Naturwissenschaften 85, 343–346 (1998) Springer-Verlag 1998

A Royal Jelly Protein Is Expressed in a Subset of Kenyon Cells in the Mushroom Bodies of the Honey Bee Brain

R. Kucharski, R. Maleszka

Visual Sciences, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra, ACT 0200, Australia

D.C. Hayward, E.E. Ball

Molecular Genetics and Evolution Group, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra, ACT 0200, Australia

Received: 26 February 1998 / Accepted in revised form: 30 April 1998

Mushroom bodies (MBs), which are thought to represent centres of multimodal processing and integration in arthropod brains, are most evolved in social Hymenoptera. Experimental evidence suggests that they are involved in learning and the formation and storage of memory. In the honey bee (*Apis mellifera*) the MBs undergo an internal structural reorganisation associated with behavioural development. We here show that the expression of a recently discovered, highly conserved royal jelly protein (RJP-3) is concentrated in a defined population of the Kenyon cells (intrinsic neurons) in honey bee MB. Thus, in addition to their poorly understood role via the digestive system, RJPs may have a novel, previously unsuspected function in the brain. The restricted distribution of RJP-3 message within the MBs supports the view that the Kenyon cells, despite their isomorphic appearance, are divided into functionally distinct sub-compartments.

In arthropods, phylogenetically conserved brain centres, known as mushroom bodies (MBs), have been implicated in higher sensory integration and other complex brain functions and are thought to correspond functionally to a vertebrate centre of learning, the hippocampus (Davis and Han 1996; Strausfeld et al. 1995). These symmetrically paired neuropils, two on each side of the brain, are highly ordered and are connected by neuronal pathways, both afferent and efferent, to other neuropils of the central nervous system (Withers et al. 1993; Fahrbach and Robinson 1995; Rybak and Menzel 1993; Ito et al. 1997). Experimental evidence suggests that the fundamental computational properties of MBs are provided by their intrinsic neurons, known as Kenyon cells, that range in numbers from 5000 in *Drosophila* to approximately 350–400,000 in the honey bee, *Apis mellifera*, and the cockroach, *Periplaneta americana* (Yang et al. 1995; Davis and Han 1996). Kenyon cell dendrites are localised within the calyces that form cup-like structures densely packed with cell bodies. Beneath each calyx the Kenyon cell axons project into the bipartite pedunculus (Rybak and Menzel 1993). Contrary to some ideas portraying Kenyon cells as isomorphic arrays of functionally equivalent intrinsic neurons (Laurent and Davidowitz 1994), a growing body of evidence suggests that MBs are not entirely isomorphic (Menzel et al. 1994; Yang et al. 1995). In particular, recent studies in *Drosophila* revealed a previously unsuspected structural complexity within the MBs, each of which appears to be subdivided longitudinally into concentric rings (Yang et al. 1995).

MBs are particularly well developed in social hymenopteran insects, such as the honey bee. Anatomical studies have shown that MBs in the honey bee undergo striking structural alterations that may be correlated with behavioural maturation, specifically with the transition from within-hive to foraging tasks (Withers et al. 1993; Fahrbach and Robinson 1995). The most notable is an increase in the volume of the neuropil of the MBs in foraging bees. Some authors emphasise that both age and experience are important factors in the structural development of different sub-compartments of MBs (Durst et al. 1994). A link between the changes of the neuropil volume of MBs and orientation flights (learning the location of the nest, or place memory) has also been proposed (Fahrbach and Robinson 1995). Since there is no evidence for neurogenesis in the adult bee brain (Fahrbach et al. 1995), volumetric remodelling of the MB neuropile is consistent with the addition of new synapses (Withers et al. 1993; Fahrbach and Robinson 1995).

We were curious to determine whether molecular signals that link behavioural development with the anatomical remodelling of MBs could be detected by comparing patterns of gene expression in brains of bees representing various developmental stages such as young, newly emerged individuals, and adult foragers. We have employed the differential-display reverse transcriptase– polymerase chain reaction (DD-RT-PCR) technology (Liang and Pardee 1992) to visualise gene expression in the bee brain. DD-RT-PCR is a gel-based approach that combines parallel analysis of many samples with the sensitivity of PCR and is best described as an mRNA finger-printing technique that allows for an identification of differentially expressed genes by comparative display of arbitrarily amplified cDNA subsets.

