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ORIGINAL PAPER

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Age-related changes in dopamine receptor densities in the brain of the honey bee, *Apis mellifera*

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Abstract Changes in the levels of binding of ^{3}H-SCH-23390, a vertebrate D1 dopamine receptor ligand, and ^{3}H-spiperone, a vertebrate D2 dopamine receptor ligand were investigated in the brain of the worker honey bee during metamorphic adult development and during the lifetime of the adult bee. Age-related fluctuations in binding levels were markedly different for these two ligands. ^{3}H-SCH-23390 and ^{3}H-spiperone binding sites were present at low levels during metamorphic adult development. After adult emergence, however, ^{3}H-SCH-23390 binding levels, in contrast to those of ^{3}H-spiperone, increased significantly. Within the first 48 h of adult life ^{3}H-SCH-23390 binding reached a level not significantly different from that detected in forager bees. No significant fluctuations in the levels of ^{3}H-spiperone binding were observed during the adult lifetime of the bee. Measurements of dopamine levels in the brains of pupal and adult bees revealed no direct correlation between fluctuations in endogenous amine levels and the amount of binding of either ^{3}H-SCH-23390 or ^{3}H-spiperone. These results provide evidence for subtype-specific patterns of expression of dopamine receptors in the insect brain and show that D1- and D2-like receptors are expressed not only in the adult CNS, but also in the developing brain of the bee.

Key words Dopamine · Insect · Age-related · SCH-23390 · Spiperone

Introduction

Honey bees are highly social insects and their rich behavioural repertoire has been a focus of attention for many years (reviews: Free 1977; Seeley 1985; Winston 1987; Robinson 1992). It is well established that members of the worker caste that perform duties within the hive such as cleaning, nursing and comb-building, are generally younger than those involved in foraging (Winston 1987). Foraging behaviour is associated with high levels of juvenile hormone in the haemolymph of the bee (Fluri et al. 1982; Robinson et al. 1987, 1991) and is accompanied by site-specific changes in the structure of the brain neuropil (Withers et al. 1993; Durst et al. 1994; Winnington et al. 1996). Interestingly, the shift to duties outside the hive is also accompanied by a significant increase in the brain dopamine levels (Taylor et al. 1992). In the vertebrate CNS, changing endogenous amine levels can result in significant up- or down-regulation of dopamine receptor populations (Creese et al. 1977; Rosengarten and Friedhoff 1979; Seeman 1980; Porceddu et al. 1985; Savasta et al. 1988; Marshall et al. 1989; Gelbard et al. 1990; Radja et al. 1993) and the developmental and behavioural consequences of such changes are wide ranging (Creese and Fraser 1987). In contrast, surprisingly little is known about fluctuations in the receptor populations that mediate the actions of dopamine in the insect CNS.

The presence of dopamine in the insect CNS is well documented (reviews: Evans 1980; Brown and Nestler 1985; Pitman 1985; Mercer 1987). In the fruitfly *Drosophila melanogaster*, dopamine-immunoreactive neurons have been detected early in embryonic development (Lundell and Hirsh 1994) and larval dopamine-containing neurons have been shown to persist throughout the ontogeny of the fly (Budnik and White 1988). Dopamine has also been reported in the larval and pupal nervous systems of other insects, including the fly, *Calliphora erythrocephala*, the moth, *Manduca sexta* and the honey bee, *Apis mellifera* (Nässel and Laxmyr 1983; Krueger et al. 1990; Taylor et al. 1992; Geng et al. 1993) and there is increasing evidence that dopamine may play a role in neuronal development (McCobb et al. 1988; Kirchhof and Mercer 1995; Neckameyer 1996) as well as in the adult CNS of invertebrates (reviews: Walker and Holden-Dye 1989; Bicker and Menzel 1989).

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Recently, two different dopamine receptor clones which show structural or pharmacological similarities to the vertebrate D1 receptor family have been isolated from *Drosophila* (Gotzes et al. 1994; Sugamori et al. 1995; Feng et al. 1996) confirming that in insects, as in vertebrates, multiple D1-like dopamine receptor subtypes exist. D2-like dopamine receptors have yet to be cloned and characterised in insects, but pharmacological analysis and in vitro radioligand binding assays indicate that other dopamine receptor subtypes also are present in the insect CNS (Orr et al. 1987; Davis and Pitman 1991; Blenau et al. 1995; Kokay and Mercer 1996).

In the brain of the honey bee, binding sites for the vertebrate dopaminergic receptor ligands ^{3}H-SCH-23390 and ^{3}H-spiperone have been described (Kokay and Mercer 1996). In brain homogenate preparations ^{3}H-SCH-23390 labels a high-affinity site with a K_d of 6.3 nmol $\cdot$ l^{-1}. ^{3}H-Spiperone also binds with high-affinity (K_d 0.2 nmol $\cdot$ l^{-1}) to honey bee brain membranes. Results of experiments using a range of dopaminergic agonists and antagonists suggest that ^{3}H-SCH-23390 and ^{3}H-spiperone label D1- and D2-like dopamine receptors, respectively, in bee brain (Kokay and Mercer 1996) but ^{3}H-spiperone, unlike ^{3}H-SCH-23390, exhibits a phenolaminergic as well as a dopaminergic binding component. In this study, binding assays are used to examine changes in the density of ^{3}H-SCH-23390- and ^{3}H-spiperone-binding sites in the brain of the bee, firstly, during metamorphic adult development and secondly, during the lifetime of the adult worker bee. Levels of dopamine present in the brains of pupal and adult workers are also examined to explore possible correlations between changes in binding site densities and the levels of dopamine in the brain of the bee. Our results reveal marked differences between the ontogeny of ^{3}H-SCH-23390 and ^{3}H-spiperone binding site densities and suggest that in addition to its actions in the adult CNS, dopamine may play a role in the developing brain of the bee.

