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Structural and proteomic analyses reveal regional brain differences during honeybee aging

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SUMMARY

Among insects, learning is particularly well studied in the fruit fly *Drosophila melanogaster* and the honeybee *Apis mellifera*. A senescence-dependent decline in classic pavlovian conditioning is demonstrated for both species. To understand how aging affects learning, genetic approaches used with *Drosophila* can benefit from complementary studies in *Apis*. Specifically, honeybees have a larger brain size allowing for compartment-specific approaches, and a unique life-history plasticity. They usually perform within-nest tasks early in life (nest bees) and later they collect food (foragers). Senescence of learning performance is a function of the bees' foraging duration but underlying causes are poorly understood. As cognitive aging is commonly associated with structural and biochemical changes in the brain, we hypothesized that brain areas implicated in learning change in synaptic and biochemical composition with increased foraging duration. First, we used synapse-specific immunohistochemistry and proteomics to screen for alterations in the calyx region of the mushroom body, a key structure for memory formation. Using proteomics, we next profiled the central brain, which comprises all higher-order integration centers. We show that, with increased foraging duration, levels of kinases, synaptic- and neuronal growth-related proteins decline in the central brain while the calyx region remains intact both in structure and biochemistry. We suggest that proteome-level changes within major anatomical sites of memory formation other than the calyx region could be central to learning dysfunction. These include large compartments of the central brain, such as the mushroom body's output regions and the antennal lobes. Our data provide novel information toward heterogeneity in the aging insect brain, and demonstrate advantages of the honeybee for invertebrate neurogerontological research.

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Key words: senescence, aging, neuronal degradation, learning, immunohistochemistry, proteomics.

INTRODUCTION

Senescence of brain functions is a characteristic feature of aged individuals of the fruit fly (*Drosophila melanogaster*) and the honeybee (*Apis mellifera*) (Behrends et al., 2007; Mery, 2007; Tamura et al., 2003). These species are the most commonly used models in insect neuroscience (Berry et al., 2008; Fiala, 2007; Menzel et al., 2006). Among the different compartments of the insect central brain, the paired mushroom bodies (MB) are key sites of memory formation and are believed to be crucial for normal brain function (Hammer and Menzel, 1998; Szyszka et al., 2008; Yu et al., 2006; Zars et al., 2000). The MB's input region, the calyx, has traditionally been a focus of studies addressing experience-dependent changes during maturation of synaptic structures (Fahrbach et al., 2003; Farris et al., 2001; Menzel, 2001; Strausfeld et al., 1998).

At the molecular level, several proteins are known to contribute to the different forms of memory (e.g. long-, mid- and short-term) in insects. For example, protein kinase C (PKC) influences mid-term memory (Grunbaum and Muller, 1998) whereas the cyclic AMP (cAMP)/protein kinase A (PKA) cascade is required for long term memory formation (Muller, 2000). However, little is known about how brain structure and biochemistry change in aging insects and how these changes can contribute to functional decline.

In honeybees, an emerging model in aging research (Münch et al., 2008), senescence of associative olfactory- and tactile learning is linked to a division of labor (Behrends et al., 2007; Scheiner and Amdam, 2009). Initially, workers perform within-nest tasks (nest bees) and later in life they forage outside (foragers). Foragers exhibit neuronal outgrowth during behavioral maturation (Farris et al., 2001), yet they are generally characterized by reduced somatic maintenance and show impaired learning performance after an extended period of foraging (Behrends et al., 2007). This suggests that brain structures and biochemical pathways central to learning and memory can be negatively affected by foraging duration after neuronal outgrowth is complete.

In the present study, we used honeybees to examine structural and proteomic features of identified brain areas after short (≥5 days) and extremely long (≥15 days) foraging durations. We focused on the mushroom body's calyx and the central brain – the latter with all major sites of memory formation included but the large optical lobes excluded (Fig. 1) – which allowed us to investigate characteristics of individual bees. We documented foraging-dependent changes in the central brain protein matrix that resemble senescence-related patterns in *Drosophila* and vertebrates. By contrast, the structure and biochemistry of the calyx remained intact.

