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Rho GTPase activity in the honey bee mushroom bodies is correlated with age and foraging experience

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ABSTRACT

Foraging experience is correlated with structural plasticity of the mushroom bodies of the honey bee brain. While several neurotransmitter and intracellular signaling pathways have been previously implicated as mediators of these structural changes, none interact directly with the cytoskeleton, the ultimate effector of changes in neuronal morphology. The Rho family of GTPases are small, monomeric G proteins that, when activated, initiate a signaling cascade that reorganizes the neuronal cytoskeleton. In this study, we measured activity of two members of the Rho family of GTPases, Rac and RhoA, in the mushroom bodies of bees with different durations of foraging experience. A transient increase in Rac activity coupled with a transient decrease in RhoA activity was found in honey bees with 4 days foraging experience compared with same-aged new foragers. These observations are in accord with previous reports based on studies of other species of a growth supporting role for Rac and a growth opposing role for RhoA. This is the first report of Rho GTPase activation in the honey bee brain.

1. Introduction

The honey bee, *Apis mellifera,* provides a powerful insect model for the study of experience-dependent brain plasticity (Fahrbach and Dobrin, 2009; Giurfa, 2007; Groh and Meinertzhagen, 2010). Studies of neural plasticity in the honey bee have focused on neuropils associated with the mushroom bodies and the antennal lobe. Typical occurrences in the daily lives of honey bees such as foraging or exposure to a queen lead to measureable changes in the structure of the brain.

The first 3 weeks of a worker honey bee's adult life are spent performing tasks inside the hive (Winston, 1987). Workers then transition to foraging. As foragers, worker honey bees take multiple flights from the hive each day in search of resources: primarily pollen and nectar, but also water and propolis. The neuropils associated with the mushroom bodies are significantly larger in more experienced foragers than in less experienced foragers (Durst et al., 1994; Withers et al., 1993). Foraging experience has also been linked to changes in mushroom body dendritic spine morphology and to changes in the number and volume of areas of synaptic contact in the calyces of the mushroom bodies called microglomeruli (Coss et al., 1980; Krofczik et al., 2008). Studies of precocious foragers indicated that the changes in the mushroom body neuropils reflect primarily foraging experience rather than age (Farris et al., 2001; Withers et al., 1993).

Signaling via neurotransmitter receptors has been linked to changes in neuron structure in honey bees. Pharmacological activation of muscarinic cholinergic receptors in worker honey bees prevented from foraging resulted in growth of the mushroom body neuropil and increases in dendritic complexity that matched changes observed in honey bees foraging under natural conditions (Dobrin et al., 2011; Ismail et al., 2006). Other small molecule neurotransmitters – glutamate, octopamine, acetylcholine, dopamine, and serotonin – have been demonstrated to modulate associative learning in honey bees, and it is possible and even likely that many transmitters regulate experience-induced structural changes in the honey bee brain (Hammer and Menzel, 1998; Locatelli et al., 2005; Lozano and Gauthier, 1998; Maleszka et al., 2000; Müssig et al., 2010; Wright et al., 2010).

Focused analysis of candidate genes in the honey bee brain has led to the identification of several genes with expression modulated by foraging experience, including insulin/insulin-like signaling (IIS), *AmUSP*, *AmFor*, and the putative immediate early gene *kakusei* (Ament et al., 2008; Ben-Shahar et al., 2002; Kiya et al., 2008; Velarde et al., 2006). If the products of these genes do have a role in the regulation of foraging-dependent structural plasticity, they must be upstream of effector molecules directly interacting with the neuronal cytoskeleton. Many other candidate genes related to brain plasticity have been identified but not investigated. The sequencing of the honey bee genome (Honeybee Genome Sequencing Consortium, 2006) offered the opportunity for large

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scale studies of associations between brain gene expression and behavioral plasticity. Microarray-based comparisons of differences in abundance of specific mRNAs between nurses and foragers revealed thousands of candidate plasticity genes (Whitfield et al., 2003, 2006), nearly 500 of which are correlated with foraging experience (Lutz et al., 2011).

Rho GTPases are small, monomeric G proteins that regulate cellular morphogenesis through direct interactions with actin and microtubule-organizing proteins (Ponimaskin et al., 2007). Because the cytoskeleton determines dendritic morphology, the regulation of microtubule and actin dynamics is the driver of dendritic structural plasticity. Cycling between the inactive GDP-bound and the active GTP-bound forms is controlled by guanine nucleotide exchange factors, GTPase activating proteins, and guanine nucleotide dissociation inhibitors (Nobes and Hall, 1994). Rac and RhoA, two of the best characterized Rho GTPase family members, have been shown to regulate dendritic reorganization in the insect *Drosophila melanogaster*, the amphibian *Xenopus laevis*, and other vertebrates including rodents and humans (Bakal et al., 2007; Lee et al., 2000; Nadif Kasri and Van Aelst, 2008; Nakayama et al., 2000; Newey et al., 2005; Sin et al., 2002; Threadgill et al., 1997). Studies in which constitutively-active or dominant negative forms of the GTPases were expressed in *D. melanogaster* implicated Rho GTPase proteins in the development of neuron morphology, with Rac supporting and RhoA opposing growth (Genova et al., 2000; Hakeda-Suzuki et al., 2002; Lee et al., 2000; Lundquist, 2003).