Materials and methods

Identification of bees of different ages

Frames with combs of sealed honey bee brood were transferred from hives kept at the University of Otago, Department of Zoology and placed in an incubator maintained at 30–35 °C. Using well-defined morphological characteristics associated with metamorphic adult development (Jay 1962), pupal bees removed from the brood cells with forceps were assigned to one of the following age groups: 1- to 3-day-old pupae (P2), 4- to 5-day-old pupae (P4), 6- to 7-day-old pupae (P6), 8-day-old pupae (P8), and pupae that had completed metamorphosis but were still in sealed brood cells (P9). Adult bees were marked on the dorsal thorax with a dot of enamel paint (Testors, Rockford, USA) as they emerged from the brood comb. Samples of newly-emerged bees (NE) were collected immediately, but most of the marked bees were returned to the outside hive and collected 1–6 days later. Bees that were to be sampled 12 h after adult emergence were retained in the incubator along with a number of older (non-sampled) adult bees. Bees designated as foragers (F) were also examined. These bees of unknown age were collected at the entrance of the hive as they returned from flights. Although these bees were assumed to be returning from foraging trips, it is possible some preforager bees on orientation flights may have been sampled. As dopamine undergoes diel fluctuations in insect brain (Linn et al. 1994; I. C. Kokay et al., unpublished observations), all adult bees, including those emerging from the comb, were marked and collected between 1200 and 1430 hours.

Radioligand binding assays

In the case of young pupal bees (P2–P6), binding assays were performed on homogenates of intact head capsules. For binding assays performed on tissue from older pupal bees (P8–P9) or from adult bees, isolated brains were used. Each brain (plus suboesophageal ganglion) was cleared of glandular tissue, dissected from the head capsule and immediately frozen in liquid N_2. As collection of sufficient tissue for a single binding assay took several days, individual batches of tissue were stored at −80 °C. The tissue was then pooled before being homogenised for ca. 10 s in a 50-fold volume of ice-cold 50 mmol $\cdot$ l^{-1} TRIS-HCl buffer (pH 7.4) containing 1 mmol $\cdot$ l^{-1} EDTA, 10 mmol $\cdot$ l^{-1} $MgCl_2$ and 200 µmol $\cdot$ l^{-1} phenylmethylsulfonyl fluoride (PMSF) using a glass-Teflon tissue homogeniser (Gallenkamp SS-425). Homogenates prepared from intact head capsules were sieved through muslin at this point. All tissue homogenates were then centrifuged at 400 *g* for 10 min to remove retinal pigments and fragments of connective tissue. The resulting supernatant was centrifuged twice (20 000 *g*, 45 min) before resuspension in incubation buffer. For ^{3}H-SCH-23390 assays 50 mmol $\cdot$ l^{-1} TRIS-HCl buffer (pH 7.5) containing (mmol $\cdot$ l^{-1}): 120 NaCl, 1 $MgCl_2$, 5 KCl and 200 µmol $\cdot$ l^{-1} PMSF was used. For ^{3}H-spiperone binding assays, the pellets were resuspended in 20 mmol $\cdot$ l^{-1} 3-[n-morpholino]propanesulfonic acid (MOPS) buffer containing (mmol $\cdot$ l^{-1}): 120 NaCl, 20 $MgCl_2$, 10 KCl, 2 $CaCl_2$, 1 EDTA and 200 µmol $\cdot$ l^{-1} PMSF, and adjusted to pH 7.5 with TRIS base. Homogenates were stored at −80 °C for no more than 3 weeks before use.

To examine age-related changes in the amount of binding of ^{3}H-SCH-23390 and ^{3}H-spiperone a single concentration of each radioligand (2 and 0.5 nmol $\cdot$ l^{-1}, respectively) was used. In accordance with previous work (Kokay and Mercer 1996), non-specific binding of ^{3}H-SCH-23390 was defined as binding in the presence of $5 \cdot 10^{-6}$ mol $\cdot$ l^{-1} *cis*-(Z)-flupentixol. Domperidone (10^{-5} mol $\cdot$ l^{-1}) was used to define non-specific binding of ^{3}H-spiperone. Aliquots of membrane preparation containing 100–200 µg protein were added to tubes containing incubation buffer with or without competing drugs and the reaction was started by addition of radioligand. The total volume used was 0.5 ml. After incubation for 45 min at 20 °C (^{3}H-SCH-23390), or 90 min at 20 °C (^{3}H-spiperone), the reaction was quenched by addition of 3 ml ice-cold TRIS-HCl buffer (50 mmol $\cdot$ l^{-1}, pH 7.3) then rapidly filtered through GF/C filters presoaked in 0.5% polyethylenimine. Filters were washed three times in 3 ml TRIS-HCl buffer, extracted overnight in 10 ml of toluene-based scintillation fluid and the radioactivity counted using a Packard 1900CA liquid scintillation analyser with a counting efficiency of 53–56%.

To compare the binding affinity of ^{3}H-SCH-23390 in brains of forager bees with that in brain homogenates of newly emerged bees, Scatchard analyses of saturation binding isotherms were carried out. In these experiments, 2 nmol $\cdot$ l^{-1} ^{3}H-SCH-23390 was added to triplicate assay tubes together with unlabelled ligand to give final concentrations of SCH-23390 ranging from 2.2 nmol $\cdot$ l^{-1} to 250 nmol $\cdot$ l^{-1}.

