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Comparison of RNAi knockdown effect of tyramine receptor 1 induced by dsRNA and siRNA in brains of the honey bee, *Apis mellifera*

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ABSTRACT

RNA interference (RNAi) is a powerful tool for artificially manipulating gene expression in diverse organisms. In the honey bee, *Apis mellifera*, both long double stranded RNA (dsRNA) and small interference RNA (siRNA) have been successfully used to reduce targeted gene expression and induce specific phenotypes. However, whether dsRNA and siRNA have different effects and efficiencies in gene silencing has never been investigated in honey bees. Thus, we tested the effect of dsRNA and siRNA on the tyramine receptor 1 (*tyr1*), which encodes a receptor of neurotransmitter tyramine, in honey bee brains at mRNA and protein levels over time. We found that both dsRNA and siRNA achieved successful gene knockdown. The siRNA mixes affected *tyr1* gene expression faster than dsRNA, and the duration of the knockdown between dsRNA and siRNA varied. We also found that the turnover rate of TYR1 protein was relatively fast, which is consistent with its role as a neurotransmitter receptor. Our study reveals the different efficiencies of dsRNA and siRNA in honey bee brains. We show that consideration of the gene regions targeted by RNAi, prior screening for RNAi molecules and combing siRNAs are important strategies to enhance RNAi efficiency.

1. Introduction

RNA interference (RNAi) has been successfully used to investigate gene functions, and it has important applications in plants and various animals (Bosher and Labouesse, 2000; Fire et al., 1998; Milhavet et al., 2003; Xue et al., 2012). In honey bees, RNAi has made significant contributions to understanding gene functions in sex determination (Beye et al., 2003), immune response (Aronstein and Saldivar, 2005), aging (Seehuus et al., 2006), social behavior and caste differentiation (Ament et al., 2008; Wang et al., 2013b,c, 2010), learning (Farooqui et al., 2003) and memory formation (Mussig et al., 2010).

RNAi based gene knockdown can be achieved by an artificial introduction of long double-stranded RNAs (dsRNA) or small interference RNA (siRNAs) (Cole and Young, 2017; Philip and Tomoyasu, 2011; Pitino et al., 2011). A long dsRNA, which usually has 300–500 bp, is cut into double-stranded siRNAs (20–25 bp) by an enzyme called Dicer in the cytoplasm. The siRNA binds to an RNA-induced silence complex (RISC). Consequently, the two strands are separated into passenger and guide strands. The guide strand pairs with the targeted mRNA in a sequence-specific manner, and its binding enzyme complex, RISC, cleaves the targeted mRNA to achieve the specific gene silencing (Wilson and Doudna, 2013). Since siRNA is the key inducer for RNAi, exogenous synthetic or viral siRNA has been used in the research on functional genomics (Philip and Tomoyasu, 2011; Pitino et al., 2011).

Efficiency and stability of RNAi are of great importance for studying target gene functions. The efficiency of RNAi varies in different organisms and for different genes, and it also depends on the type and dosage of RNAi molecule (Scott et al., 2013). Quantifying mRNA level with real-time quantitative polymerase chain reaction (qPCR) has been widely used to characterize the efficiency of RNAi in numerous studies (Jarosch and Moritz, 2011; Li-Byarlay et al., 2013; Scott et al., 2013). However, phenotypes resulting from the RNAi rely not on the change in mRNA but on the change of the protein encoded by the mRNA. If a protein has a slow turnover rate, the efficiency of RNAi can be affected and even masked during the gene translation process (Wu et al., 2004). In honey bees, both dsRNA and siRNA have been intensively employed for gene functional studies. However, to date there has not been a study investigating whether the effect and efficiency of dsRNA and siRNA on both mRNA and protein levels differ. Understanding these questions can help us more efficiently perform RNAi in honey bees and also have valuable implication in other organisms. In this study, we chose tyramine receptor 1 (*tyr1*) gene as the target gene in honey bee brains to

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investigate RNAi efficiencies.

