typical colonies (typical foragers and typical nurses) are shown. Values are means ± s.e.m.; sample size is indicated in each bar. *Significant differences between nurses and foragers (ANOVA and Fisher PLSD *post-hoc* tests). Results of two-way ANOVA for Tβh expression as a function of behavior and colony type are also shown. Three trials were conducted (the same trials as in Fig. 1, and corresponding to Trials 1, 2 and 3 of Fig. 3).

results suggest either that Tβh activity levels in the brain are not correlated with octopamine levels, or that our Tβh activity assay was not sufficient to detect a correlation. Regarding the first possibility, mRNA and protein levels are not always correlated (Gygi et al., 1999); perhaps upregulation of other components necessary for OA synthesis, including tyrosine decarboxylase (Livingston and Temple, 1983), cofactors [e.g. copper, ascorbate and pyridoxal (Lehman et al., 2000a)] and transporters (Malutan et al., 2002) are more related to OA levels. If this is the case, perhaps these components are regulated differently in different species, which could account for why there is a clear relationship between Tβh activity and octopamine levels in *Manduca sexta* (Lehman et al., 2000b) but not in *Apis mellifera* (this study). On the other hand, perhaps the Tβh activity assay was not sufficient to detect a correlation. This may have occurred because of the differences between what occurs *in vivo* and what occurred in our *in vitro* assay. The Tβh *in vitro* assay contains cofactors at high concentrations and the reaction is run under conditions where the enzyme produces product at a linear rate. These conditions may not reflect the conditions *in vivo*. In addition, neurotransmitter production and catabolism is critically dependent on the subcellular localization of all cofactors, enzymes, reactants and products. For example, the creation and degradation of 5-HT is critically dependant on cellular location. After 5-HT is synthesized and released, it is taken up from extracellular space by a specific serotonin transporter (SERT) (Blakely and Bauman, 2000). Monoamine oxidase-A (MAO-A) is located on the mitochondrial surface and converts intracellular 5-HT to 5-hydroxyindole-3-acetaldehyde (5-HIAL), which is then transported out of the cell and becomes 5-hydroxyindole acetic acid (5-HIAA) (Shih et al., 1999; Squires et al., 2006). Although less is known about the precise cellular location of enzymes and transporters controlling OA synthesis and degradation, it is clear that the cellular distribution of these factors is disrupted in the Tβh *in vitro* assay and this loss of cellular integrity may have negatively influenced the Tβh assay. It is difficult to image that there would be species-specific differences in the subcellular location of these processes; however, there could be differences in the amounts of these proteins in *Manduca* and *Apis* as described above. Ultimately, further analysis of Tβh protein with specific honey bee Tβh antisera will be necessary to resolve these issues.

Our observation that the location and number of *Tβh* expressing neurons are similar in number and location to previously identified octopamine immunoreactive neurons supports the conclusion that *Tβh* gene expression is linked to octopamine levels in the honey bee brain. We identified four groups of *Tβh* expressing neurons that resemble previously identified octopamine immunoreactive neurons. However, seven distinct clusters of octopamine immunoreactive neurons in the bee brain and subeosphageal ganglia have been reported (Kriessl et al., 1994; Spivak et al., 2003; Sinakevitch et al., 2005). We may have missed some sections that contained octopaminergic clusters, or the sensitivity of immunocytochemistry may be greater than the sensitivity of our *in situ* hybridization study. Nevertheless, because there were no obvious differences in the cellular location of *AmTβh* mRNA between foragers and nurses for the cell populations that we did identify, our results indicate that elevated *AmTβh* mRNA levels are a result of up-regulation of Tβh expression