High performance liquid chromatography

Reverse phase high-performance liquid chromatography with electrochemical detection (HPLC) was used to determine the levels of dopamine in the brains of pupal and adult bees. The first set of assays was carried out during the late summer of 1993/early autumn of 1994. The assays were repeated the following summer. Bees were immobilised by chilling at 4 °C prior to removing the

brain from the head capsule. The brain (supraoesophageal ganglion) of each bee was carefully cleared of surrounding glandular tissue and retinal pigment then dissected from the head capsule as quickly as possible. The optic lobes of the brain were separated from the rest of the supraoesophageal ganglion for separate analysis. All tissue samples were placed in Eppendorf tubes and immediately frozen in liquid N_2. The tissue was stored at −80 °C for 1–4 weeks.

Samples were analysed individually (one brain or one pair of optic lobes per sample) or they were pooled (five brains or five pairs of optic lobes per sample). The tissue was sonicated for 6–8 s in 50 μl ice-cold 0.4 mol·l^{-1} $HClO_4$ containing 2.6 mmol·l^{-1} sodium metabisulphite and 2.7 mmol·l^{-1} EDTA, then centrifuged for 20 min at −4 °C. Supernatant (20 μl) was injected directly onto the HPLC column. The HPLC equipment used in this study consisted of a Shimadzu LC-10AD pump, a Rheodyne injector, a C8 column (4.6 × 100 mm with 5 μm packing) and an ESA model 5100A coulometric detector. The mobile phase consisted of (mmol·l^{-1}): 20 sodium acetate, 100 sodium dihydrogen orthophosphate, 0.3 EDTA, 0.9 octanesulfonic acid (sodium salt) and 11% (v/v) acetonitrile adjusted to pH 2.5. The working potential was set at +0.3 V and a flow rate of 1.5 ml min^{-1} was used.

Calibration curves using dopamine HCl and *N*-acetyldopamine monohydrate standards were determined at the beginning of each assay run. Standards were also included at intervals during the assay run to confirm sample peak retention times. Stock solutions of standards were prepared in 0.4 mol·l^{-1} $HClO_4$ and frozen at −80 °C. These were further diluted into 0.4 mol·l^{-1} $HClO_4$ prior to each assay run. To take account of changes in the protein content of the brain during metamorphic adult development and to adjust for differences in brain size between individual adult bees, dopamine levels are expressed in pmol per mg protein.

Protein analysis

Protein content of the membrane homogenates was measured using a modification (Peterson 1977) of the method of Lowry et al. (1951), which incorporates an initial trichloroacetic acid precipitation step. For protein determinations of samples used for HPLC, the pellets obtained after sample centrifugation were dissolved in 250 μl 1 mol·l^{-1} NaOH containing 2.5% sodium dodecyl sulphate before analysis. Bovine serum albumin was used as the standard.

Data analysis

Estimates of equilibrium dissociation constants (K_d) and the total number of sites (B_{max}) were calculated from saturation experiments using the computer program EBDA/LIGAND (BIOSOFT, Cambridge, UK). To test the significance of age-related changes in the amount of radioligand binding, the levels of protein in brain homogenates, and dopamine levels in the brain, data were subjected to ANOVA followed by Tukey's tests for post hoc comparisons. Significance was defined as $P < 0.05$. Where necessary data were log transformed before analysis. To test for relationships between amine levels and receptor levels, Pearson's correlation coefficients were calculated. Student's *t*-tests were used to test for differences in binding parameters (K_d and B_{max} values) between newly-emerged and forager bees.

Radioligands and drugs

^{3}H-SCH-23390 (78–79 Ci mmol^{-1}) and ^{3}H-spiperone (96–107 Ci mmol^{-1}) were purchased from Amersham (Buckinghamshire, UK). Domperidone and *cis*-(Z)-flupentixol 2HCl were purchased from Research Biochemicals (Natick, Mass., USA). Dopamine HCl and *N*-acetyldopamine monohydrate were purchased from Sigma (St. Louis, Mo., USA).

Results

Binding sites for ^{3}H-SCH-23390

The radioligand ^{3}H-SCH-23390 was used to label D1-like dopamine receptors in the brain of the honey bee. Figure 1 shows the amount of specific ^{3}H-SCH-23390 binding detected in brain homogenates of worker bees from 13 distinct age groups. Specific binding, defined here as binding that could be displaced by 10^{-5} mol·l^{-1} *cis*-(Z)-flupentixol, was around 85% of total binding for forager bee samples and on average 74% of total binding for pupal bees. The level of specific binding in forager bees was four- to six-fold higher than in bees shortly after adult emergence. In the brains of pupal bees, relatively low amounts of ^{3}H-SCH-23390 binding were detected. Furthermore, remarkably little variation in ^{3}H-SCH-23390 binding was apparent during the course of metamorphic adult development (P2–P9). After adult emergence however, specific binding increased significantly. This increase was rapid. Twenty-four hours after adult emergence ^{3}H-SCH-23390 bind-