Here we report that one of the differentially expressed MB transcripts encodes a recently described royal jelly protein (RJP). This clone, referred to as RJP-3, encodes a 432 amino acid polypeptide that is almost identical (see legend to Fig. 1) to a recently reported 56-kDa protein expressed in the hypopharyngeal gland of the

Correspondence to: R. Maleszka
e-mail: maleszka@rsbs.anu.edu.au

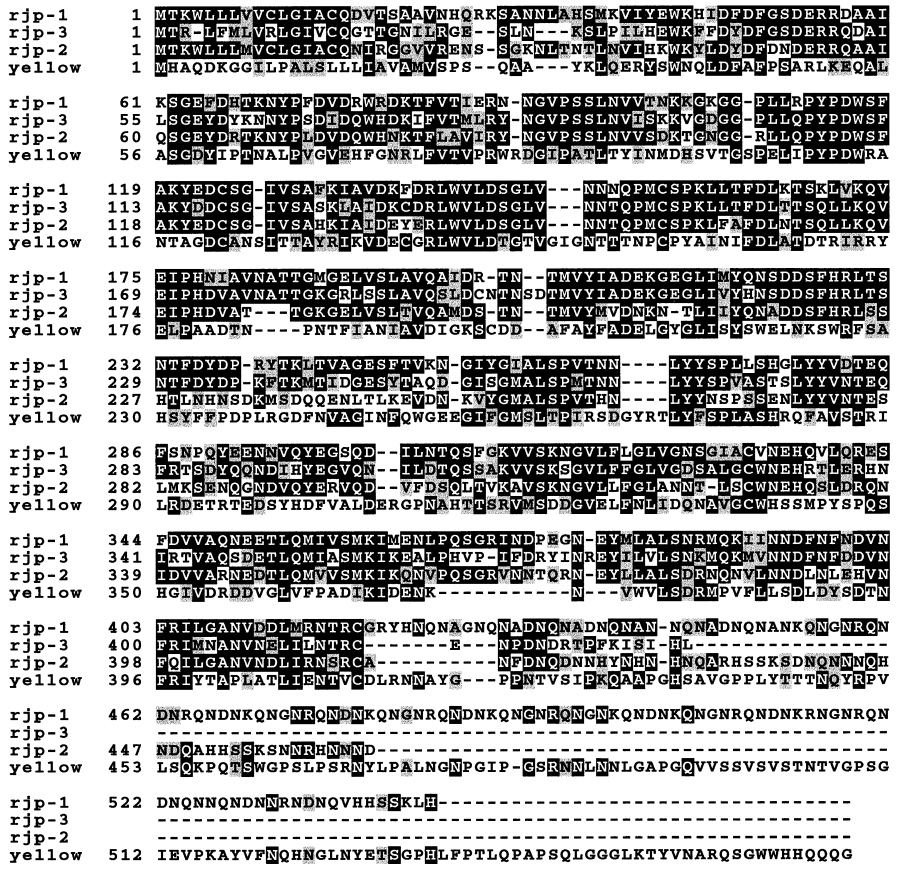

Fig. 1. Alignment of the predicted polypeptides of the honey bee RJPs and the yellow protein from *Drosophila*. *Black boxes*, identical amino acids in at least three sequences; *grey boxes*, conservative changes. The most likely cleavage sites for signal peptides are: RJP-1:21/22 (TSA-AV), RJP-2:20/21 (IRG-GV), RJP-3:19/20 (TTG-NI) and yellow protein: 21/22 (SWA-AY). Note: the RJP-3 sequence used in this alignment represents a conceptual translation of our own clone, and it differs in position 9 from the published one (C instead of R). The alignment was generated with CLUSTAL W (Thompson et al. 1994). Database searches were performed at the National Center for Biotechnology Information using the BLAST server. The presence of signal peptides and the most likely cleavage sites were predicted with SignalP VI.I (Nielsen et al. 1997). Other bioinformatics and molecular methods are described elsewhere (Maleszka and Stange 1997; Kucharski and Maleszka 1998). GenBank accession numbers are: RJP-1:Z26318; RJP-2:Z26319; RJP-3:D79207, yellow protein: P09957

worker bee (Ohashi et al. 1997; GenBank, D79207). RJP-3 shows a high level of identity to the previously published major RJPs, RJP-1 and RJP-2 (63% and 56% identity, respectively; Fig. 1). Thus, these three proteins are clearly paralogs and belong to a multiple protein family that evolved by gene duplication. RJP-3 also exhibits 28% amino acid identity with the *Drosophila* yellow protein (Fig. 1). Although the yellow protein may represent a structural ortholog of RJPs, its involvement in cuticle pigmentation (Geyer et al. 1986, Kornezos and Chia 1992) implies a distinct function. Hence, RJPs may be unique to Hymenoptera.