MATERIALS AND METHODS

Animals

Experiments were conducted at the University of Life Sciences, Norway. Honeybees (*Apis mellifera* L.) are characterized by a

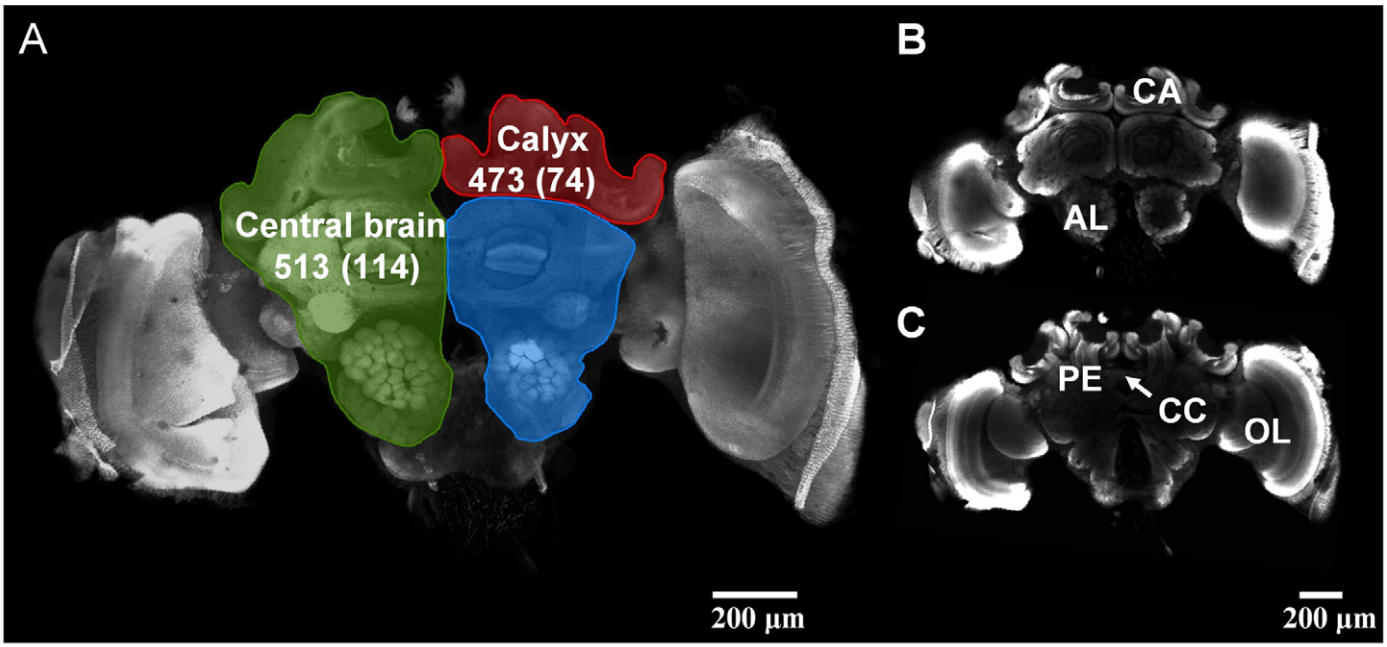

Fig. 1. Overview of the honeybee brain. The central brain (green) includes the calyx (CA, red), the antennal lobes (AL), the central complex (CC) and the mushroom bodies with pedunculi (PE). The large optical lobes (OL) with mainly lower-order brain centers for visual information processing were excluded from central brain analyses. Shown are maximum projection view (A) and single optical sections (B,C) of confocal image stacks. Neuropiles were visualized with an anti-synapsin antibody. Numbers refer to proteins identified in a proteomics analysis. Numbers in parentheses refer to protein identifications exclusive to the respective tissue. White bars: 200 µm.

decline in learning performance after ≥15 days of foraging (Behrends et al., 2007; Scheiner and Amdam, 2009). In order to assure a clear separation of similarly aged forager cohorts, we first assessed the phenotype (Table 1) and demographical characteristics (survivorship) of bees after ≥5 and ≥15 days of foraging. Foraging age was determined as follows: newly emerged bees (N>4000) were individually paint-marked and introduced into three colonies. Following foraging onset (2–3 weeks later), bees were re-marked when returning from first foraging flights (N=1500) and thereafter recaptured after 5 or 15 days of foraging. After 15 days of foraging, only a minor fraction (<10%) of confirmed foragers could be retrieved. For further analyses, we used specimens that met the profiled criteria of bees with overall short foraging duration (≥5 days) *vs* extended foraging duration (≥15 days; Table 1). In addition, for the demographically under-represented group with ≥15 foraging days, we only accepted individuals where the foraging duration was confirmed by paint marks.