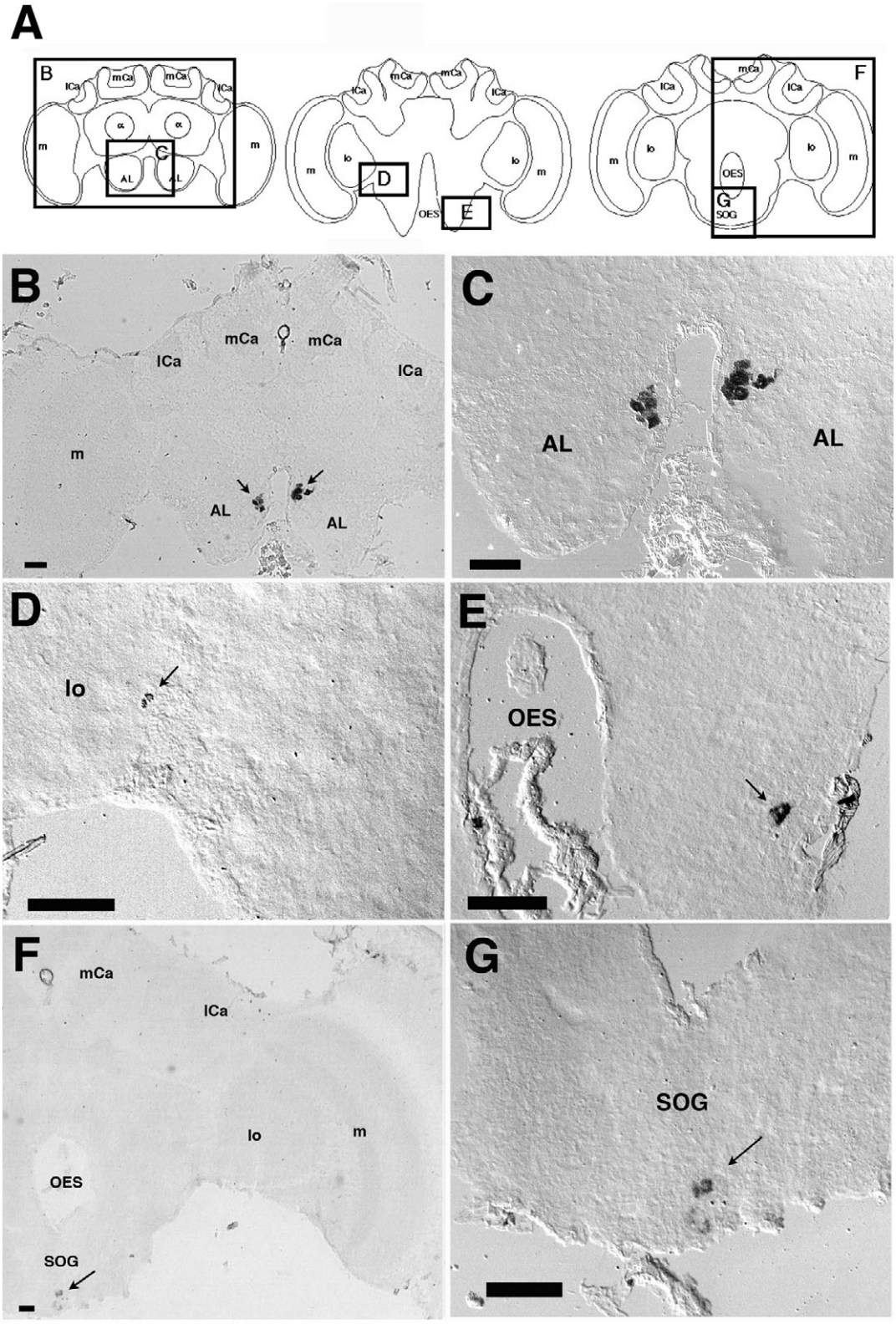

Fig. 6. *Tβh* gene localization in honey bee brains revealed by *in situ* hybridization. *In situ* hybridization was performed on nurses and foragers using 10 μm sections but only brains from nurses are shown here. Control experiments using DIG-labeled sense probes gave no significant signals in any of the *in situ* hybridization experiments (data not shown). (A) Frontal view of anterior (left), middle (middle) and posterior portion of the section (right). Areas corresponding to B–G are boxed. (B–G) Signals are indicated by arrows. m, medulla; lo, lobula; AL, antennal lobe; OES, esophagus; SOG, subesophageal ganglion. ICa, mushroom body lateral calyces; MCa, mushroom body medial calyces. Bars indicate 100 μm.

in existing octopaminergic cells, and not due to the appearance of new neurons that begin to synthesize OA later in life.

Although our *in situ* hybridization results did not reveal all known octopamine immunoreactive neurons in the honey bee brain, we did identify specific neurons that express *Tβh* and that apparently also contain octopamine. We suggest that one or more of these groups of neurons is involved in honey bee behavioral maturation. Those related to the antennal lobes are of special interest, because the antennal lobes appear to be the region of the bee brain that is especially important in octopamine-mediated regulation of division of labor in honey bees (Schulz and Robinson, 1999; Barron et al., 2002; Barron and Robinson, 2005). These are the neurons emanating from octopamine-immunoreactive cell groups 3 (G3a), 5 (G5a) and the VUM neurons that invade the honey bee antennal lobes where they branch profusely within the olfactory glomeruli and in the coarse neuropil central to the glomeruli (Spivak et al., 2003; Sinakevitch et al., 2005). *Tβh* expression was detected in two of these three cell groups that innervate the antennal lobes (cell group 3 and the VUMs). Octopamine plays important roles in regulating responsiveness to foraging-related stimuli (Mercer and Menzel, 1982; Bicker and Menzel, 1989; Hammer, 1993; Hammer and Menzel, 1995; Barron et al., 2002; Barron and Robinson, 2005) and foraging-related learning and memory (Menzel and Muller, 1996; Farooqui et al., 2003). Perhaps some of the neurons associated with the antennal lobes identified in our study are involved in the acquisition and retention of foraging-related olfactory information. Future studies of *Tβh* in the bee brain may lead to the identification of specific neurons involved in division of labor.

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