All RJP paralogs have a predicted N-terminal signal peptide (Fig. 1), indicating that these proteins are secreted. In addition, RJPs contain consensus target sites for N-glyocosylation, as well as consensus phosphorylation sites recognised by protein kinase C, Ca-calmodulin dependent protein kinase II and tyrosine-protein kinase (Klaudiny et al. 1994). Southern blots indicate that in addition to the three already sequenced genes encoding RJ proteins the honey bee genome contains other, unknown RJP-related genes that are likely to encode proteins belonging to the royal jelly family (not shown). Developmental northern blots spanning the interval from late pupae to older foragers show that RJP-3 transcription begins shortly after emergence, and reaches a high level approximately 48 h later (Fig. 2A). The elevated level of RJP-3 expression continues for at least 2–3 weeks, and the message is still detectable in bees 22 days old (Fig. 2B).

All RJP transcripts are abundant in the cephalic, hypopharyngeal gland (Ohashi et al. 1997, Kucharski and Maleszka unpublished), but the RJP-3 transcript is also detectable in the brain. In order to corroborate our northern blots and PCR results we have investigated the pattern of RJP-3 expression in the brain by using in situ hybridisation. The distribution of RJP-3 mRNA in the mushroom bodies, shown in Fig. 3, not only confirms its presence in the brain but also reveals that the expression of the RJP-3 message is concentrated in a defined population of Kenyon cells (intrinsic neurons of MBs). This result suggests a novel, previously unsuspected brain function for the RJP(s).

The RJPs are components of the mandibular secretion of worker bees that is used to feed the larvae. The food of the larval queen, the so-called royal jelly, is believed to act as a phagostimulant which triggers a neuroendocrine cascade resulting in queen development (Moritz and Southwick 1992). However, little is known about the mode of action of royal jelly, in particular the functional significance of the various RJP paralogs. Unlike stingless bees (Meliponinae) which utilise two genetic loci to determine the development of queens, honeybees (Apinae) can rear queens from any female larva by feeding it exclusively with royal jelly (Moritz and Southwick 1992). The honeybee queens are not only morphologically and anatomically distinct but also live 20–30 times longer than workers or drones. It is generally accepted that large quantities of royal jelly consumed by the larval queen accelerate feeding and/or metabolic rates and

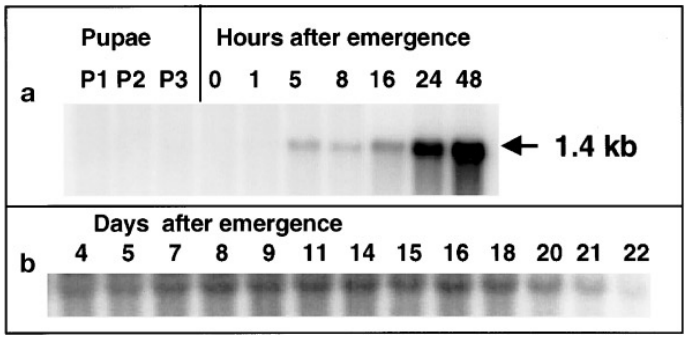

Fig. 2a,b. Northern blot hybridisation, generated using Imagequant software on a Molecular Dynamics PhosphorImager, showing expression of RJP-3 message in the heads of honey bees of various ages. a) Illustration of the pattern of expression in late pupae and in newly emerged bees. b) Later stages of expression during adult life. The amount of RNA loaded per lane in a) and b) corresponds to 3 and 1 heads, respectively. To standardise the amount of RNA loaded per lane all samples were stained with the RNA dye, SybrGreen II (FMC Bioproducts) and quantified with a Vistra FluorImager SI. We estimate the loading accuracy to be 10–15%. RNA ladder from NEN BioLabs was used as a size marker. Other experimental details are described by Kucharski and Maleszka (1998)