TYR1 is a receptor for a biogenic amine, tyramine (TA), which is produced in the nervous system. TA acts as a neurotransmitter, neuromodulator and neurohormone, playing important roles in learning (Nisimura et al., 2005), behavior (Roeder, 2005), energy metabolism (Roeder, 2005; Sasaki and Harano, 2007; Thompson et al., 2007), and reproductive physiology (Sasaki and Harano, 2007; Thompson et al., 2007) in diverse insect species (Blenau and Baumann, 2001; Roeder, 2002). Less than two hundred neurons produce TA in honey bee brains and project in all neuropilar areas of the brain (Sinakevitch et al., 2017; Thamm et al., 2017). It has been shown that TA in honey bees is involved in learning (Scheiner et al., 2002), locomotion (Fussnecker et al., 2006), and sucrose responsiveness (Scheiner et al., 2014). Furthermore, one of its receptors, *tyr1*, is a key candidate gene found in a genomic region identified by multiple QTL studies associated with learning (Chandra et al., 2001) and foraging division of labor in honey bee workers (Hunt et al., 2007). Recently, *tyr1* in worker abdominal fat body was successfully down-regulated by injecting its dsRNA constructs into the hemolymph (unpublished data, Y Wang and R Page). In contrast, gene knockdown in the honey bee brain can only be achieved by local injection (Farooqui et al., 2003, 2004; Mustard et al., 2010) because of the brain blood brain barrier (Jarosch and Moritz, 2011). In this study, we investigated efficacy of RNAi by injecting dsRNA and siRNA of *tyr1* into the brain of honey bees.

2. Materials and methods

2.1. Animals

For this study, bees were collected from the colony with open-mated New World Carniolan queens. Returning foragers were captured at the entrance of the hive in the morning. The bees were briefly cold anesthetized and restrained in custom harnesses that left their proboscis and antennae free to move normally. After they recovered from the anesthetization, the bees were divided into control and treatment groups.

2.2. DsRNA preparation and injection

We selected the fragment that was non-homologous with other genes in the genome database to avoid non-specificity during RNAi knockdown. The *tyr1* CDS was partially cloned with the T7 RNA polymerase promoter sequence. The plasmid was used as templates in PCR reactions to produce pure *tyr1* DNA templates. The PCR primers were listed in Table 1. PCR products were purified using Qiaquick PCR purification kit (Qiagen, Frederick, MD, USA). DsRNA was synthesized using RiboMax Large Scale T7 RNA Production Systems (Promega, Madison, WI, USA) following the manufacturer's protocol. DsRNA for green fluorescent protein (gfp), which is not found in bee genomes, was synthesized as a control from AF097553 template (Wang et al., 2013b,c). DsRNA toward *tyr1* and *gfp* was purified using a phenol extraction. Aliquots were run on a 1% agarose gel for verification of dsRNA size and purity. For injections, the dsRNA was diluted to 10 μg/μl (30 μM) in nuclease free water.

We directly injected 2 μg (10 μg/μl × 207 nl) of dsRNA into the brains of the foragers through the median ocellus. All injection procedures were performed under a stereo-dissection microscope using a NANOLITER injector 2000 (World Precision Instruments, Sarasota, FL, USA) with a glass micropipette tip. After injection for 2 h, 4 h, 8 h, 12 h, and 24 h, the brains of dsRNA-injected honey bees were directly dissected and the effects of RNAi were detected through qPCR and western blot.

Table 1
T7 primers for *tyr1* dsRNA.