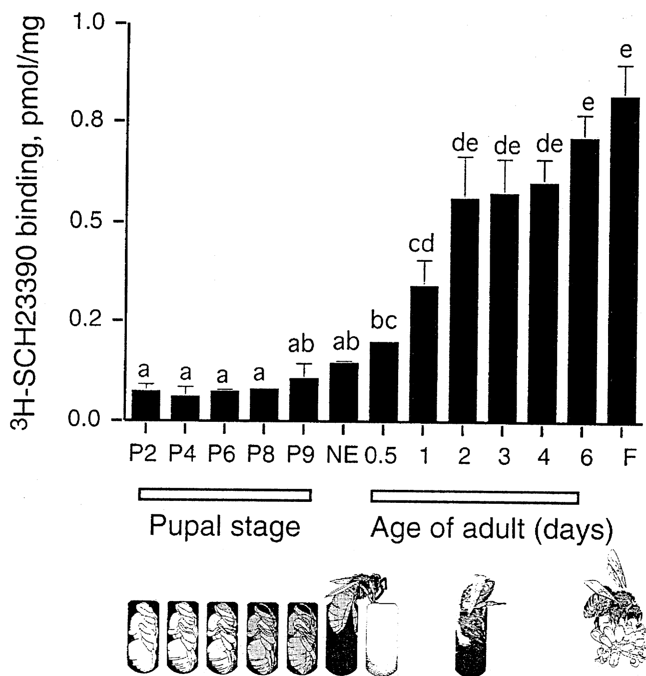

Fig. 1 Age-related changes in ^{3}H-SCH23390 binding in worker honey bee brains. Values represent mean specific binding (±SEM) of 2 nmol·l^{-1} ^{3}H-SCH23390 in brain homogenates of pupal bees (*P2–P9*), newly-emerged adults (*NE*), adults of different ages (0.5–6 days) and foragers (*F*) of unknown age. Specific binding was calculated by subtracting non-specific binding (binding in the presence of $5 \cdot 10^{-6}$ mol·l^{-1} *cis*-(Z)-flupentixol) from total binding. Overall statistical significance was determined by ANOVA followed by Tukey's tests for post hoc comparisons. *Letters* that appear above each bar on the graph indicate whether or not differences between groups are significant. Groups that do not differ significantly share at least one letter. Groups with significantly different values have different letters

ing levels were significantly higher than binding levels during pupal stages and 1 day later the amount of binding had reached a level not significantly different from that detected in forager bees.

To investigate whether the increased amount of binding observed in the brains of adult bees was attributable solely to an increase in the density of ^{3}H-SCH-23390 binding sites, or also reflected a change in receptor affinity for the radioligand, saturation experiments were performed. The density of receptors (B_{max}) and the affinity (K_d) of the radioligand for the D1-like dopaminergic receptor in brain homogenates of newly emerged adult bees and adult forager bees were compared. The saturation data were analysed by the non-linear curve-fitting programme, EBDA/LIGAND. One-site and two-site models were fitted to the data and the goodness of fit compared by the programme with an *F* test. Representative Scatchard plots are presented in Fig. 2. Saturation curves of forager bee brain homogenates were best fitted by a one- site model yielding a mean affinity constant ($K_d = 9.5 \pm 2.0$ nmol·l^{-1}) within the same range as that calculated previously ($K_d = 6.3$ nmol·l^{-1}; Kokay and Mercer 1996). Saturation data derived from newly emerged bees, however, could be fitted to both a one-site and a two-site model. A two-site model was selected as, in each of four independent experiments, it gave a slightly better fit for the data. The apparent K_d of the ^{3}H-SCH-23390 binding sites in forager bee brain was not significantly different from that of the apparent high-affinity site detected in newly emerged bee brain (mean K_d 9.5 ± 2.0 and 12.5 ± 0.5 nmol·l^{-1}, respectively). The mean density of the high-affinity binding sites for ^{3}H-SCH-23390 was significantly higher in brain homogenates of forager bees ($B_{max} = 3.6 \pm 0.5$ pmol mg protein^{-1}) compared to newly emerged bees ($B_{max} = 1.0 \pm 0.2$ pmol mg protein^{-1}), consistent with the trend of increased amounts of specific ^{3}H-SCH-23390 binding with age as shown in Fig. 1. The low-affinity site detected by Scatchard transformation of the saturation data prepared from brain homogenates of newly emerged bees had an apparent mean affinity constant of 127 ± 25 nmol·l^{-1} and a calculated mean B_{max} of 2.9 ± 0.5 pmol mg protein^{-1}.

Binding sites for ^{3}H-spiperone

The binding of ^{3}H-spiperone was also investigated in bees of different ages. Figure 3 shows the amount of binding of this radioligand in brain homogenates prepared from bees ranging in age from pupal stage 6 through to 6-day-old adults and foragers. Specific binding was defined as total binding minus binding in the presence of 10^{-5} mol·l^{-1} domperidone. Binding sites for ^{3}H-spiperone were present in all age groups but they were detected at consistently lower levels than were ^{3}H-SCH-23390 binding sites. The amount of ^{3}H-spiperone binding in brain homogenates of foragers and 4-day-old adult bees is significantly higher than that