Dissection

Individuals were chilled on ice until they became motionless. Brain tissue was dissected in phosphate buffered saline (PBS) and immediately transferred into either fixative [4% paraformaldehyde (PFA) in PBS] for histology or into protein extraction buffer (see below) for proteomic analysis.

Histology

Brains were fixed overnight in PFA at 4°C and then rinsed three times, 10 min each, in PBS. Fixed brains were incubated overnight in 30% sucrose/PBS solution, then covered with Tissue-Tek O.C.T. (Miles Laboratories, Elkhart, IN, USA), shock-frozen in liquid nitrogen and stored at –80°C. After thawing and rinsing stored samples in PBS, lipids were extracted in an ascending ethanol series (50, 70, 90, 100%; 10 min each) followed by a reverse rehydration procedure. To allow for the antibody to penetrate even whole brain preparations, brains were microwaved five times, 2 min each time. To prevent excessive heating, the tubes containing the brain tissue were constantly cooled in 1 liter of ice water. Brains were then pre-incubated for 1 h in 10% bovine serum albumin/PBS solution and incubated for six days with a primary antibody directed against synapsin [SYNORF1, 1:10 (Klagges et al., 1996)]. To avoid tissue deterioration during the long incubation time, 0.1% sodium azide was added to the antibody solution. Subsequently, a Cy5 coupled goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA, 1:200 in PBS) was applied for one day. Brain tissue was then rinsed for 30 min in PBS, cleared and mounted in methyl

Table 1. Foraging-dependent physiological classification of bees

Phenotype	Foraging duration ≥5 days	Foraging duration ≥15 days
Wingwear	Intact wings	Worn wings
Hair coat	Dense on thorax and head	Hairless patches on thorax and head
Head capsule tissue	Hypertrophied hypopharyngeal glands	Atrophied hypopharyngeal glands

Bees caught after different foraging durations (≥5 days and ≥15 days) were characterized based on wing wear, gland size and hair coat. This classification provided a framework of physiological markers for subsequent sample collections.

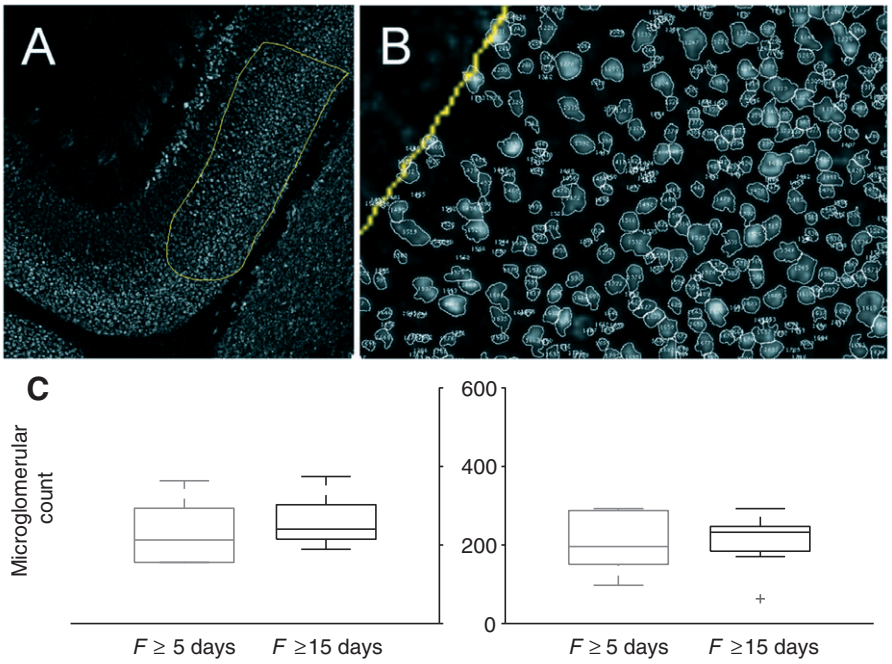

Fig. 2. The number of presynaptic complexes within the calyx is not related to foraging duration. (A) Maximum projection view of a representative image stack (depth [*Z*]=5 μm) that shows microglomerular presynaptic complexes labeled with an anti-synapsin antibody. Outlined (yellow line) is the analyzed area representing the volume of interest with [*Z*]=5 μm depth. (B) Depiction of the quantification method used. The original gray-level image and the connected pixel components (singly recognized presynaptic complexes) are superimposed. (C) Two volumes from defined depth levels, separated by *Z*=20 μm, were analyzed. In both volumes the number of presynaptic complexes did not differ between groups (Mann–Whitney *U*-test, $P>0.05$, $N=6/7$ for $F\geq5$ days/$F\geq15$ days). The number of presynaptic complexes was normalized to 5000 μm^3 for the outlined regions of each individual preparation. Boxplots represent medians and 25–75 percentiles. $F\geq5$ days=foragers with at least 5 days of foraging experience. $F\geq15$ days=foragers with at least 15 days of foraging experience.

salicylate.

