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Imidacloprid changes *period* mRNA level in the brain of forager honey bee, *Apis mellifera*

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Honeybee foragers depend on circadian clock of their brain for sun compass navigation, dance communication and timing visits to flowers. Circadian clock is regulated by several clock genes of which *period (per)* is the most important because of its interaction with other components of the clock. Foragers possess high levels of brain *per* mRNA relative to younger bees and higher levels of *per* are required to engage clock-controlled systems in sun compass navigation. Foragers are exposed to pesticides while foraging on different blooming crops. As a consequence, sun compass oriented navigation, return flight to hive and dance communication may disturb due to the bad effect of pesticides on clock system. In this study, we quantify brain *per* mRNA level of imidacloprid intoxicated foragers to observe whether it hampers the *per* expression. Imidacloprid treated foragers showed significantly low levels of brain *per* mRNA compared with untreated foragers. Based on our previous study of homing failure of imidacloprid treated foragers, it may be concluded that imidacloprid disturbed the key clock gene, *period* and thus caused homing failure of foragers.

Keywords: Circadian clock, foragers, imidacloprid, *period* mRNA

INTRODUCTION

Using the sun as a celestial compass, honeybee foragers orient towards food source during the maximal availability of pollen and nectar in a day (Moore-Ede *et al.,* 1983; Willmer and Stone, 2004; von Buttel-Reepen, 1900; von Frisch, 1967). Foragers can accurately measure the angles between the relative positions of the sun, the food source and the hive (von Frisch, 1967). And thus they are able to reach the correct location of the food, able to come back to the hive and can provide the information of location of food source to other foragers by performing waggle dance inside the hive (Lindauer, 1960, 1961; vonFrisch, 1950, 1967). However, all these processes might have gone wrong because of sun's movement over time. In fact, the sun moves 15° per hour. Hence, foragers need to compensate the shift of the sun and accordingly adjust the angle for navigation, home coming and performing the waggle dance (Lindauer, 1961; von Frisch, 1967). Foragers have a highly developed internal circadian clock and they consult with this clock for sun compass navigation, dance communication, and timing visits to flowers (Beier and Lindauer, 1970; Crailsheim *et al.,* 1996; vonFrisch, 1967; Moore *et al.,* 1998; Moore, 2001; Toma *et al.,* 2000).

The central circadian clock is located in the tiny brain of

the honey bee and is regulated by interplay of several clock genes namely *period (per), clock (clk), cycle (cyc) and timeout (tim 2)*(Bloch, 2009; Bloch, 2010). Among these clock genes *period (per)* is the most important because of its direct or indirect interaction with all other known components of the circadian clock (Dunlap, 1999; Wager-Smith and Kay, 2000; Williams and Sehgal, 2001). Foragers (3 weeks of age or older bees) always possess higher levels of *per* mRNA relative to younger bees (Bloch *et al.,* 2001, 2002; Bloch and Robinson, 2001; Toma *et al.,* 2000, 2003). PER-like immunoreactivity in the bee brain is higher in foragers than younger bees (Bloch *et al.,* 2003). There are speculations that levels of brain *per* mRNA increase in anticipation of the challenges of foraging and higher levels of *per* are required to engage clock-controlled systems in sun compass navigation (Toma *et al.,* 2000).

Foragers require multiple cognitive faculties for navigation and communication. Memories requires for recognition of the sun compass, visual distance estimation, learning of multisensory cues inside and outside the hive and translating as well as reading the codes of the waggle dance and finally integration of these processes are performed in consultation with the internal clock (Decourtye and Devillers, 2009; Fischer *et al.,* 2014; Galizia*et al.,* 2012; Gruter and Farina, 2009; Menzel *et al.,* 1998, 2006, 2012). Foragers exposed to pesticides during foraging trip can have a wrong acquisition or integration of these processes because pesticides may impair important processes involved in cognitive functions and behavior (Belzunces *et al.,* 2012; Decourtye and Devillers, 2009).