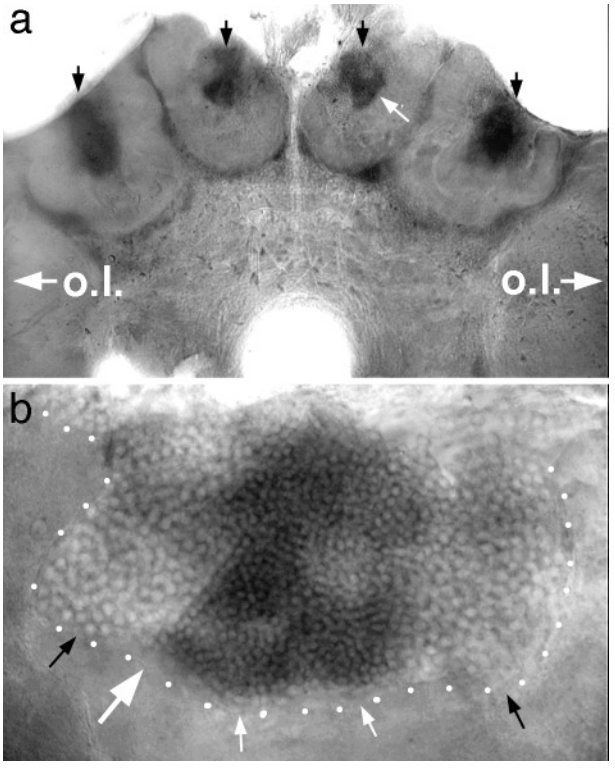

Fig. 3a,b. In situ hybridisation (Broadus and Doe 1995) with an anti-sense riboprobe corresponding to the 3' region of the RJP-3 message reveals that the transcript is localised within the brain of worker bees. a) Thick section of the medial portion of a bee brain reveals message in the Kenyon cells within and surrounding the mushroom bodies (*black arrows*). *o.l.*, Lateral position of the optic lobes; *white arrow*, vertical midline of b). b) Differential distribution of the message within the mushroom body. *White dots*, limits of the Kenyon cells. Lateral Kenyon cells contain little message (*black arrows*), while medial Kenyon cells contain high levels. Thick freehand sections were cut of the brains of 15 bees (three bees per probe, two anti-sense probes and three sense controls) which were then treated as whole mount in situ preparations

lead to the induction of midgut receptors. These receptors are thought to stimulate the corpora allata-mediated secretion of juvenile hormone, a major factor controlling the queen's development (Robinson and Vargo 1997). This cascade leads to the synthesis of largely unknown, "queen-specific" proteins (Winston 1987, and references therein).

Our results indicate a possible dual role for RJPs. In addition to their poorly understood role via the digestive system the expression of RJP-3 in a sub-population of Kenyon cells leads to the suggestion that RJP-3 may have a role in the central brain. One alternative is that RJPs regulate the experience- and/or age-related structural plasticity of the MB in conjunction with, or under the control of, juvenile hormone, which has been implicated as an organiser of the MB (Fahrbach and Robinson 1995, 1996; Robinson and Vargo 1997). It is conceivable that RJP in the brain is part of a complex regulatory network that combines inputs from a number of body compartments.

The sharp cut-off of RJP-3 message corresponding to the straight line of cells along the axis of the large white arrow in Fig. 3 appears to indicate a physical or developmental compartment within the MB. Thus, our finding is consistent with the model for MB function recently proposed in *Drosophila* whereby parallel sub-compartments exhibit discrete patterns of gene expression (de Belle 1995; Yang et al. 1995) rather than being isomorphic arrays of functionally equivalent Kenyon cells. Recent studies in *Drosophila* have shown that genes whose expression is restricted to MBs, or MB compartments, are rare. An extensive survey of 6000 enhancer detector lines revealed only 15% of transcripts to be preferentially expressed in one brain region, including those with enhanced expression in the MBs, antennal lobes and optic lobes (Han et al. 1996). Until now MB-specific markers have not been reported in the honey bee. Although the neuronal, type II cAMP-dependent protein kinase has been found to be concentrated largely in the MBs, it is also detectable, to a lesser extent, in other regions of the

brain that are likely to utilise the cAMP second messenger pathway (Muller 1997). In this context our finding that the RJP-3 message is concentrated in a defined area of Kenyon cells is particularly exciting. RJP-3 may therefore prove to be useful as a unique resource for the future correlation of brain structure and function, specifically to study changes occurring in the bee brain during behavioural development, learning and memory formation.

We thank Paul Helliwell for technical assistance, Shao-Wu Zhang, Miriam Lehrer and M.V. Srinivasan for advice on the use of bee facilities and the ANU Electron Microscopy Unit for use of facilities.