Because the functions of these proteins appear to be conserved, the present study tested the prediction that foraging experience alters levels of activated Rac and RhoA in the worker honey bee brain. We used the model of foraging-dependent growth of Kenyon cell dendritic arborizations, hypothesizing that foraging-associated dendritic growth is initiated and/or regulated by Rho family GTPase activity. We predicted that growth-supporting Rac activity would increase in parallel with increasing foraging experience, which is associated with dendritic growth; conversely, growth-opposing RhoA activity would decrease in the mushroom bodies of honey bees with increasing foraging experience. We assayed worker honey bees that had initiated foraging at different ages and at different periods in the summer to examine experience-, age-, and period-based changes in mushroom body Rho GTPase activity.

2. Materials and methods

2.1. Honey bee collection and experimental design

Honey bees (*A. mellifera*) were obtained from research apiaries maintained at Wake Forest University (Forsyth County, NC, USA) using standard commercial techniques. To obtain newly emerged bees, brood comb containing pharate adult workers was removed from multiple field colonies and placed in an incubator (Percival Scientific, Inc., Perry, IA, USA) maintained at 33 °C, 35–45% relative humidity. To obtain known-age, known-experience foragers, 6000–8000 honey bees (<12 h post-emergence; "focal bees") were marked individually over the course of two days on the dorsal thorax with a single dot of enamel paint (Testors PLA, Rockford, IL, USA). Marked honey bees were returned to a typical field colony containing a naturally-mated queen, where they were allowed to develop as foragers without further manipulation. Twenty-one days later, when a large number of the marked bees had initiated foraging, the hive entrance was observed for 5–7 h. Any focal honey bee observed returning to the hive entrance with a load of pollen or nectar was marked at that time with a second color of paint on the abdomen. These foragers were excluded from the experiment because it is unknown when they initiated foraging. Beginning two days later, 300–400 focal foragers (those returning to the hive entrance bearing a single paint dot on the thorax) were painted with a new color on the abdomen ("marked foragers"). These honey bees were allowed to forage under natural conditions in the field for 1, 4, 8, or 12 days before being collected as they returned to the hive entrance from a foraging trip. To collect new foragers for comparison with experienced foragers, the hive entrance was observed for 5–7 h the day prior to each collection. Each marked forager with a single paint mark noted (those which initiated foraging 1, 2, or 3 days before) was marked with a second color of paint on the abdomen and excluded from future collections. On the day of collection, bees with a single paint mark (those which initiated foraging that day) were collected as new foragers. This experiment was repeated two times in the summer of 2010, once beginning in May and again in July. A timeline of the studies is provided (Fig. 1).

Pollen and nectar foragers were collected as they returned to the hive with full pollen baskets or distended abdomens. To facilitate collections, a wire screen (3 mm spacing) was placed temporarily over the entrance of the hive to prevent bees from entering. Experienced and new marked foragers were collected using forceps and immediately submerged (within seconds of collection) into liquid nitrogen. Immediate freezing was required because inactivation of the Rho family of GTPases can occur extremely rapidly (Murakoshi et al., 2011). Once collections for a day had been completed, the frozen bees were individually transferred to a bed of dry ice and decapitated. The frozen heads (containing the brains) were then immediately transferred to individual prechilled microcentrifuge tubes, placed in a bath of liquid nitrogen, and finally stored at −80 °C until preparation of mushroom body lysates. It took approximately 1–2 min per bee from removing the whole bee from liquid nitrogen to placing the microcentrifuge tube in liquid nitrogen, and approximately 30–45 min to process all foragers and store the frozen heads at −80 °C.

2.2. Group definitions

The experiment was run twice, once beginning in May with forager collections in June (June collection) and again beginning in July with forager collections in August (August collection). Summer in the region of North America where this study was performed can be separated into three periods, each associated characteristic honey bee behaviors. Early summer is primarily a period of growth – brood production is paramount, resulting in large strong colonies. Mid-summer is when the colony size and foraging intensity is greatest. In the late summer, the weather remains warm but is typically drier. As a consequence, resource availability decreases and honey bees begin to undergo physiological and behavioral changes in preparation for the winter. The bees collected in June are considered representative of the end of early-summer and those collected in August as representative of the end of mid-summer.

Marked foragers were collected every 4 days beginning at 24 days old. On each collection day, experienced foragers (those with more than one day of foraging experience) and new foragers (those with less than 24 h of foraging experience) of the same age were collected. When referring to groups within a collection, the age of the bee will be followed by the number of days of foraging experience. For example, 32/1 indicates a 32 day old new forager whereas 32/8 indicates a 32 day old forager with 8 days of foraging experience.

2.3. Mushroom body lysate collection

Whole heads were lyophilized using a VirTis BenchTop 2K freeze dryer (SP Industries, Warminster, PA, USA) for 45 min at