Honey bees are exposed to different agricultural chemicals while foraging on the flowers of blooming crops regularly treated with pesticides (Mullin *et al.,* 2010). Imidacloprid, an insecticide of neonicotinoid group, has become a widely used chemical which are directly connected to current bee decline known as colony collapse disorder (Banmatin *et al.,* 2005; Bortolotti *et al.,* 2003; Henry *et al.,* 2012). Neonicotinoid exposures in foragers have been reported to compromise behavior and cognitive abilities including memory formation and retrieval (Aliouance *et al.,* 2009; El Hassani *et al.,* 2008), social interactions, navigation and communication (Belzunces *et al.,* 2012; Bortolotti *et al.*, 2003; Medrzycki *et al.,* 2013). It has been reported that sub-lethal doses of the three neonicotinoids either block the retrieval of exploratory navigation memory or alter this form of navigation memory (Fischer *et al.,* 2014). Neonicotinoid exposures induce abnormal foraging activity (Schneider *et al.,* 2012; Yang *et al.,* 2008), adversely affect olfactory and visual learning (Decourtye *et al.,* 2004; Han *et al.,* 2010; Williamson and Wright, 2013), impair the ability to perform the waggle dance (Eiri and Nieh, 2012) as well as impaired orientation skills and caused homing failures of foragers (Bortolotti *et al.,* 2003; Fischer *et al.,* 2014; Nahar and Ohtani, 2015).

Based on the literature review we hypothesize imidacloprid may disturb a major clock gene, *per* production of foragers which may cause homing failures by hampering the memory and cognition. Based on our previous study of homing failure of imidacloprid treated foragers (Nahar and Ohtani, 2015), in this study, we quantify brain *per* mRNA level of imidacloprid intoxicated foragers to observe whether it hampers the *per* expression.

MATERIALS AND METHOD

Study was conducted at Kobe University, Japan from July to September 2011. Honey bee, *Apis mellifera* colony was maintained in the university garden according to standard commercial techniques. A glass-walled observation hive was placed inside a room in the garden. One day old honey bees were regularly caught from main hive, marked them with number and put inside the observation hive for different study purpose. For this study, day old 100 bees were marked at thorax with red paint and put on observation hive. The hive was regularly monitored to see whether the marked bees turned into foragers. Foragers were identified as bees returning to the hive with loads of pollen in their pollen basket of hind legs. They were caught from the entrance of the observation hive at 14.00 h by forceps, put on transparent plastic box and carried to the laboratory for imidacloprid injection. Foragers were injected with imidacloprid 10ng/bee in accordance with Nahar and Ohtani, 2015 for consecutive three days. Control group foragers were injected with 0.1% di methyl sulfoxide. After injection foragers were kept on transparent plastic box and put on incubator (LD=12:12, temp. 27°C). They were provided with sugar syrup as food. Foragers were be-headed and brains were dissected in phosphate buffered saline (PBS). Compound eyes, ocelli, hypopharyngeal glands and any other glandular tissues were removed during dissection. Only intact dissected brains were collected in liquid nitrogen individually and stored at -80^{o}C until *per* mRNA quantification. Three foragers were used in three replication for treatment and control group.

Isolation of total RNA and cDNA preparation

Isolation of total RNA was performed using the RN easy total RNA isolation kit (Qiagen, Tokyo, Japan) according to manufacturer's instruction. Total RNA from a single brain (about 350 ng) was reverse-transcribed in 0.5 microlitre 5xRT buffer, 0.5 microlitre RT enzyme mix with random primers and oligo d(T) primers using Rever Tra Ace (Toyobo Co., Ltd., Osaka, Japan). Reverse transcription was carried out at 65^{o}C 5 minutes, 37 ^{o}C 30

minutes, 95 ^{o}C 5 minutes and then incubated at 4 ^{o}C.