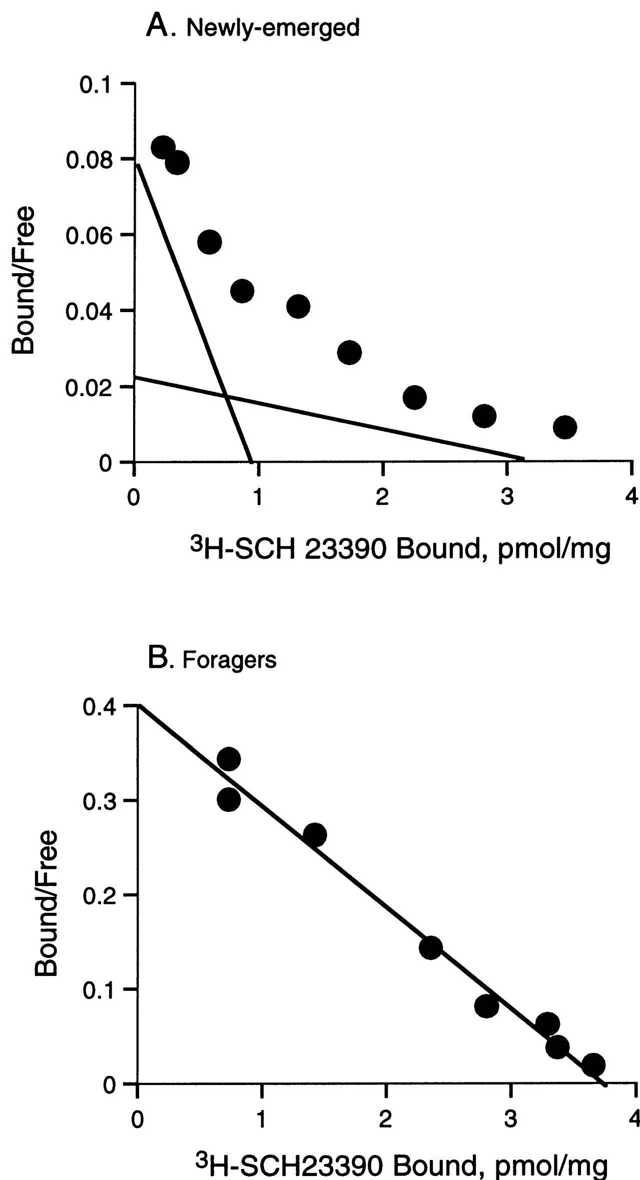

Fig. 2 A Representative Scatchard plot of ^{3}H-SCH23390 saturation curve data derived using brain homogenates prepared from newly emerged adult honey bees. A fixed concentration of ^{3}H-SCH23390 (2 nmol·l^{-1}) and varying amounts of unlabelled SCH23390 were used to produce final concentrations of SCH23390 ranging from 2.4 to 250 nmol·l^{-1}. Each experiment was repeated four times in triplicate. Scatchard analysis by LIGAND gave a two-site binding model. For the high-affinity site the calculated binding parameters for this representative plot were $K_d = 13.1$ nmol·l^{-1} and $B_{max} = 0.9$ pmol per mg membrane protein, and for the low-affinity site $K_d = 152$ nmol·l^{-1} and $B_{max} = 3.1$ pmol per mg protein. Mean parameter values (±SEM) of the four saturation experiments are provided in Results. **B** Representative Scatchard plot of ^{3}H-SCH23390 saturation curve data derived using brain homogenates prepared from forager honey bees. A fixed concentration of ^{3}H-SCH23390 (2 nmol·l^{-1}) and varying amounts of unlabelled SCH23390 were used to produce final concentrations of SCH23390 from 2.2 to 250 nmol·l^{-1}. Each experiment was repeated three times in triplicate. Data were best fitted by LIGAND to a one-site binding model. The calculated binding parameters for this plot were $K_d = 9.1$ nmol·l^{-1} and $B_{max} = 3.6$ pmol per mg^{-1} membrane protein. See Results for mean parameter values (±SEM) of the three saturation experiments

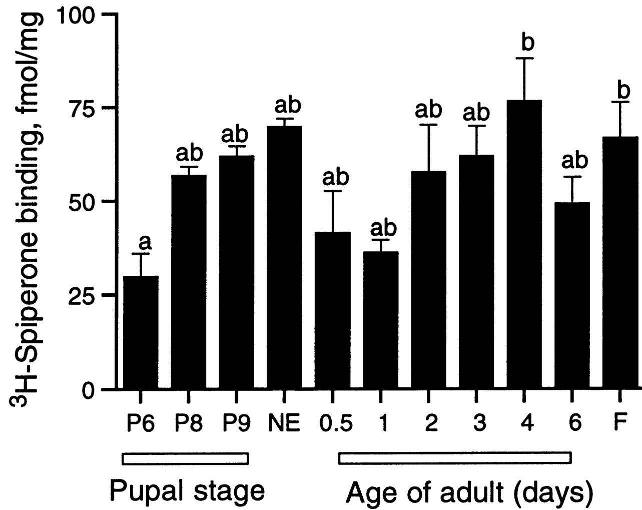

Fig. 3 Age-related changes in ^{3}H-spiperone binding in worker honey bee brains. Values represent mean specific binding (±SEM) in fmol per mg of protein of 0.5 nmol·l^{-1} ^{3}H-spiperone in brain homogenates of pupal bees (*P6–P9*), newly emerged adults (*NE*), adults of different ages (0.5–6 days) and foragers (*F*) of unknown age. Specific binding was calculated by subtracting non-specific binding (binding in the presence of 10^{-5} mol·l^{-1} domperidone) from total binding. Overall statistical significance was determined by ANOVA followed by Tukey's tests for post hoc comparisons. Letters that appear above each bar on the graph indicate whether or not differences between groups are significant. Groups that do not differ significantly share at least one letter. Groups with significantly different values have different letters

observed at pupal stage P6. Although additional fluctuations in specific binding of ^{3}H-spiperone are evident (Fig. 3), these changes are not statistically significant. The low numbers of ^{3}H-spiperone binding sites made it difficult to evaluate the significance of fluctuations in ^{3}H-spiperone binding with age. Saturation curves of newly emerged bee brain homogenates were not carried out using this ligand.