Forward	TAATACGACTCACTATAGGGCGAATCCTGAGCGTGTTCACGTA
Reverse	TAATACGACTCACTATAGGGCGAGGACAGCGTTTATCCTGCTC

Table 2
siRNA sequences for RNAi.

siRNA	Sequence	Position
siRNA-1	UCGUCGUAUGCUGGUUGCCAUUCUU	ORF
siRNA-2	CCCUCCGAUCGUAUGGUCUACUUCA	ORF
siRNA-3	UCGUUACGCGUUGAGUUCAGCUGAA	3′-UTR
siRNA-4	CCGAGAAUUCGAAUAUUCUUGAUGA	3′-UTR

2.3. Synthesis of siRNAs and injection

The *tyr1*–specific siRNAs (Table 2) were designed using Invitrogen's Block-iT™ RNAi Designer 5 that uses standard and proprietary algorithms based on individual base composition to design and synthesize with Invitrogen's proprietary Stealth technology. The top four non-overlapping siRNA sequences were list in Table 2.

We injected siMix-1 (siRNA-1 and siRNA-3, 207 nl × 50 μM each) or siMix-2 (siRNA-2 and siRNA-4, 207 nl × 50 μM each) into the brains of foragers through the median ocellus. GFP-siRNA (100 μM) was injected into different animals as a control. After injection for 2 h, 4 h, 8 h, 12 h, and 24 h, the brains of siRNA-injected honey bees were directly dissected and knockdowns were verified through qPCR and western blot.

2.4. RNA preparation and qPCR assay

Total RNA was extracted from brain tissues following the protocol for use of Trizol (Life technology, Camarillo, CA). DNase (Qiagen) was applied to eliminate DNA contamination in RNA samples. To analyze the expression levels of target genes, we reverse transcribed 2 μg total RNA in every sample using reverse transcriptase (Thermo Fisher, Waltham, MA, USA) and following the manufacturer's instructions. PCR amplification was conducted in ABI 7500 using Real Master-Mix (SYBR Green) kit (Roche, Basel, Switzerland). The amplification procedure was initiated with a 15 min incubation at 95 °C, followed by 40 cycles of 20 s at 95 °C; 20 s at 58 °C; 20 s at 72 °C. Melting curve was detected to confirm the amplification specificity of target genes. The primers for qPCR assay were provided in Table 3.

2.5. Protein preparation and western blot analysis

Total protein for western blot analysis were extracted through Trizol (Life Technology). For protein analyses, rabbit affinity-purified polyclonal antibodies against TYR1 were used. These anti-AmTyr1 antibodies developed in rabbit by four immunizations using two different peptide conjugates 21st Century Biochemical Incorporation (Marlboro, MA, USA), the specificity of the antibodies were analyzed in detail (Sinakevitch et al., 2017). We used the anti-AmTyr1 raised against peptide-2 (H_2N-PEELEPGTPCQLTRRQG-amide). The protein samples (30 μg) were separated by gel electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) by Wet transfer apparatus (Bio-Rad). Non-specific binding sites on the membranes were blocked with 5% bovine serum albumin for 1 h at room temperature (BSA) (Sigma-Aldrich). The blots were incubated with the primary antibodies (rabbit anti-AmTYR1 antibodies, 1:500; mouse anti-

Table 3
Primer sequences for quantitative PCR.

Genes	Forward primer	Reverse primer
tyr1	GTTCGTCGTATGCTGGTTGC	GTAGATGAGCGGGTTGAGGG
actin	GTTACAATAATTTCCGTTCC	GGCTTTACACCGTTCTCAT

actin antibody, 1:2000, Sigma-Aldrich) in 0.1% Tween 20 in 1 × Tris buffered saline (TBS-T, pH 7.4, Sigma-Aldrich) overnight at 4 °C, respectively. After incubation, the membranes were washed, incubated with anti-rabbit IgG-HRP or anti-mouse IgG-HRP secondary antibody (1:5000) (Santa Cruz Biotechnology) for 1 h at room temperature, and then washed again. Immunological detection was subsequently carried out using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Thermo Fisher, Waltham, MA, USA). The intensity levels of the western blot signals captured by image system (Bio-Rad) were quantified using densitometry (Image J software). The relative expression of TYR1 protein was calculated as the intensities of TYR1 divided by the corresponding intensities of Actin.