Image acquisition and analyses

A Leica TCS SP5 Laser Scanning Microscope (LSCM) (Leica Microsystems, Wetzlar, Germany) equipped with an Ar/Kr Laser served to acquire image stacks of the labeled specimens. Images for quantitative analyses were viewed at high resolution with a ×40 oil immersion objective (numerical aperture 1.25). The voxel size for all image stacks was set to 0.3×0.3×0.5 μm.

To assure consistency between specimens, neuropilar volumes were obtained from identified collar areas (Fig. 2A) within whole brain preparations at defined distances from the dorsal most end of each medial calyx cup. To achieve high-throughput quantification of large numbers of presynaptic complexes, we used algorithms that automatically count connected pixel components in binary 2-D images (ImageJ, http://rsbweb.nih.gov/ij). Image stacks of 5 μm depth were first merged into a single 2-D image using a 5 μm depth value, which minimized superposition of microglomeruli from other levels (Fig. 2B). A person without prior knowledge of the identity of individual images assigned thresholds for gray-level image segmentation. A watershed algorithm (http://rsb.info.nih.gov/ij/docs/index.html) was used to remove overlap of particles due to limited optical resolution. The number of connected pixel components was then counted automatically (Fig. 2B) and further analyzed with Excel (Microsoft Corporation, Redmond, WA, USA) and Statistica (Statsoft, Tulsa, OK, USA).

Protein extraction and digestion

Samples (central brains or calyces from individual bees) were each homogenized in 150 μl of protein extraction buffer (50 mmol l^{-1} Tris, pH 8.5, 2% SDS, 5% beta-mercaptoethanol, 0.15 mol l^{-1} NaCl, 30% glycerol) per sample. Samples were then vortexed vigorously, boiled at 95°C for 5 min, vortexed again and centrifuged for 2 min at 10,000 ***g***. The supernatant was subjected to methanol/chloroform precipitation (Wessel and Flugge, 1984). The pellets were air-dried for 10 min and proteins were redissolved in 50 μl of extraction buffer (EB; containing 50 mmol l^{-1} Tris, pH 8.5, 6 mol l^{-1} urea, 2 mol l^{-1} thiourea, 0.15 mol l^{-1} NaCl, 1 mmol l^{-1} $CaCl_2$). Then, 150 μl of EB without urea/thiourea was added and the samples were vortexed. Proteins were digested over night at 30°C using 1 μg of trypsin in digestion buffer (50 mmol l^{-1} Tris, pH 8.5, 0.15 mol l^{-1} NaCl, 1 mmol l^{-1} $CaCl_2$). The next day peptide desalting was performed as previously described (Rappsilber et al., 2003; Wolschin and Amdam, 2007). Peptides were redissolved in 10 μl of 5% acetonitrile/2% trifluoroacetic acid (TFA), and approximately 10 μg per sample were used in a non-targeted LC–MS/MS (liquid chromatography–mass spectrometry) analysis. Six samples were analyzed per group.

LC–MS/MS analysis

LC–MS/MS analysis was essentially carried out as described before (Wolschin and Amdam, 2007), with the following modifications: peptides were separated on a picofrit column (75 μm ID, New Objective, Woburn, MA, USA) using a 105 min gradient ranging from 95% A (0.1% formic acid, 99.9% H_2O) to 80% B (0.1% formic acid, 99.9% acetonitrile), followed by a 15 min equilibration step. Blanks were run between samples, and samples were randomized. Samples were measured on a linear ion trap (LTQ, Thermo Electron, San Diego, CA, USA). Database search (OMSSA 2.0.0, http://pubchem.ncbi.nlm.nih.gov/omssa/) was conducted using a 0.8 Da fragment and precursor tolerance and a maximum of two missed cleavages. Methionine oxidation and deamidation of N and Q were included as variable modifications. Only tryptic sequences were allowed, and the program was set to initially allow for eight possible peptide hits per spectrum, which were then filtered to one peptide hit per spectrum.