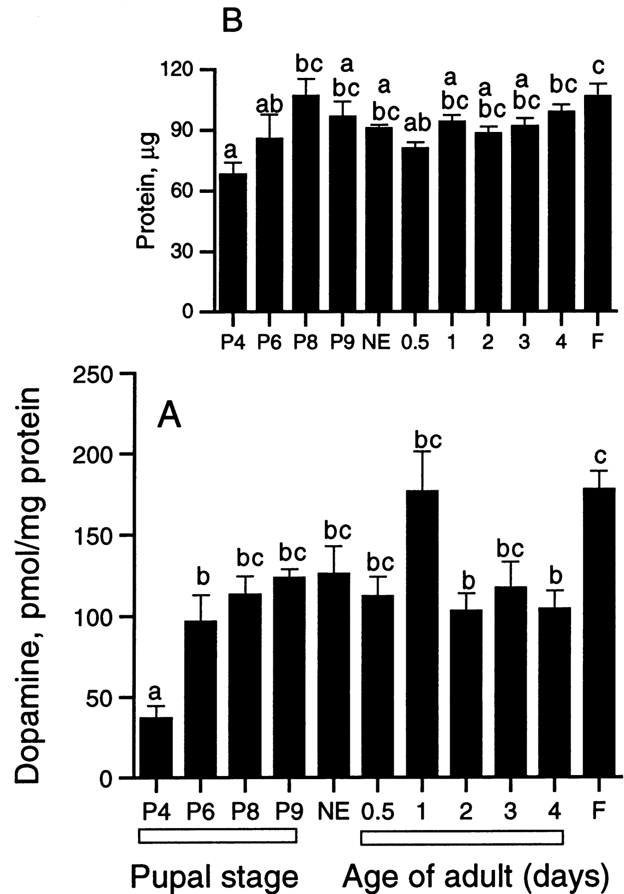

Fig. 4 A Levels of dopamine in the brains (minus the optic lobes) of pupal and adult worker honey bees. Each value represents the mean level (±SEM) of dopamine in pmol per mg protein. For each age group, five to ten samples (one brain or five pooled brains per sample) were analysed in two independent runs. *NE*, Newly emerged adults; *F*, foragers. Overall statistical significance was determined by ANOVA followed by Tukey's tests for post hoc comparisons. Letters that appear above each bar on the graph indicate whether or not differences between groups are significant. Groups that do not differ significantly share at least one letter. Groups with significantly different values have different letters. **B** Mean (±SEM) amount of protein per brain (minus the optic lobes) in pupal and in adult worker honey bees. At each stage five to ten brains were analysed

Brain dopamine levels

Dopamine levels in the brain (minus the optic lobes) of the bee increased significantly as bees progressed from P4 to P6 of metamorphic adult development (Fig. 4A). From P6 to adult emergence the levels of dopamine per mg of brain protein remained relatively stable. At 1 day after adult emergence there was an apparent surge in dopamine levels but this increase was transient and was not statistically significant. Relatively high levels of dopamine were also detected in the brains of forager bees, significantly higher than those detected in the brains of 2- and 4-day-old bees.

The mean level of dopamine detected in the optic lobes of the brain also increased significantly after pupal stage 4. Relatively high levels of dopamine were present in the optic lobes of older pupal bees (P6–P9 , Fig. 5A), with levels measured at P8 significantly higher than in 2-day-old adults. Dopamine levels in the optic lobes of 2-day-old adults were also significantly lower than those detected in 1-day-old adult bees.

Large variations in dopamine levels were found within some groups, for example, in pupal optic lobe samples (Fig. 5A) and in samples of the brain minus optic lobes of newly emerged and 1-day-old adults (Fig. 4A). These variations reflect marked differences between the dopamine levels recorded in mid summer and in late summer/early autumn for some groups. However, mean dopamine levels are presented here because for most groups the dopamine levels at the two sampling times were remarkably similar. Within group variations made it difficult to evaluate the significance of certain trends, such as the apparent rise in brain dopamine levels 1 day after adult emergence. No significant correlations were found between the amounts of binding