Broadus J, Doe CQ (1995) Evolution of neuroblast identity: seven-up and prospero reveal homologous and divergent neuroblast fates in *Drosophila* and *Schistocerca*. Development 121:3989–3996

Davis RL, Han K-A (1996) Neuroanatomy: mushrooming mushroom bodies. Curr Biol 6:146 –148

de Belle JS (1995) *Drosophila* mushroom body subdomains: innate or learned representations of odor preference and sexual orientation? Neuron 15:245–247

Durst C, Eichmuller S, Menzel R (1994) Development and experience lead to increased volume of sub-compartments in the honey bee mushroom body. Behav Neural Biol 62:259–263

Fahrbach SE, Robinson GE (1995) Behavioural development in the honey bee: toward the study of learning under natural conditions. Learning Memory 2:199–224

Fahrbach SE, Robinson GE (1996) Juvenile hormone, behavioural maturation, and brain structure in the honey bee. Dev Neurosci 18:102–114

Fahrbach SE, Strande JI, Robinson GE (1995) Neurogenesis is absent in the brains of adult honey bees and does not explain behavioural neuroplasticity. Neurosci Lett 197:145–148

Geyer PK, Spana C, Corces VG (1986) On the molecular mechanism of gypsy-induced mutations at the yellow locus of *Drosophila melanogaster*. EMBO J 5:2657–2662

Han P-L, Meller V, Davis RL (1996) The *Drosophila* brain revisited by enhancer detection. J Neurobiol 31:88–102

Ito K, Awano W, Suzuki K, Hiromi Y, Yamamoto D (1997) The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurons and glial cells. Development 124:761–771

Klaudiny J, Hanes J, Kulifajova J, Albert S, Simuth J (1994) Molecular cloning of two cDNAs from the head of the nurse honey bee (*Apis mellifera L.*) for coding related proteins of royal jelly. J Apicul Res 33:105–111

Kornezos A, Chia W (1992) Apical secretion and association of the *Drosophila* yellow gene product with developing larval cuticule structures during embryogenesis. Mol Gen Genet 235:397–405

Kucharski R, Maleszka R (1998) Arginine kinase is highly expressed in the compound eye of the honey bee, *Apis mellifera*. Gene (in press)

Laurent G, Davidowitz H (1994) Encoding of olfactory information with oscillating neural assemblies. Science 265:1872–1874

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967–971

Maleszka R, Stange G (1997) Molecular cloning, by a novel approach, of a cDNA encoding a putative olfactory protein in the labial palps of the moth *Cactoblastis cactorum*. Gene 202:39–43

Menzel R, Durst C, Erber J, Eichmuller S, Hammer M, Hildebrandt H, Mauelshagen J, Muller U, Rosenboom H, Rybak J (1994) The mushroom bodies in honeybees: from molecules to behaviour. In: Schildberger K, Elsner N (eds) Neural basis of behavioural adaptations. Fischer, Stuttgart, pp 81–102

Moritz RFA, Southwick EE (1992) Bees as superorganisms – an evolutionary reality. Springer, Berlin Heidelberg New York

Muller U (1997) Neuronal cAMP-dependent protein kinase type II is concentrated in mushroom bodies of *Drosophila melanogaster* and the honeybee *Apis mellifera*. J Neurobiol 33:33–44

Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering 10:1–6

Ohashi K, Natori S, Kubo T (1997) Change in the mode of gene expression of the hypopharyngeal gland cells with age-dependent role change of the worker honeybee *Apis mellifera* L. Eur J Biochem 249:797–802

Robinson GE, Vargo EL (1997) Juvenile hormone in adult eusocial Hymenoptera: Gonadotropin and behavioural pacemaker. Arch Insect Biochem Physiol 35:559–583

Rybak J, Menzel R (1993) Anatomy of the mushroom bodies in the honey bee brain: The neuronal connections of the alpha-lobe. J Comp Neurol 334:444 –465

Strausfeld NJ, Buschbeck EK, Gomez RS (1995) The arthropod mushroom body: Its functional roles, evolutionary enigmas and mistaken identities. In: Breidbach O, Kutsch W (eds) The nervous system of invertebrates: an evolutionary and comparative approach. Birkhauser, Basel, pp 349–382

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Winston ML (1987) The biology of the honey bee. Harvard University Press, Cambridge

Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honey bee. Nature 364:238 –240

Yang MY, Armstrong JD, Vilinsky I, Strausfeld NJ, Kaiser K (1995) Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. Neuron 15:45–54