Statistical analyses including Mann–Whitney *U*-test and control for type 1 error inflation by bootstrapping were essentially performed as described previously using total spectral count as a reference for normalization (Wolschin and Amdam, 2007). In order to be considered for quantification, proteins had to be identified with a spectral count of ≥3 and a peptide count of at least two (e-value ≤0.1) in at least four of the replicates of one group. In addition,

protein changes were only ranked significant if the mean corrected spectral count between groups varied by at least twofold. For protein identifications, at least two peptides (e-value ≤0.1) were required. False discovery rate (FDR) for peptide identifications was <0.95% (and 0 for protein hits) as determined by searches against a database with reversed sequences {FDR = [hits to reversed database / (hits to original database + hits to reversed database)] × 100}.

RESULTS

In honeybees, age-related functional deficits develop as a function of foraging duration, so that learning performance is reduced after ≥15 days of foraging (Behrends et al., 2007; Scheiner and Amdam, 2009). In our experiment, cohorts with short (≥5 days) and extended (≥15days) foraging durations were verified by an established paint-marking scheme as well as by individual phenotyping (see Materials and methods section for details). All experiments were carried out on calyces or central brains of individual bees.

(1) Examination of the calyx compartment of the honeybee brain

The calyx is a well defined and easily accessible brain structure with a stereotypical synaptic organization that makes it an ideal candidate for structural studies (Groh et al., 2004). See Fig. 1 for an overview of the brain regions analyzed in our study. The presynaptic boutons of the calyx are surrounded by a shell of postsynaptic specializations from Kenyon cells (intrinsic MB neurons) forming microglomeruli that can be easily distinguished and analyzed even by means of light microscopy.

(1a) Structural analysis of pre-synaptic complexes within the calyx

To calculate the number of presynaptic complexes within an identified calyx compartment (Fig. 2A,B), we selected two volumes that were separated by 20 µm depth level. Fig. 2C shows the counts of microglomerular presynaptic boutons per 5000 μm^3 for calyces visualized with laser scanning microscopy after removal from bees of ≥5 (short) and ≥15 days (long) foraging duration. The two selected regions did not reveal significant differences with respect to the number of presynaptic complexes (see figure legend for statistics).

(1b) Proteomic screening of the calyx

Semi-quantitative proteomics of calyx samples detected 473 proteins (Fig. 1 and Table S1 in supplementary material). Statistical analysis using the conditions described in experimental procedures did not reveal significant differences in this protein matrix between bees after ≥5 and ≥15 days of foraging duration.

(2) Proteomic examination of the entire central brain

We next conducted a less spatially restricted proteomic screening of the entire central brain, including the output regions of the MB and the antennal lobes (Fig. 1). The majority (77.8%) of the 513 proteins identified in this experiment overlapped with the ones identified in the calyx (Fig. 1 and Table S1 in supplementary material). The fact that not all proteins identified in the calyx were re-identified in the central brain samples, even though these samples included calyx tissue, can be explained by the characteristics of the technique. The proteomics approach favors detection of highly abundant proteins, and thus our result on relative protein identification suggests that proteins that are highly abundant in the calyx can be relatively less abundant in the entire central brain.

