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Chapter 9

IMMEDIATE EARLY GENES AS SEARCH TOOL FOR FINDING CELLULAR AND MOLECULAR EVENTS UNDERLYING SOCIAL BEHAVIORS OF HONEYBEES: A BRIEF COMMENTARY

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ABSTRACT

The daily foraging of honeybees is one of the most well organized social behaviors that exist among social insects. Honeybees are extensively used model animals in behavioral studies for understanding the time-space learning, landmark use and concept of learning etc. Highly systematic social interaction and communication are well decorated behavioral components of honeybee foraging. However, understanding the molecular

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and cellular modulators of these behaviors is limited to a few studies. Available reports have already demonstrated that immediate early genes (IEGs) can be used as neural markers in honeybee brain functions and behaviors. Moreover, IEGs are known to be first activated genes by a stimulus that link to membrane events and nucleus. Its dramatic roles in phenotypic changes of neurons have been executed in many studies. Therefore it is thoughtful to select IEGs to begin a search experiments towards finding molecular and cellular events that participate in a specific behavior. Taking this thought forward, we recently studied two IEGs *Egr-1* and *Hr38*. We found transient over-expression of the two genes during the daily foraging of honeybees (Singh et al, 2018). We also observed over-expression of the Egr-1 downstream genes, ecdysone receptor (*EcR*), dopamine decarboxylase (*Ddc*), dopamine receptor 2 (*DopR2*), which are known to have involved in ecdysteroid signalling and dopaminergic pathway. The finding provides an an avenue to further explore and identify the regulatory genes/proteins and neurons that underlie a specific behavior such as learning, memory, communication and interaction by using IEGs as search tool while the honeybees are freely moving in a more or less like natural environment.

Key Words: Immediate early genes (IEG), honey bee foraging, neural marker, social behaviors, search tool, molecular & celluar mechanism.

HONEYBEE FORAGING CONSTITUTE SEVERAL BEHAVIORAL COMPONENTS

The foraging of bees includes several well-known behavioral components such as search of food, food source location, food identification, food taste, food source memorization, social interaction, communication and recruitment among foragers etc. These behaviors are observed while bees forage to collect nectar/pollen and store it to the hive for the colony (Singh et al., 2018; Frisch 1965; Seeley 1995). In an artificially made bee house, the bees flied back and forth several times from the hive to the feeder during foraging which is a highly repetitive behavior. Very interestingly, during our behavioral experiments (Singh et al., 2018), we also observed that bees used round flights while locating the food source, then tasted the food, and after collecting the food they made again few round flights over the feeder before

flying back to the hive. After the first few trips, these round flights got disappeared. It thus appears that bees used these round flights as a means/mechanism for memorizing the food source location and food identification (we humans too use several means for memorizing new places). One can observe all these behaviors by feeding them in a specific place while they are freely moving during the foraging. It's easy to work with cape honeybees *Apis mellifera* and one can just enjoy watching it by sitting close to the feeder. A continuous food reward is required for continuous foraging. To identify a specific bee, pen marking on bees head is easy to do and it is common practice in honeybee research.

TOWARDS FINDING IEGS THAT INVOLVE IN DAILY FORAGING OF HONEYBEES

The IEGs role had been found to persist from the first stages of brain development unto the adulthood. It showed possible inherent features in everyday brain activity (Loebrich and Nedivi, 2009) and its dramatic roles in phenotypic changes that occurred in neurons (Dijkmans et al., 2009). Many IEG encoded transcription factors were rapidly induced within the neurons while some were delayed (Friedman et al., 1992; Kaczmarek 1993) in response to different stimuli and cellular scenarios leading to short- and long-lasting phenotypic changes within neurons (Loebrich and Nedivi, 2009; Hughes and Dragunow, 1995). After the stimulation, the early response neurons reacted from milliseconds to minutes and it involved 1st and 2nd messenger system along with phosphatases; whereas late response continues from hours to days, and even leads to permanent changes which require gene expression changes (Hughes and Dragunow, 1995; Clayton 2013). Moreover, the late response was related to learning, memory and sensitization processes and even to drug tolerance habits etc. (Hughes and Dragunow, 1995; Clayton 2013). Subsequently, the IEGs were the first activated genes that link to membrane events and nucleus in the process of nerve stimulation (Beckmann and Wilce, 1997). Thus, the regulation in the

IEGs expression is being considered as first part in general neuron response to a natural stimulus. Interestingly, IEGs encoded proteins can be individually regulated in different regions of the brain depending on the type of the stimuli ((Beckmann and Wilce, 1997). This suggests that the same/different IEG expression at different parts of the brain induced by different stimuli may signal to perform different behavioral tasks. In other words, different behaviors correspond to IEGs expressed at different parts of the brain depending on the type of the stimulus. The IEGs are also rapidly and transiently induced within minutes of stimulation in the absence of de-novo protein synthesis, and regulation of IEG production is necessary for the cells. Because in turn it can activate the downstream targets that typically function as a part of a network of constitutively expressed proteins (Perez-Cadahia et al., 2011). It has also been reported that different IEG reaches their pick levels at different times even though they expressed immediately after the stimulation (Bottai et al., 2002; Vazdarjanova et al., 2002). This further reveals involvement of different IEGs in the different behavioral tasks. Therefore, understanding the details of region-wise IEG expression pattern in the brain could provide a remarkable tool in finding various cellular and molecular paths that link to specific behavioral features more precisely. Therefore it is a thoughtful way to start with IEGs in the strategy of the experimental designs towards finding specific molecular and neuronal pathway leading to a specific behavior.

