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Vitellogenin in the honey bee brain: Atypical localization of a reproductive protein that promotes longevity

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ABSTRACT

In comparative gerontology, highly social insects such as honey bees (*Apis mellifera*) receive much attention due to very different and flexible aging patterns among closely related siblings. While experimental strategies that manipulate socio-environmental factors suggest a causative link between aging and social signals and behaviors, the molecular underpinnings of this linkage are less well understood. Here we study the atypical localization of the egg-yolk protein vitellogenin (Vg) in the brain of the honey bee. Vg is known to influence honey bee social regulation and aging rate. Our findings suggest that Vg immunoreactivity in the brain is specifically localized within the class of non-neuronal glial cells. We discuss how these results can help explain the socially-dependent aging rate of honey bees.

1. Introduction

Honey bees are wild or semi-domesticated animals that live in colonies with complex labor division and individuals that belong to different female castes: workers and the queen (Winston, 1987). Colonies are maintained by the worker caste, helpers that perform brood care, food collection, thermoregulation, hygienic and guarding behaviors. These distinct social care behaviors enable the production of new workers and male drones from eggs laid by a single queen. In contrast to the queen, the life-histories of workers can be very plastic during adulthood, i.e. individuals change from one social care task to another ('temporal castes', Winston and Fergusson, 1985). In summer, workers typically progress from tending the brood ('nursing') to food-collection tasks ('foraging'). In autumn, however, when brood production ceases, bees develop into so-called winter bees (Maurizio, 1950). Apart from physiological specializations that enable the different helper behaviors, the three major worker types differ vastly in lifespan and aging rate (Fig. S1). Foragers typically die within two weeks and are the shortest-lived individuals, whereas bees that continue nursing can reach intermediate lifespans of more than 50 days. The longest-lived workers, winter bees, however survive the months from late summer to next year's spring and are only outlived by queens (Page and Peng, 2001; Remolina et al., 2007; Dukas, 2008; Münch et al., 2013).

Research on brain aging in honey bee workers has largely focused on decline in learning function and on cellular senescence patterns in the brain. This focus is likely explained by the bee's status as a traditional neurobiological model with well-developed experimental tools for these lines of investigation (Galizia et al., 2012). For worker bees, odor-learning capacity rapidly declines (within 2 weeks) in the shortest-lived foragers, while such decline remains undetectable for more than 6 months in the longest-lived winter bee worker type ('negligible senescence') (Behrends et al., 2007; Münch et al., 2013). Moreover, patterns of slowed, accelerated and also reversed behavioral aging can be experimentally evoked, when a change in the social environment pushes workers to transition from one worker-, and hence aging-type, to another (Baker et al., 2012; Münch et al., 2013). The social cues that are experimentally coopted to "push" workers to change their tasks include those that convey the presence or absence of brood, or those cues that signal a skewed social demography in which colonies lack a certain worker type (Nelson, 1927; Maurizio, 1950).

At the level of the honey bee brain, cellular senescence progresses at different rates in the different worker types and occurs at different rates in different regions (Münch and Amdam, 2013), thus resembling the spatial heterogeneity in senescence that is commonly found in other systems, including humans (Double et al., 2008; Raz et al., 2010; Münch and Amdam, 2013). Rapid, slowed or reversed behavioral senescence rates are linked to cellular changes in the brain, including:

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an altered abundance of synaptic and other signaling proteins (synapsin, protein kinase C), of proteins involved in cellular maintenance and lipid transport (heat shock proteins, peroxiredoxin, and fatty acid binding protein), the accumulation of residuals ('waste') from incomplete lysosomal degradation (lipofuscin), and changes in the brain's epigenetic state that differ between worker types (Baker et al., 2012; Herb et al., 2012; Münch et al., 2013) review in, (Münch and Amdam, 2013). Combined, these changes may contribute to learning decline. Yet, it remains unclear whether well-studied anti-aging signals with known functions in honey bee body maintenance and homeostasis may also be active in the brain.