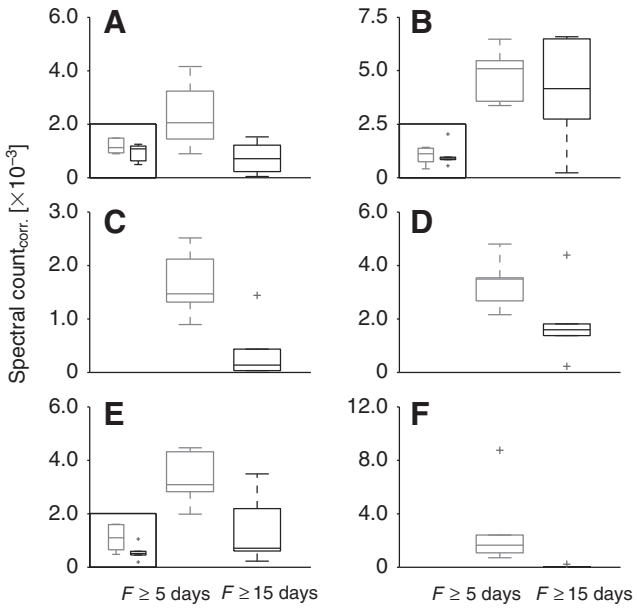

Fig. 3. Age-related protein changes in the honeybee central brain. Main boxplots denote protein abundances in the central brain while nested boxplots show protein abundances in the calyx region only. (A) Protein kinase C, (B) protein kinase A subunit R2, (C) nervous wreck, (D) synapsin, (E) failed axon connections (fax), (F) retinoid and fatty acid binding protein. *F*≥5 days=foragers with at least 5 days of foraging experience. *F*≥15 days=foragers with at least 15 days of foraging experience. *Y*-axis: individual spectral count corrected for total spectral count. Boxplots represent medians and 25–75 percentiles. Significant differences were detected for proteins A and C–F found in the central brain (Mann–Whitney *U*-test, $P<0.05$, $N=6$ per group).

In contrast to the calyx, significant protein abundance differences were observed when comparing central brain samples obtained from bees collected after ≥5 days and ≥15 days of foraging (Fig. 3). Overall, five proteins were negatively correlated with foraging duration. These included proteins involved in synaptic and intracellular signaling (synapsin and PKC), in regulation of neuronal growth [nervous wreck, failed axon connections (fax)] and in general metabolism (retinoid-and fatty acid-binding protein).

DISCUSSION

Uno et al. previously showed that specific areas of the honeybee brain have different protein profiles (Uno et al., 2007). Here, we present the first attempt to use synapse-specific immunohistochemistry and proteomics to profile brain regions for senescence-associated changes that could explain reduced learning performance in aged bees. We speculated that, after neuronal outgrowth is complete, foraging duration is associated with aging traits, such as synaptic degradation and changes in protein composition (Yankner et al., 2008) potentially explaining the decline in learning performance in older foragers.

Surprisingly, using immunohistochemistry and proteomics on samples derived from individual bees, we found that a brain region implicated in learning (the calyx) was unaffected by senescence both on the structural and protein level. This negative outcome does not exclude that aging occurs at the level of the calyx but demonstrates that changes cannot be detected in this region with the methods we used. Using the same procedure, however, proteomics data on the entire central brain revealed effects that could contribute to learning deficits observed in bees after ≥15 days of foraging.

Honeybee aging is more strongly influenced by foraging duration than by chronological age (Behrends et al., 2007; Scheiner and Amdam, 2009). Our study, therefore, focused on animals of known foraging duration rather than of known age. Yet, the observed proteomic patterns are strikingly similar to previous reports of age-related changes in other species. Explicitly, it was shown before that PKC levels in vertebrate and invertebrate heads can drop with increasing age (Humphries et al., 2003; Pascale et al., 2007), while PKA subunit abundance is not affected (Humphries et al., 2003; Yamazaki et al., 2007). Both kinases are key proteins for memory formation in insects and in vertebrates (Menzel, 2001; Nogues, 1997; Skoulakis et al., 1993), and their levels can thus have a major impact on learning performance.

Apart from changes in kinase levels, changes within the synaptic machinery and neuronal wiring can lead to signal transduction deficiencies within entire neuronal networks (Yankner et al., 2008). Interestingly, proteins that are directly linked to synaptic structure and function (fax, nervous wreck, synapsin) showed a foraging-dependent decline in our study. Failed axon connections (fax), is known to regulate neural outgrowth (Liebl et al., 2000; Much et al., 2000). Lower levels of fax in honeybees after ≥15 days of foraging, thereby, may indicate that the neuronal outgrowth during adulthood (Fahrbach et al., 2003) is limited, resulting in a senesced state in foragers after prolonged foraging duration. Likewise, synapsin abundance was reduced after ≥15 days of foraging, suggesting a decrease in the availability of neurotransmitter-containing vesicles and, hence, impaired neurotransmission (Klagges et al., 1996).

The abundance of a protein similar to nervous wreck was also lower in the bees with extended foraging experience. In *Drosophila*, nervous wreck was suggested to prevent synapses from growing excessively (Coyle et al., 2004), and thus the lower abundance levels of the homologous protein in bees after ≥15 days of foraging could be regarded as indicative of sustained growth. Yet, *Drosophila* mutants with downregulated nervous wreck expression are characterized by aberrant synapse morphology (hyperbranching), smaller synaptic boutons with fewer active zones and severe locomotor defects (Coyle et al., 2004). Accordingly, lower levels of nervous wreck are probably indicative of a deregulation of neuronal growth-control during senescence, in contrast to well-regulated outgrowth of synapses.