Measurement of mRNAs levels of forager bee brain

Quantitative real-time RT-PCR (qPCR) was performed to measure mRNA levels by ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using THUNDERBIRDTM qPCRMix (Toyobo Co., Ltd., Osaka, Japan). The level of *per* mRNA was measured relative to elongation factor 1 alpha (EF-1 alpha). It has already proven that the amount of EF-1 alpha mRNA in the honey bee brain does not vary with age, task or time of day (Bloch *et al.,* 2001). Primers for *per* were as follows. F: 5-CACTATGTACGGCAGCGATGAA-3 (anneals between residue 1689 and 1710); R: 5-ACCACTGCTAAGGTTTTCTGCACTA-3(1839-1815). Primers for EF-1 alpha were as follows. F: 5-GCAGTTGATCGTTGGAGTGAAC-3 (153-174); R: 5 CCTCTTTCTTGATCTCCTCGAAAC-3 (anneals between residue 235 and 212). Amplification reaction (25 µl) contained 22.5 µl PCR master mix (nuclease-free water, primers, ROX dye and MIX) and 2.5 µl cDNA. Negative control had no reverse transcriptase. Amplification thermal profiles were: 95 ^{o}C 1 minute, 95 ^{o}C 15 seconds and 60 ^{o}C 1 minute x 40 cycles. Standard curves for the transcripts were generated by serial (5×) dilutions of amplified cDNAs. After 40 cycles, samples were run for melting curve analysis, and in every case, a single expected amplicon was confirmed. The results were analyzed using the software associated with the instrument. Results of independent samples were used to calculate the mean ± SEM both for control and treated group. Statistical comparison between treated and control group was made using Mann-Whitney U test.

RESULTS AND DISCUSSION

We tested our hypothesis by comparing *per* mRNA levels of imidacloprid treated and untreated foragers. Imidacloprid treated foragers showed significantly low levels of brain *per* mRNA compared with untreated foragers (Figure 1).

Several authors reported that foragers always possess high level of brain per mRNA which is required for clock-controlled systems in sun compass navigation (Bloch *et al.,* 2001, 2002, 2003; Bloch and Robinson, *2001*; Toma *et al.,* 2000). We speculate from our result that circadian clock of foragers cannot function normally due to this low expression of *per* mRNA and so the cognitive functions and thus in return trip foragers fail to come back hive. However, in this study, we could not show how the per mRNA changes influenced other clock genes. Because, circadian clock functions due to the interaction between *per* and other clock genes. There are several studies

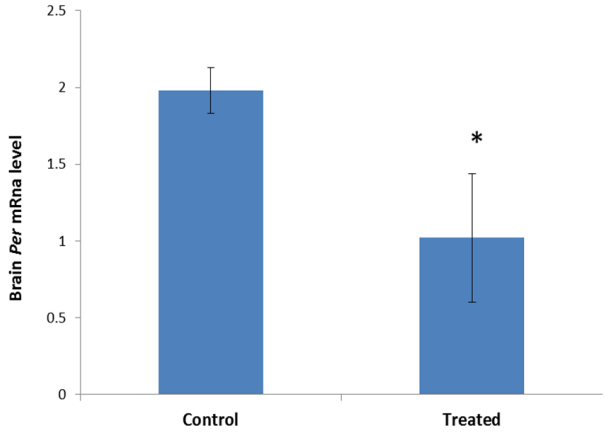

Figure 1. Relative brain *per* mRNA level of imidacloprid treated and control foragers measured by quantitative RT-PCR. The value shown in relative to the amount of EF-1 alpha mRNA.

which proved that learning and memory of foragers have been disturbed by neonicotinoids (Aliouane *et al.*, 2009; Decourtye *et al.* 2004, 2005; El Hassani *et al.* 2008; Han *et al.* 2010). Fischer *et al.,* 2014 concluded neonicotinoids applied at sub-lethal doses block or alter the retrieval of navigation memory of foragers. Exposure to imidacloprid and clothianidin leads to a reduction of foraging activity and longer foraging flights and reduced visual learning capacities (Colin *et al.*, 2004; Han *et al.,* 2010; Nielsen *et al.* 2000; Ramirez-Romero *et al.* 2005; Schneider *et al.,* 2012; Yang *et al.,* 2008). In another studies we showed imidacloprid treated foragers fail to return home (Nahar and Ohtani, 2015) which is in agreement with the study of Bortolotti *et al.,* 2003.

CONCLUSION

The brain relative *per* mRNA level of imidacloprid treated and untreated foragers did differ significantly. There is a limitation of our study that we could not capture foragers at different time points. Collection in multiple time points are important because *per* oscillates in circadian fashion. However, in light of our other study (Nahar and Ohtani, 2015) it may be concluded that imidacloprid disturbed the key clock gene, *period* and caused homing failure of foragers. To understand more clearly the disturbance of circadian clock by neonicotinoids, study on other clock genes (*tim, cyc* and *clk* expression) and their interactions with *period* after neonicotinoid intoxication is suggested.

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