We hypothesized that the protein vitellogenin (Vg) influences the level of somatic investment in the bee brain. Vg is a large lipid transport protein that functions as an egg-yolk precursor in nearly all egg-laying animals (see Havukainen et al., 2013 and references therein). However, it is also found in the non-reproductive worker castes of many social insects. In insects, Vg is produced in the fat body tissue, which is functionally homologous to the white adipose tissue and liver of vertebrates. Insects may have fat bodies in the abdomen, the thorax and in the head, but worker honey bees only have a sizeable fat body in the abdomen (Snodgrass, 1956). Vg is generally released from fat body, circulates in the insect blood (hemolymph) and is taken up by the ovary through receptor-mediated endocytosis (Amdam et al., 2003; Guidugli et al., 2005). Honey bee workers typically do not import Vg to ovaries, however, Vg can be taken up for example by the hypopharyngeal glands in the head of nursing workers that synthesize food jelly for young larvae, other workers and the queen (Amdam et al., 2003). In addition, honey bee Vg shields cells from oxidative damage and protects both workers and queens from oxidative stress (Seehuus et al., 2006; Corona et al., 2007; Havukainen et al., 2013). Hemolymph and fat body levels of the Vg protein are highest in the longest-lived winter bee workers (up to 60–90 μg/μl hemolymph), and lowest in short-lived foragers (0–5 μg/μl hemolymph, Seehuus et al., 2006).

The fact that honey bee Vg can protect against oxidative insult, and that its levels in workers are negatively correlated with aging rate, have fueled the hypothesis that Vg is a central regulator of honey bee lifespan (Seehuus et al., 2006; Havukainen et al., 2013). This hypothesis also takes in account that Vg can suppress levels of juvenile hormone (JH) in workers. JH is a pro-aging hormone in *Drosophila melanogaster* and in honey bees (reviewed by Flatt et al., 2013). In honey bee workers, JH promotes foraging, and the down-regulation of Vg by RNA interference-mediated gene knockdown leads to increased JH levels and speeds up the transition to the short-lived forager stage (see Flatt et al., 2013 for a review and further references).

Despite knowledge about the central roles of honey bee Vg in social regulation and aging, its presence in the brain has not been confirmed so far. Thereby, it is uncertain whether this protein can influence the brain directly. To begin addressing our hypothesis that Vg can directly impact the function and integrity of the brain, we studied whether the Vg protein can be identified in worker bee brains, and whether the *vitellogenin* (*vg*) gene is expressed by cells in the brain of workers.

2. Material and methods

2.1. Animals

All experiments were performed using honey bees (*Apis mellifera carnica* Pollmann). For coherency among results from different analysis techniques, all experiments were performed using an identified worker type that exhibits the highest levels of Vg protein synthesis: nurse bees (Amdam et al., 2003 for further references). All tested individuals had a chronological age range of 9 to 25 days. This age range was stipulated to represent fully mature individuals, thereby avoiding early-adulthood maturational (<8 days) and late-life (≥30 days) aging effects (Whitfield et al., 2006; Remolina et al., 2007). Individual chronological age was confirmed by paint marks that young bees received on the day they emerged as adults from wax combs collected from colonies.

2.2. Histology, Western blotting, in situ hybridization and imaging to identify Vg protein and vg gene expression in the honey bee brain

Tissue preparation for anatomic studies of Vg distribution was carried out essentially as described before (Seehuus et al., 2007). Briefly, ultrathin sections of brains infiltrated with London Resin-White were labeled with a primary antibody against honey bee Vg, followed by the incubation with a fluorescence labeled secondary antibody and the DNA stain DAPI to visualize neuronal and glial cell somata. For glia specific immunohistochemistry, we used an anti-REPO serum (gift from J. Urban, Mainz) that was raised in rabbit, similar to anti-Vg. To further confirm the presence of Vg in the brain, dissected brains and control tissues (head, abdominal carcasses) were processed using standard electrophoresis and Western blot protocols, followed by incubating the blots with the Vg specific antibody that was used in anatomic studies. In situ hybridization was performed by applying standard protocols on semithin brain sections (50 μm) and on fat body tissue (positive control).

Images of anatomic sections labeled with the Vg specific antibody were acquired with a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems), Western blots were imaged with a Typhoon Variable Mode Imager 8600 (GE Healthcare), and in situ samples were imaged with a Leitz Aristoplan microscope equipped with Leica DFC425 camera (Leica Microsystems).

Detailed protocols for histology, Western blotting and in situ hybridization are provided as supplemental material (M1).