Lastly, a retinoid and fatty acid-binding protein was exclusively found in the bees with ≥5 days of foraging experience. This protein could stem from secretory glands that surround the brain and would thus qualify as a contaminant. The glands are hypertrophic in nest bees, and although the glands rapidly shrink after the transition to foraging activity, bees collected after short foraging duration typically have more developed glands than bees that have foraged for ≥15 days (D.M., personal observation). However, care was taken to remove all gland tissue and contamination is therefore unlikely. Among taxa, there is an established relation between the levels of retinoids and nerve growth (Dmetrichuk et al., 2006). Thus, retinoid and fatty acid-binding protein might provide the brains of bees with short foraging duration with retinoids that aid in neural development.

Positive correlations between protein abundance and foraging duration were observed but are not reported due to the conservative selection criteria used in this study. Our quality control included correction for type 1 errors (overestimation of differences). However, we cannot exclude the occurrence of type 2 errors (an underestimation of the differences).

The heterogeneity of senescence-related patterns that we observe between compartments of the honeybee brain is a phenomenon similar to what is documented in primates (Fraser et al., 2005). Heterogeneity, and the particular changes we found in the central brain, led us to propose that distinct brain areas are differently affected by senescence in honeybees, and that regions other than the calyx are primarily responsible for foraging-dependent performance decline. These other regions can include the antennal lobes and the output regions of the MB, which are important sites for memory formation (Hammer and Menzel, 1998; Mauelshagen, 1993; Yu et al., 2006). We conclude that the honeybee can become a useful tool for studies aimed at understanding the molecular basis for senescence of learning performance.

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REFERENCES

Behrends, A., Scheiner, R., Baker, N. and Amdam, G. V. (2007). Cognitive aging is linked to social role in honey bees (*Apis mellifera*). *Exp. Gerontol.* **42**, 1146-1153.
Berry, J., Krause, W. C. and Davis, R. L. (2008). Olfactory memory traces in Drosophila. *Prog. Brain Res.* **169**, 293-304.
Coyle, I. P., Koh, Y. H., Lee, W. C., Slind, J., Fergestad, T., Littleton, J. T. and Ganetzky, B. (2004). Nervous wreck, an SH3 adaptor protein that interacts with Wsp, regulates synaptic growth in Drosophila. *Neuron* **41**, 521-534.
Dmetrichuk, J. M., Carlone, R. L. and Spencer, G. E. (2006). Retinoic acid induces neurite outgrowth and growth cone turning in invertebrate neurons. *Dev. Biol.* **294**, 39-49.
Fahrbach, S. E., Farris, S. M., Sullivan, J. P. and Robinson, G. E. (2003). Limits on volume changes in the mushroom bodies of the honey bee brain. *J. Neurobiol.* **57**, 141-151.
Farris, S. M., Robinson, G. E. and Fahrbach, S. E. (2001). Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. *J. Neurosci.* **21**, 6395-6404.
Fiala, A. (2007). Olfaction and olfactory learning in Drosophila: recent progress. *Curr. Opin. Neurobiol.* **17**, 720-726.
Fraser, H. B., Khaitovich, P., Plotkin, J. B., Paabo, S. and Eisen, M. B. (2005). Aging and gene expression in the primate brain. *PLoS Biol.* **3**, e274.
Groh, C., Tautz, J. and Rossler, W. (2004). Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development. *Proc. Natl. Acad. Sci. USA* **101**, 4268-4273.
Grunbaum, L. and Muller, U. (1998). Induction of a specific olfactory memory leads to a long-lasting activation of protein kinase C in the antennal lobe of the honeybee. *J. Neurosci.* **18**, 4384-4392.
Hammer, M. and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn. Mem.* **5**, 146-156.
Humphries, M. A., Muller, U., Fondrk, M. K. and Page, R. E., Jr (2003). PKA and PKC content in the honey bee central brain differs in genotypic strains with distinct foraging behavior. *J. Comp. Physiol. A. Neuroethol. Sens. Neural. Behav. Physiol.* **189**, 555-562.
Klagges, B. R., Heimbeck, G., Godenschwege, T. A., Hofbauer, A., Pflugfelder, G. O., Reifegerste, R., Reisch, D., Schaupp, M., Buchner, S. and Buchner, E. (1996). Invertebrate synapsins: a single gene codes for several isoforms in Drosophila. *J. Neurosci.* **16**, 3154-3165.
Liebl, E. C., Forsthoefel, D. J., Franco, L. S., Sample, S. H., Hess, J. E., Cowger, J. A., Chandler, M. P., Shupert, A. M. and Seeger, M. A. (2000). Dosage-sensitive, reciprocal genetic interactions between the Abl tyrosine kinase and the putative GEF trio reveal trio's role in axon pathfinding. *Neuron* **26**, 107-118.
Mauelshagen, J. (1993). Neural correlates of olfactory learning paradigms in an identified neuron in the honeybee brain. *J. Neurophysiol.* **69**, 609-625.
Menzel, R. (2001). Searching for the memory trace in a mini-brain, the honeybee. *Learn. Mem.* **8**, 53-62.
Menzel, R., Leboulle, G. and Eisenhardt, D. (2006). Small brains, bright minds. *Cell* **124**, 237-239.
Mery, F. (2007). Aging and its differential effects on consolidated memory forms in Drosophila. *Exp. Gerontol.* **42**, 99-101.
Much, J. W., Slade, D. J., Klampert, K., Garriga, G. and Wightman, B. (2000). The fax-1 nuclear hormone receptor regulates axon pathfinding and neurotransmitter expression. *Dev.* **127**, 703-712.
Muller, U. (2000). Prolonged activation of cAMP-dependent protein kinase during conditioning induces long-term memory in honeybees. *Neuron* **27**, 159-168.
Munch, D., Amdam, G. V. and Wolschin, F. (2008). Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. *Funct. Ecol.* **22**, 407-421.
Nogues, X. (1997). Protein kinase C, learning and memory: a circular determinism between physiology and behaviour. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **21**, 507-529.