One of the most extensively studied IEGs is the early growth response gene type-1 (*Egr-1*) which codes for a transcription factor protein having Cys2His2 zinc finger motif. It has been proposed that *Egr-1* played a role in plasticity events in adults rather than during development (Nikam et al., 1995; Veyrac et al., 2013). The *Egr-1* along with *Arc* and *Fos* are among most widely used markers for neuronal activation and plasticity during memory formation (Loebrich and Nedivi, 2009; Lanahan and Worley, 1998). In honeybees, *Egr-1* had been suggested to be one of the major transcription factors regulating gene expression changes during the transition of nursing bees to foraging bees (Khamis 2015; Lutz and Robinson, 2013). This reveals that *Egr-1* upregulation is a consequence of higher sensory processing and learning instead of motor activity. Moreover,

Egr-1 expression in the mushroom bodies of Apis mellifera was executed with its participation in spatial learning and time memory in honey bees (Lutz and Robinson, 2013; Shah et al., 2018). Further, study on mice also found that *Egr-1* involves in object and object-place recognition and it was rapidly induced after the object sampling (Davis et al., 2010). Moreover, deletion of *Egr-1* affected long term memory (Li et al., 2007). Another recently discovered IEG which had been implicated as a neural marker in honeybee Apis mellifera is the noncoding RNA kakusei gene whose expression level in the mushroom body differed with the number of foraging trips (Kiya et al., 2012). In addition to it, in silk moth *Bombix mori* and fruit fly *Drosophila melanogaster*, an IEG hormone receptor *Hr38* had been shown to be a neural marker where the expression of the gene was implicated in female stimulation (Chen et al., 2016). The *HR38* is a downstream candidate gene of *Egr-1* and it demonstrated to be a binding partner of ultraspiracle (Usp) in which HR38 binds to Usp by competing with ecdysone receptor. This binding was shown to be regulated by neural activation and was suggested to involve in the proper signaling of ecdysone pathway (Chen et al., 2016; Fujita et al., 2013; Baker et al., 2003; Zhu et al., 2000). Further studies on vinegar flies had shown that the participation of ecdysone signaling pathway in the memory formation was certain (Ishimoto et al., 2009). It may be noted that both the *Egr-1* and *Hr38* were preferentially expressed in the Kenyon cells of the mushroom bodies (Yamazaki et al, 2006; Ugajin et al., 2013), a central area of honeybees and other insect brains where the neuronal function in that region was shown to be actively involved in learning and memory. Subsequently, the studies in vertebrates further reported that, while the neural activity induced the expression changes of IEGs, the products of IEGs regulated the expression of downstream genes that involved in neural homeostasis and synaptic plasticity (Loebrich and Nedivi, 2009; Beckmann and Wilce, 1997; Clayton 2000). These several lines of evidences promised us to believe that IEGs could surely partake in the daily routine foraging of honeybees which accommodates several behavioral features.

OUR RECENT FINDING ON IEGS ROLE IN DAILY FORAGING OF HONEYBEES

That belief and imagination came to a real state when our recent experiments evidenced that Egr-1 and Hr38 genes in honeybee brain were transiently expressed and sustained for about 2 hours, during their daily foraging (Figure 1).[1] The upregulation of the two genes was declined within a short time (few minutes) when the feeder was presented without food, indicating that the genes involvement in associative learning. And a continuous food reward was required to sustain the genes high expression level. This further supports to evidence the role of the two genes during foraging. Moreover, a sustained up-regulation of the *Egr-1* and *Hr38* was still observed when the feeder was presented at a different feeding time of the day. These results clearly demonstrated that IEGs role in the daily foraging of bees is prominent. In addition to it, few *Egr-1* downstream genes, *EcR, Ddc,* and *DopR2* were also involved in the foraging. Further details of these observations could be found in our recent article by Singh et al., 2018.

TO USE FORAGING RESPONSE IEG AS TOOL IN THE CONSTITUENT BEHAVIORS AND BEYOND

Several previous works have regarded IEGs as neural markers, and our resent finding[1] has further witnessed again. Some other genes like *nf-kb, pax6* and *hairy* were also reported to involve in foraging (Khamis et al., 2015). However their role in discrete foraging behavioral components has not been studied. Because of the beauty of presence of several behavioral components, our attention was drawn to examine IEGs involvement in honeybee foraging across two hours of foraging time and we found significant role of *Egr-1* and *Hr38* during the daily foraging (Singh et al., 2018). We believed there may be many more of foraging IEGs yet to be discovered. Finding their associate partner genes and/or proteins that function during honeybee foraging and further testing their roles during

foraging and examining the changes that takes place in each of those behavioral components, and tracing the neurons and brain regions where the actions of those genes/proteins occur, would be a promising direction towards identification of molecular players and the signaling pathways that leads to each behavioral component of the daily foraging of honeybees (graphical cartoon view is shown in Figure 2).