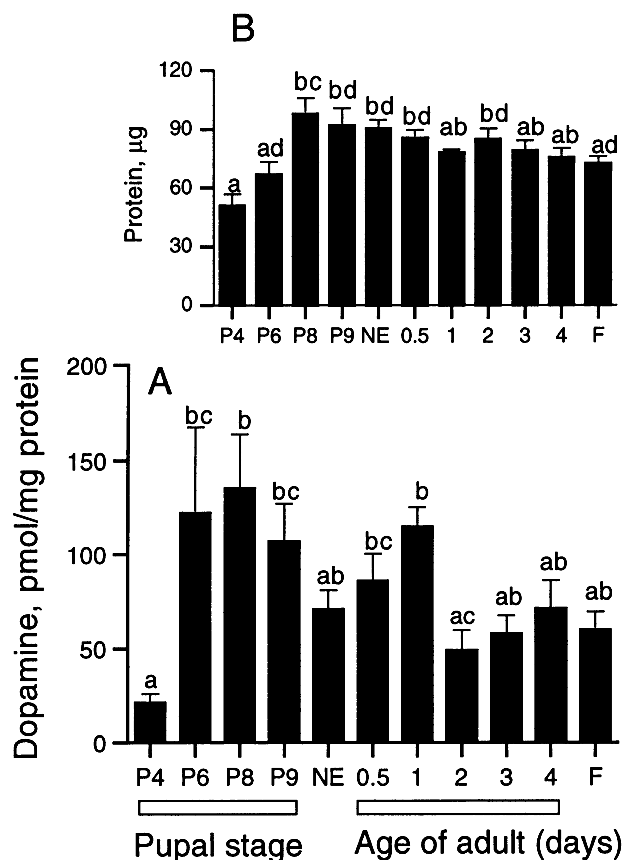

Fig. 5 A Levels of dopamine in the optic lobes of pupal and adult worker honey bees. Each value represents the mean level (±SEM) of dopamine in pmol per mg protein. At each age group, four to ten samples (one pair of optic lobes or five pooled pairs of optic lobes per sample) were analysed in two independent runs. *NE*, newly-emerged adults; *F*, foragers. Overall statistical significance was determined by ANOVA followed by Tukey's tests for post hoc comparisons. Letters that appear above each bar on the graph indicate whether or not differences between groups are significant. Groups that do not differ significantly share at least one letter. Groups with significantly different values have different letters. **B** Mean (±SEM) amount of protein per pair of optic lobes in pupal and in adult worker honey bees. At each stage four to ten pairs of optic lobes were analysed

of either ^{3}H-SCH-23390 or ^{3}H-spiperone and dopamine concentrations in the brain or the optic lobes of the bee.

Protein levels

Protein assays reveal a significant increase in the amount of protein present in the brain (minus optic lobes) of the bee during metamorphic adult development (Fig. 4B). The level of protein detected in the brains of pupal stage 4 bees is significantly lower than at pupal stage 8 and lower also than the protein levels detected in the brains of 4-day-old adults and foragers. Although from P8 to adult emergence the levels of protein in the brain appear to fall, this trend is not statistically significant. Protein levels in the brains of bees assayed 12 h after adult emergence however, are significantly lower than those detected in forager bees.

Mean protein levels detected in the optic lobes also increased significantly during pupal life (Fig. 5B). The amount of protein detected at pupal stage 4 was significantly lower than that found at pupal stages P8–P9. The mean amount of protein detected at P4 is also significantly lower than that detected in newly emerged adults, in adult bees 12 h after emergence, and in 2-day-old adults. Protein levels in the optic lobes were highest at pupal stage 8 (98 µg per pair of optic lobes), significantly higher than at stages P4–P6 and also higher than in forager bees. Mean protein content of the optic lobes did not fluctuate significantly after adult emergence.

Discussion

In the brain of the honey bee ^{3}H-SCH-23390 and ^{3}H-spiperone recognise D1- and D2-like dopamine receptors, respectively (Kokay and Mercer 1996). Here we show, firstly, that levels of binding sites in the brain for these dopaminergic ligands fluctuate significantly during the lifetime of the honey bee, and secondly, that the pattern of these changes is markedly different for the two dopamine receptor subtypes.

The appearance of D1- and D2-like dopamine receptors early in metamorphic adult development (Figs. 1, 3) is consistent with the possibility that in addition to its actions in the adult CNS, dopamine may play a role in the developing brain of the bee. In vitro studies suggest that dopamine may act as a growth regulatory signal in the developing nervous system (reviews: Lipton and Kater 1989; Whitaker-Azmitia 1991; Lauder 1993) and that the specific morphogenic effects of this amine depend on the receptor subtype activated (Lauder 1993). Stimulation of D1 dopamine receptors in cultured retinal cells, for example, has been shown to inhibit neurite outgrowth (Lankford et al. 1988; Santos Rodrigues and Dowling 1990), whereas in fetal cortical neuronal cultures and neuronal cell lines, activation of D2 receptors promotes the outgrowth of neurites (Todd 1992; Swarzenski et al. 1994). Analysis of the effects of dopamine on the structural and functional development of honey bee central neurons awaits further investigation. Recent studies reveal, however, that dopaminergic neurons in the brain of the bee express their transmitter phenotype early in metamorphic adult development and are well placed to influence the growth and reorganization of the brain neuropil (B.S. Kirchhof et al. unpublished data).

As there is a phenolaminergic component to the binding of ^{3}H-spiperone in bee brain homogenates (Kokay and Mercer 1996), and a lack of selective blocking agents makes it difficult to isolate the dopaminergic component of the binding of this ligand, the density of D2-like receptors reported here may be an overestimate. However, results from this study show that throughout the lifetime of the honey bee the number of ^{3}H-spiperone-binding sites in the bee brain is consistently lower than the number of sites labelled by the D1-dopamine receptor ligand ^{3}H-SCH-23390.

Furthermore, ^{3}H-SCH-23390 binding increases significantly during the lifetime of the adult bee. These results suggest ^{3}H-SCH-23390- and ^{3}H-spiperone-binding sites are regulated independently during the development and maturation of the honey bee nervous system. This is consistent also with recent results showing that ^{3}H-SCH-23390- and ^{3}H-spiperone-binding sites in the bee brain show little overlap in their distributions (I. C. Kokay, unpublished data).

The dramatic increase in the density of ^{3}H-SCH-23390 binding early in adult life coincides with changes both in the structure of the brain and in the behaviour of the bee. For example, during the first week of adult life the responsiveness of young worker bees to olfactory cues increases (Masson and Arnold 1984; Allan et al. 1987; Robinson 1987a) and primary olfactory centres of the brain (the antennal lobes) show a significant increase in volume (Withers et al. 1993; Winnington et al. 1996). As changes in brain structure associated with the maturation of the honey bee olfactory system are influenced significantly by environmental conditions during early adult life (Gascuel and Masson 1987: S.M. Morgan, unpublished data), experiments are underway currently to determine whether altering conditions in the hive affects the rate of increase in the density of D1-like dopamine receptors in the brain of young worker bees.