3. Results

3.1. Cell-specific localization of Vg in the honey bee brain

Using Vg specific immunohistochemistry on anatomical sections, we found intense Vg immunoreactivity (VgIR) for several regions of the brain. Representative examples for N = 5 individuals are shown in Fig. 1. Intense VgIR was detected in the brain, and also in the jelly-producing hypopharyngeal glands that are located on the top of the brain (Br and HpG, respectively in Fig. 1 and S2). Vg was detected in these glands before, thus serving as a positive control (e.g., Havukainen et al., 2013). In the brain, VgIR was found in peripheral antennal and optic lobes, which process olfactory and visual receptor information, respectively. Likewise, VgIR was detectable in or around major central brain areas including the central complex and the mushroom body, a paired structure that is extensively studied for its role in adaptive behaviors (Fig. 1A–D, for general anatomy compare, e.g. Bicker, 1999; Strausfeld, 2002). As with other insects, synaptic neuropiles with axonal and dendritic projections are spatially separated from surrounding areas that contain cell bodies (soma cortices). This spatial separation is revealed by the nuclear stain DAPI (cyan in Fig. 1B, C). Conspicuously, intense VgIR (magenta in Fig. 1B–D) in the brain occurred consistently at boundary layers that surround synaptic neuropiles (SN). This is exemplified for the antennal lobes, where VgIR outlined the separate neuropiles, so-called glomeruli (NP-Gl in Fig. 1B, C). In addition, intense VgIR formed a layer that separates the soma cortex from protocerebral synaptic neuropiles (NP-Pc in Fig. 1B). For both these areas, the respective boundary layers are known locations of glial cells, and not neuronal somata (Hahnlein and Bicker, 1997). As a reference for how the distribution of VgIR and glial cells match, Fig. 1E shows ventral parts of the brain labeled with the glia specific anti-REPO antibody. In conclusion, these results strongly indicate that Vg is not simply restricted to certain brain areas in honey bees, but also to non-neuronal–glial-cells with known functions in cellular homeostasis and energy metabolism of the brain.