Pascale, A., Amadio, M., Govoni, S. and Battaini, F. (2007). The aging brain, a key target for the future: the protein kinase C involvement. *Pharmacol. Res.* **55**, 560-569.
Rappsilber, J., Ishihama, Y. and Mann, M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* **75**, 663-670.
Scheiner, R. and Amdam, G. V. (2009). Impaired tactile learning is related to social role in honeybees. *J. Exp. Biol.* **212**, 994-1002.
Skoulakis, E. M., Kalderon, D. and Davis, R. L. (1993). Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. *Neuron* **11**, 197-208.
Strausfeld, N. J., Hansen, L., Li, Y., Gomez, R. S. and Ito, K. (1998). Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn. Mem.* **5**, 11-37.
Szyszka, P., Galkin, A. and Menzel, R. (2008). Associative and non-associative plasticity in kenyon cells of the honeybee mushroom body. *Front Syst. Neurosci.* **2**, 3.
Tamura, T., Chiang, A. S., Ito, N., Liu, H. P., Horiuchi, J., Tully, T. and Saitoe, M. (2003). Aging specifically impairs amnesiac-dependent memory in Drosophila. *Neuron* **40**, 1003-1011.
Uno, Y., Fujiyuki, T., Morioka, M., Takeuchi, H. and Kubo, T. (2007). Identification of proteins whose expression is up- or down-regulated in the mushroom bodies in the honeybee brain using proteomics. *FEBS Lett.* **581**, 97-101.
Wessel, D. and Flugge, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138**, 141-143.
Wolschin, F. and Amdam, G. V. (2007). Plasticity and robustness of protein patterns during reversible development in the honey bee (*Apis mellifera*). *Anal. Bioanal. Chem.* **389**, 1095-1100.
Yamazaki, D., Horiuchi, J., Nakagami, Y., Nagano, S., Tamura, T. and Saitoe, M. (2007). The Drosophila DCO mutation suppresses age-related memory impairment without affecting lifespan. *Nat. Neurosci.* **10**, 478-484.
Yankner, B. A., Lu, T. and Loerch, P. (2008). The aging brain. *Annu. Rev. Pathol.* **3**, 41-66.
Yu, D., Akalal, D. B. and Davis, R. L. (2006). Drosophila alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. *Neuron* **52**, 845-855.
Zars, T., Fischer, M., Schulz, R. and Heisenberg, M. (2000). Localization of a short-term memory in Drosophila. *Science* **288**, 672-675.