Worker honey bees exhibit an age-based polyethism; young workers performing duties within the hive such as cleaning, nursing, comb building and food handling, and older bees performing tasks such as guarding and foraging (Free 1977; Seeley 1985; Winston 1987; Robinson 1992). Foraging duties usually commence around 3 weeks of age. This shift to duties outside the hive is accompanied by an increase in dopamine levels in the brain of the bee (Taylor et al. 1992) and is associated also with high juvenile hormone (JH) titres in the haemolymph (see Robinson 1992). In vertebrates, dopamine has been strongly implicated in the ligand-independant activation of hormone receptors (Power et al. 1991) and in the moth, *Manduca sexta*, Geng et al. (1993) report a temporal correlation between changes in dopamine levels and endocrine events. Manipulation of JH levels in bees can induce precocious foraging behaviour (Jaycox et al. 1974; Jaycox 1976; Robinson 1985, 1987b) as well as predictable changes in the structure of the brain (Withers et al. 1995; D. Sigg, unpublished data). While D1-like dopamine receptor levels increase with age and are significantly higher in forager bees than in newly emerged bees (Fig. 1), preliminary results in this laboratory suggest that changes in brain dopamine levels resulting from manipulation of JH levels are not accompanied by changes in D1-like dopamine receptor densities (C.J. Mitchell, unpublished data). The possibility that D1-like dopamine receptors play a role in mediating effects of JH in the bee, however, warrents further attention.

The dramatic increase in D1-like dopamine receptor densities in the brain of the bee following adult emergence is reflected in the B_{max} for high-affinity ^{3}H-SCH-23390-binding sites in brain homogenates of foragers (3.6 pmol mg^{-1}) and newly emerged bees (B_{max} 1.0 pmol mg^{-1}). The B_{max} for ^{3}H-SCH-23390 binding to forager brain homogenates obtained in this study is slightly higher than that reported previously (1.9 pmol mg^{-1}; Kokay and Mercer 1996). This difference is likely to reflect, at least in part, the different techniques that were used in the two studies. Here, B_{max} was derived from saturation experiments in which fixed concentrations of radioligand were used in combination with a range of concentrations of unlabelled SCH-23390, whereas B_{max} reported elsewhere was calculated from saturation isotherms in which a range of concentrations of radioligand were used and non-specific binding was defined as binding in the presence of the dopaminergic antagonist *cis*-(Z)-flupentixol (Kokay and Mercer 1996). The evidence presented in this study suggesting that D1-like dopamine receptor densities in the brain of the adult worker bee increase significantly with age is supported by autoradiographic analyses, which reveal larger numbers of ^{3}H-SCH-23390-binding sites in forager-bee brain slices than in brain slices of newly emerged adult workers (I. C. Kokay, unpublished data).

In foragers, ^{3}H-SCH-23390 labels a single high-affinity site (Fig. 2B; Kokay and Mercer 1996) with an apparent dissociation constant not significantly different from that of the high-affinity site detected in brain homogenates of newly emerged adult bees. Scatchard analysis of ^{3}H-SCH-23390 binding in the brains of newly emerged adults, however, revealed binding heterogeneity that was not apparent in brain homogenates of foragers (Fig. 2). The identity of the apparent low-affinity site detected in newly emerged bees has yet to be determined. It seems likely, however, that this low-affinity site represents binding of ^{3}H-SCH-23390 to sites other than the D1-like dopamine receptor. Ontogenic studies of D1 dopamine receptors in vertebrates indicate that the affinity of this receptor does not change significantly during development (Giorgi et al. 1987; Agui et al. 1988; Gelbard et al. 1989; Murrin and Zeng 1990; Broaddus and Bennett 1990; Rao et al. 1991).

The binding affinity of ^{3}H-spiperone also remains similar during the development of the vertebrate nervous system (Bruinink et al. 1983; Murrin et al. 1985; Murrin and Zeng 1986). The saturation binding characteristics of this radioligand in bees of different ages were not examined in this study because in bee brain homogenates levels of specific binding of ^{3}H-spiperone are low (Fig. 3) and the dopaminergic and phenolaminergic components of spiperone binding are difficult to separate (Kokay and Mercer 1996).

Changes in ^{3}H-SCH-23390 and ^{3}H-spiperone binding-site densities did not show any direct correlation with age-related changes in endogenous dopamine levels. However, the pattern of changes in dopamine levels in the optic lobes was not identical to that observed in the rest of the brain (Figs. 4, 5). Whether, as in the vertebrate brain (Noisin and Thomas 1988), changes in

dopamine receptor densities in the bee brain are region specific, has yet to be determined.

In whole-body homogenates of *Drosophila*, a transient increase in dopamine concentration coincides with adult emergence (Martínez-Ramírez et al. 1992). A surge in dopamine levels was also observed in the brain of the honey bee 24 h after emergence, but this short-lived change in dopamine levels was not statistically significant. In the bee, the magnitude of such fluctuations appears to be influenced by seasonal changes in brain amine levels (D.J. Tayor, unpublished observation), such as those described by Harris and Woodring (1992). Recent studies in our laboratory reveal circadian changes also in brain amine levels of the bee (I.C. Kokay et al., unpublished data). The effects of such changes on dopamine receptor densities are being examined.

This study provides evidence for significant differences between dopamine receptor subtypes both in their overall density and in the receptor-density fluctuations that accompany the development and aging of the adult bee. While their early appearance supports the view that dopamine receptors may play a role in the development of the honey bee CNS, the dramatic increase in D1-like dopamine receptors densities in the brain of young adult bees also warrants further attention.

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