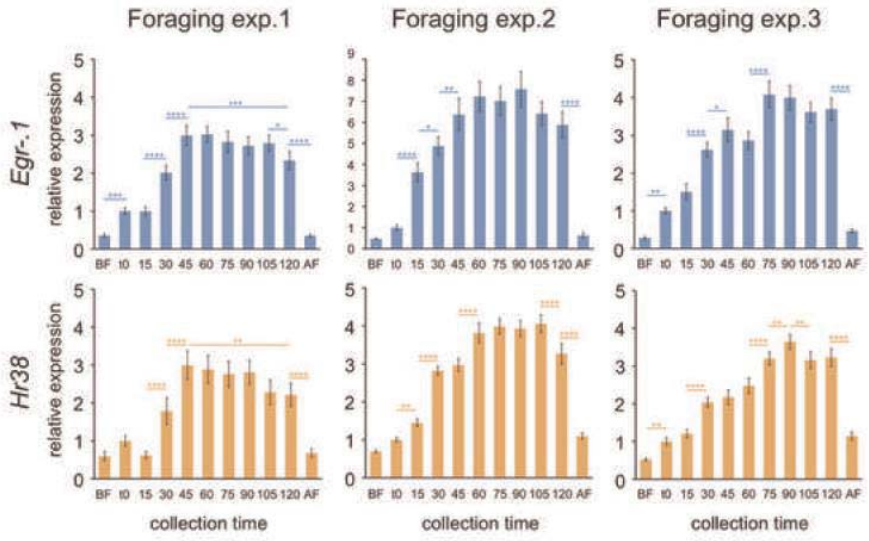

Figure 1. Bar graph representation for Egr-1 (blue) and Hr38 (orange) expression during foraging at a known feeder (modified figure from our recent paper Singh et al., 2018). Data are shown as fold changes with respect to t0 (mean value was set as 1) which indicates the presentation of the feeder and start of continuous foraging. BF = before foraging and AF = after foraging. Foraging experiment 1, 2, 3 represents three replicate experiments. One-way ANOVA with Tukey-Kramer post-hoc multiple comparison: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

The search for food, identify and taste the food, memorize its location, interact and communicate among foragers, recruit other foragers, collect and store food in the hive for the colony; any social animal and human would perform the similar kind of behavior. Therefore the honeybee foraging behaviors are widely applicable across animal kingdom. Moreover, foraging behavior is a repetitive behavior, a feature that occurs in autistic individuals, and the well-organized communication and interaction of the honeybees

during foraging, on the contrary lack communication and interaction to the autistic individuals, the findings from honeybee research from linking molecules, neurons and brain to specific behaviors may be translated even far to humans and be benefited towards finding roots for behavioral abnormalities. It may be noted that, Singh (2014) had earlier commended honeybee would be useful model system towards search and understanding molecular and cellular mechanisms in the lack of social interaction, communication of autistic behavior (Singh 2014). These research challenges are awaited and answers to those beautiful imaginations relay on the fate of future researches.

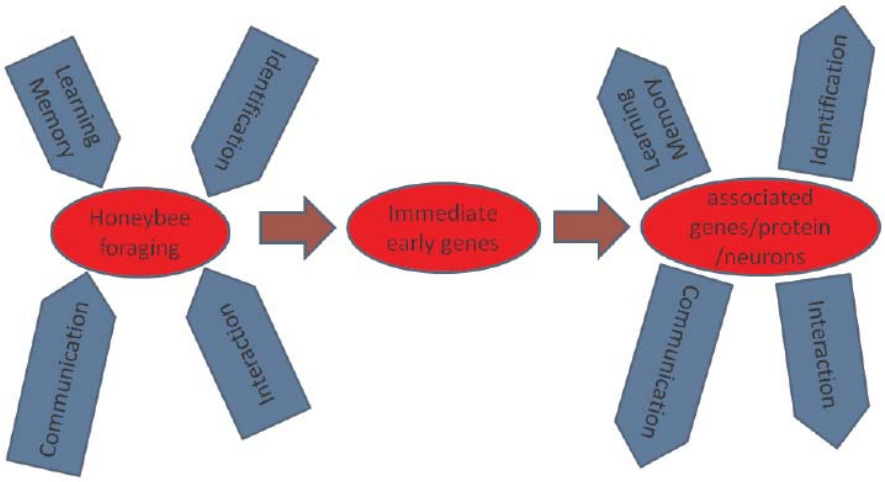

Figure 2. Diagrammatic representation of using Immediate early genes in finding the underlying cellular and molecular regulatory mechanisms of honeybee foraging behaviors.

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