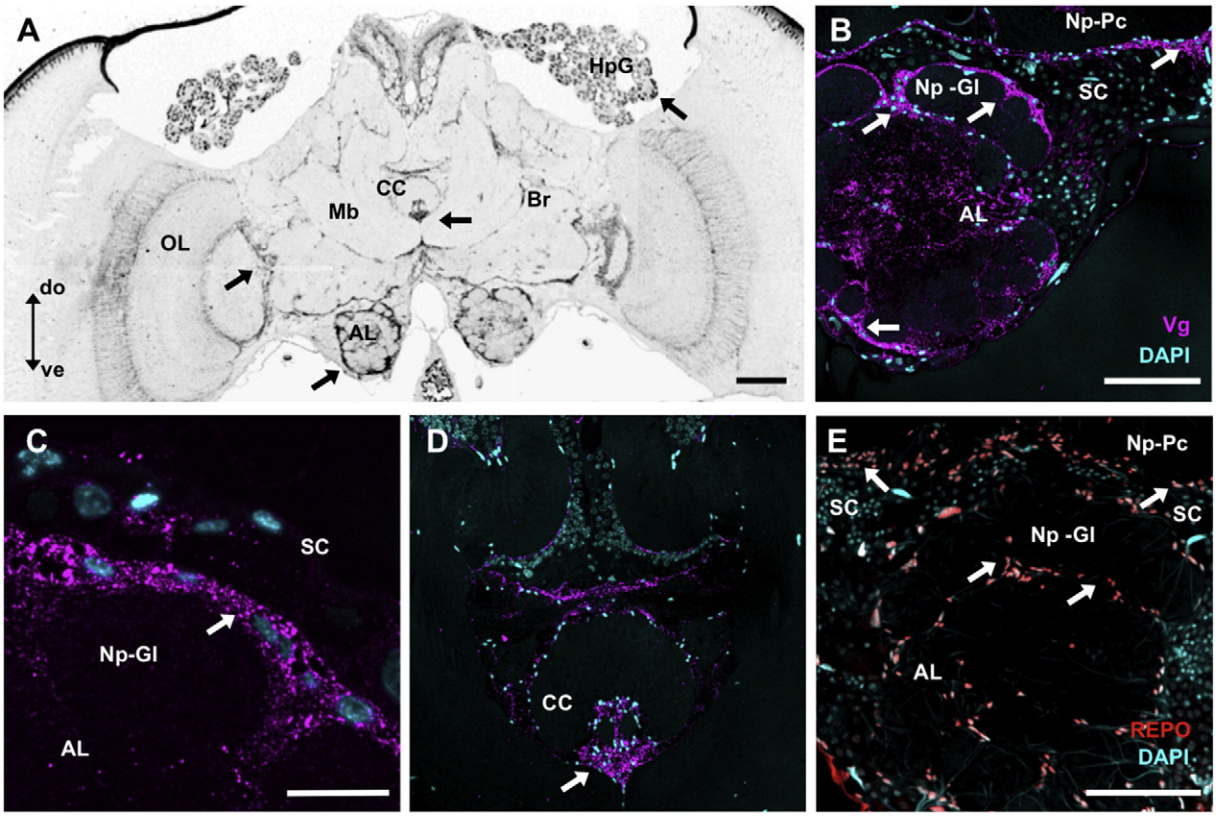

Fig. 1. Vitellogenin immunoreactivity is detectable in distinct areas and cell populations of the honey bee brain. (A) Frontal plane section of a honey bee head showing the brain's antennal lobes (AL), optic lobes (OL), the mushroom bodies (Mb) and central complex (CC). Intense Vitellogenin immunoreactivity (VgIR, arrows) is present in major brain areas as well as in hypopharyngeal glands (HpG). (B, C) High resolution images of ventral brain areas including the antennal lobe (AL) reveal intense VgIR (magenta, arrows) at boundary layers that surround synaptic neuropiles, such as single glomeruli (NP-GI), or that separate protocerebral neuropiles (NP-Pc) from soma areas (SC; DAPI stain revealing neuronal and glia somata, cyan). (D) High resolution image showing intense VgIR associated with the central complex (CC). (E) Projection view of an image stack exemplifying general distribution patterns of glial cells in the honey bee brain by using a glia specific marker (anti REPO, red). In contrast, DAPI (cyan) labels glial as well as neuronal somata. Arrows indicate that the localization of glial somata at boundary layers of glomeruli (NP-GI) and protocerebral neuropiles (NP-Pc) conforms with the distribution of VgIR shown in panel B. Abbreviations: do, dorsal; ve, ventral. Scale bars = 200 μm in A, 100 μm in B (for B and D), 20 μm in C.

3.2. Vg protein levels in different body compartments

To further validate the presence of Vg in the honey bee brain we performed Western blots with the honey bee anti-Vg antibody used in the anatomical studies (compare Sections 2.2, 3.1, N = 4 individuals). We identified a significant band at 180 kDa, corresponding to the full-length Vg protein, for brain tissue, as well as for two positive controls for which the presence of Vg was shown previously (Fig. 2A). The two positive controls include the abdomen comprising the large abdominal fat body, and head samples with the food (hypopharyngeal) glands. The full length Vg protein is known to be cleaved and is subjected to other forms of degradation, with major fragments at 150 and 40 kDa (see Fig. 2A; references in (Havukainen et al., 2013), compare also Section 4). Since we did not attempt a quantification of Vg abundance; different band intensities in Fig. 2A are not indicative of different protein abundance in the tissues.

3.3. In situ hybridization did not reveal detectable vg mRNA in the brain

Using in situ hybridization we next asked if the Vg protein could be in fact produced by brain cell populations for which positive Vg immunostaining was detected. To confirm the specificity of our anti-sense cDNA probe, we first tested abdominal fat body tissue as a positive control with known *vg* mRNA synthesis. Using the probe, *vg* mRNA transcripts were detectable in the fat body (Fig. 2B). Control fat body samples, tested with the corresponding sense cDNA probes (negative control), showed no marked staining (Fig. 2C). In brain sections, we did not detect marked *vg* mRNA transcript signals, neither for identifiable neuronal nor for glial cell populations (N = 5 individuals). Thus, in contrast to fat body tissue, *vg* mRNA signal intensity in the brain (Fig. 2D) was not distinguishable from the negative control (sense *vg* cDNA, Fig. 2E).

4. Discussion

To the best of our knowledge, we are the first to document intense Vg-like immunostaining in an insect brain, which suggests a specific localization within the class of non-neuronal glial cells in the honey bee nurse worker type. However, our data does not support Vg synthesis in the brain. It is therefore likely that glia-specific Vg accumulation relies on an uptake-mechanism that is not shared with other cell types in the brain. The Vg protein contains an α-helical and an N-sheet subunit, with both likely involving in two different Vg binding mechanisms. The affinity of Vg's α-helical domain to lipid-bilayers likely explains the abundant honey bee Vg at cellular membranes of diverse tissues, as well as Vg binding to Sf9 insect cells in vitro and even to artificial lipid bilayers (Havukainen et al., 2013). Cytosolic Vg, in contrast, appears to be limited to specific sites, and has been found in the fat body – where it is synthesized – in ovaries and hypopharyngeal glands, and now in the glial cells of the brain. In contrast to membrane binding, cytosolic Vg was attributed to receptor mediated endocytosis that relies on the recognition of Vg's highly conserved N-sheet domain (compare Li et al., 2003). Specific Vg receptors are present on queen ovaries, and have been detected in the membranes of the hypopharyngeal glands, in fat body and in the midgut, albeit at very low level (Amdam et al., 2003; Guidugli-Lazzarini et al., 2008). To better understand specific Vg uptake

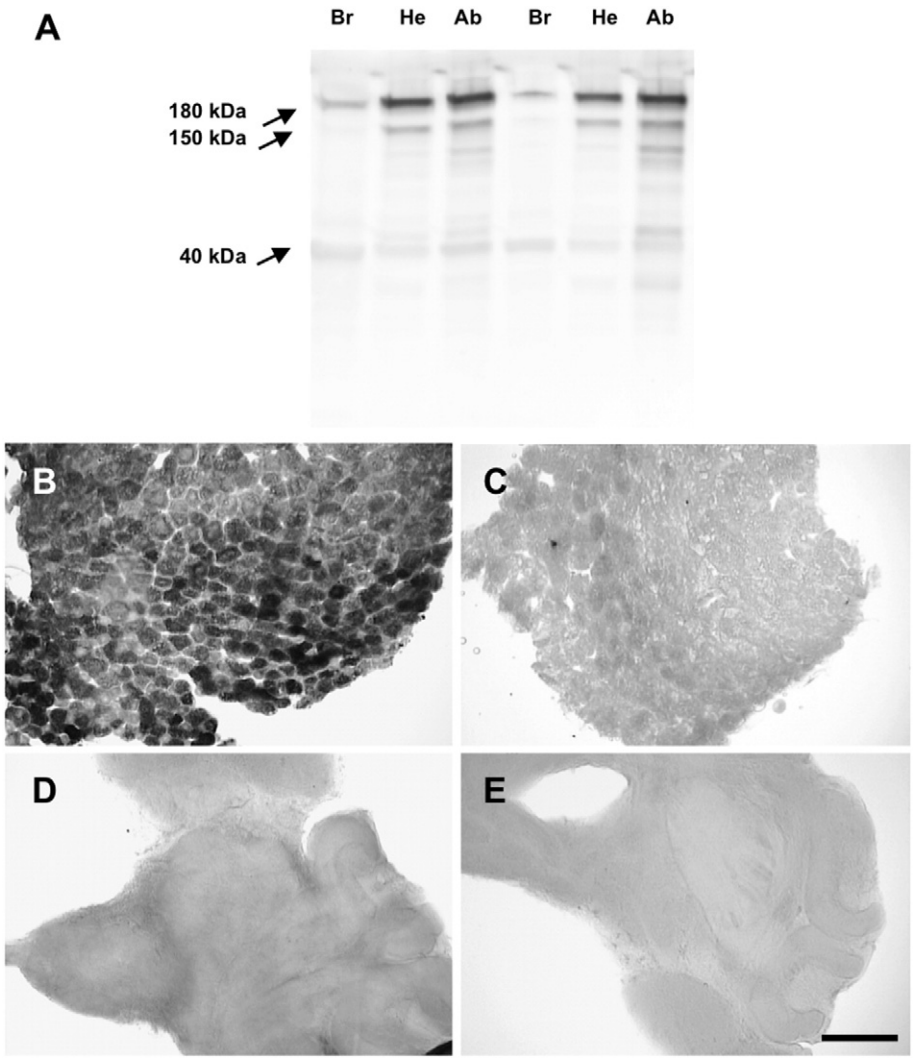

Fig. 2. Western blotting confirms the presence of the Vg protein in the honey bee brain. However, in situ hybridization data does not support that the brain is a site of abundant Vg synthesis. (A) The anti-Vg antibody used also for anatomical studies labels a dominant band at 180 kDa, the molecular weight of the full length Vg protein (arrow). This band was detected in brain samples (Br), as well as in positive controls (He, head; Ab, abdomen), for which the presence of Vg was shown previously. Marked bands at 150 kDa and 40 kDa with a lower molecular weight correspond with known fragmentation products of Vg (see Section 3.2). (B–E) Hybridization with *vg* mRNA specific cDNA reveals abundant *vg* transcription only for the positive control, i.e. abdominal fat body tissue (B). In contrast, *vg* specific signals that would indicate protein synthesis were not detected in inspected brain samples (D, representative image for N = 5 individuals). In addition, staining intensities in D were similar to samples that were probed with negative hybridization controls (non-hybridizing *vg* sense cDNA, C for abdomen, E for brain). Scale bar in D = 200 μm in B–E.

mechanisms in the brain, Vg receptor expression in honey bee glial cells remains to be tested.

Vg is well known for its protective effects against aging in honey bees. Glial cells, moreover, are generally central to brain nutritional homeostasis, detoxification and neurotransmitter uptake. In vertebrates, for example, key metabolic enzymes that are essential for glycogen and glutamate turnover are almost exclusively found in specific glial types (Hertz and Zielke, 2004; Belanger et al., 2011). Loss of these vital functions can be a factor in aging (Lynch et al., 2010). Our results, therefore, appear to be compatible with a role of Vg in brain cellular maintenance and function. However, since the classification of insect glia and their specific functional roles are not well understood, we can only speculate how the presence of Vg in honey bee glial cells might influence the functional integrity of the brain.

Honey bee neurons did not show detectable Vg immunoreactivity. The lack of abundant Vg in these neurons, in particular close to neuronal branches in the synaptic neuropiles, therefore, makes it unlikely that Vg has a direct role as a free radical scavenger in neurons, as opposed to glial cells. However, Vg shares homology with lipid transfer proteins (LLTPs) including apolipoprotein B (ApoB) that has anti-inflammatory functions in vertebrates. A similar function to ApoB is suggested for honey bee Vg, because of the protein's abilities to specifically bind to damaged and dead cells (Havukainen et al., 2013). Inflammation is a hallmark of many neurodegenerative disorders (Amor et al., 2014), and it is conceivable that an anti-inflammatory role of Vg in glial cells affects behavioral integrity. Thereby, glia specific Vg uptake may help understanding the positive correlation between honey bee brain function and levels of circulating Vg. While health benefits of Vg are reported for a number of species, a positive correlation of Vg-like proteins and lifespan does not seem to be universal. Despite functional similarities between vitellogenin and *Drosophila* Yolk proteins (YP) 1–3, a higher expression of *Drosophila* YPs correlates with reduced lifespan (Tarone et al., 2012). Such reversed relation is also reflected in the fact that the highly fecund, egg-laying queens are the longest lived individuals among honey bee castes, while high fecundity in *Drosophila* typically correlates with shortened lifespan ('cost of reproduction trade-off', Hansen et al., 2013).

Vg is prone to both unspecific degradation (Wheeler and Kawooya, 1990) and specific cutting into 150 and 40 kDa fragments

(Havukainen et al., 2011). In addition to the 150 and 40 fragments and similar to previous work, we detected other fragments in Western blots of abdominal samples, similar (Fig. 1; compare, e.g. Havukainen et al., 2011). Further studies are needed to understand, whether Vg fragments other than 150 and 40 kDa result from tissue specific Vg degradation, e.g. in the fat body, or from sample processing.

We cannot rule out that *vg* expression in the brain was simply not detectable in this study because mRNA levels were very low, or expression occurs only transiently in nurse bees. However, we suggest that our data and previous findings are more consistent with a mechanism in which Vg is synthesized elsewhere before being transported and taken up by a subset of glial cells. A targeted study of peripheral tissues surrounding the brain can be an important first step toward understanding this process. Regarding this, we occasionally observed *vg* mRNA staining in peripheral brain sections, where adhering tissue was left attached to the brain (data not shown). While such staining of membranous structures is sometimes attributed to non-specific alkaline-phosphatase activity used for probe detection, for example in trachea (Patel, 1994), the non-hybridizing probes (negative controls) did not reveal marked signals in the brain's periphery. The transcription of the *vg* gene in tissues that typically adhere to dissected brains may also explain why previous non-anatomic studies either reported *vg* mRNA or found *vg* mRNA to be not reliably detectable in nurse bee brains (Nunes et al., 2013; Wheeler et al., 2013).

5. Conclusion

We localized a longevity promoting egg-yolk protein in the glial cells of an insect for the first time. Our findings should motivate research into understanding the protective roles of glial cells and Vg in brain aging in bees and other insects, as well as studies focused on Vg uptake through the brain's blood brain barrier.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2015.08.001.

Conflict of interest

The authors declare no conflict of interest. Preliminary immunohistochemical data on the presence of Vg in the honey bee brain were previously reported in a review by two of the authors (Münch and Amdam, 2010).

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