2.6. Data analysis and statistics

Student's *t*-test was conducted to analyze the differences of *tyr1* mRNA and its protein level between control and treatment groups. The data were expressed as mean ± standard error of the mean. $P < 0.05$ was considered statistically significant. All statistical data were analyzed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. The effects of tyr1 RNAi knockdown on mRNA levels

We designed a dsRNA with 547 bp in the CDS region of *tyr1* mRNA (DQ863218.1 and NP_001011594.1). We also designed four siRNAs, each with 25 bp, in which siRNA-1 and siRNA-2 are located in the coding sequence (CDS) and siRNA-3 and siRNA-4 are located in 3′ untranslated region (3′UTR) region. We mixed siRNA-1 and 3 together as siMix-1 and mixed siRNA-2 and siRNA-4 as siMix-2. Three types of RNAi molecules were injected into the brains through the median ocellus of honey bee foragers. We then quantified mRNA transcripts of *tyr1* in the brains after 2 h, 4 h, 8 h, 12 h, and 24 h using qPCR. We found that *tyr1* expression level was significantly lowered by dsRNA 8 h after the injections, and the decrease lasted to 12 h (Student's *t*-test, $t = 1.108$, $P = 0.277$ for 2 h; $t = 0.552$, $P = 0.585$ for 4 h; $t = 2.293$, $P = 0.029$ for 8 h; $t = 3.059$, $P = 0.004$ for 12 h; $t = 0.279$, $P = 0.783$ for 24 h) (Fig. 1A). Injection of siMix-1 induced a significant decrease of *tyr1* expression starting from 2 h and lasting through 24 h ($t = 4.810$, $P < 0.001$ for 2 h; $t = 3.026$, $P = 0.003$ for 4 h; $t = 2.919$, $P = 0.006$ for 8 h; $t = 2.274$, $P = 0.029$ for 12 h; $t = 2.212$, $P = 0.034$ for 24 h) (Fig. 1B). *Tyr1* expression was also significantly lowered after injection of siMix-2 from 2 h through 8 h ($t = 3.158$, $P = 0.003$ for 2 h; $t = 0.552$, $P = 0.585$ for 4 h; $t = 2.297$, $P = 0.027$; $t = 0.312$, $P = 0.757$ for 12 h; $t = 0.520$, $P = 0.656$ for 24 h), (Fig. 1C). These results indicate that both siRNAs and dsRNA successfully induced significant reduction of *tyr1* mRNA. However, the knockdown exhibited different temporal profiles, with siMix-1 showing the highest efficiency.

3.2. The effects of tyr1 RNAi knockdown on protein levels

To facilitate studying the effects of *tyr1* on neural activities in the honey bee brain, it is necessary to know the time window when the receptor proteins are affected by RNAi. We applied western blot to determine the level of TYR1 protein after injecting dsRNA and siRNAs in the brain of honey bees during the same time course. The protein level of TYR1 significantly decreased after injection of dsRNA from 8 h to 12 h after the injections (Student's *t*-test, $t = 0.278$, $P = 0.785$ for 2 h; $t = 1.002$, $P = 0.333$ for 4 h; $t = 2.276$, $P = 0.038$ for 8 h; $t = 3.019$, $P = 0.012$ for 12 h; $t = 0.796$, $P = 0.443$ for 24 h) (Fig. 2). Therefore, the decrease of protein level of TYR1 is consistent with the reduction of *tyr1* mRNA level induced by dsRNA.

TYR1 significantly decreased 8 h after injection with siMix-1 and the effect lasted to 24 h (Student's *t*-test, $t = 0.597$, $P = 0.557$ for 2 h; $t = 0.695$, $P = 0.498$ for 4 h; $t = 2.254$, $P = 0.041$ for 8 h; $t = 2.832$, $P = 0.011$ for 12 h; $t = 2.443$, $P = 0.025$ for 24 h) (Fig. 3). The injection of siMix-2 resulted in a significant decrease of TYR1 only at 8 h ($t = 0.124$, $P = 0.902$ for 2 h; *t*-test, $t = 0.496$, $P = 0.627$ for 4 h;

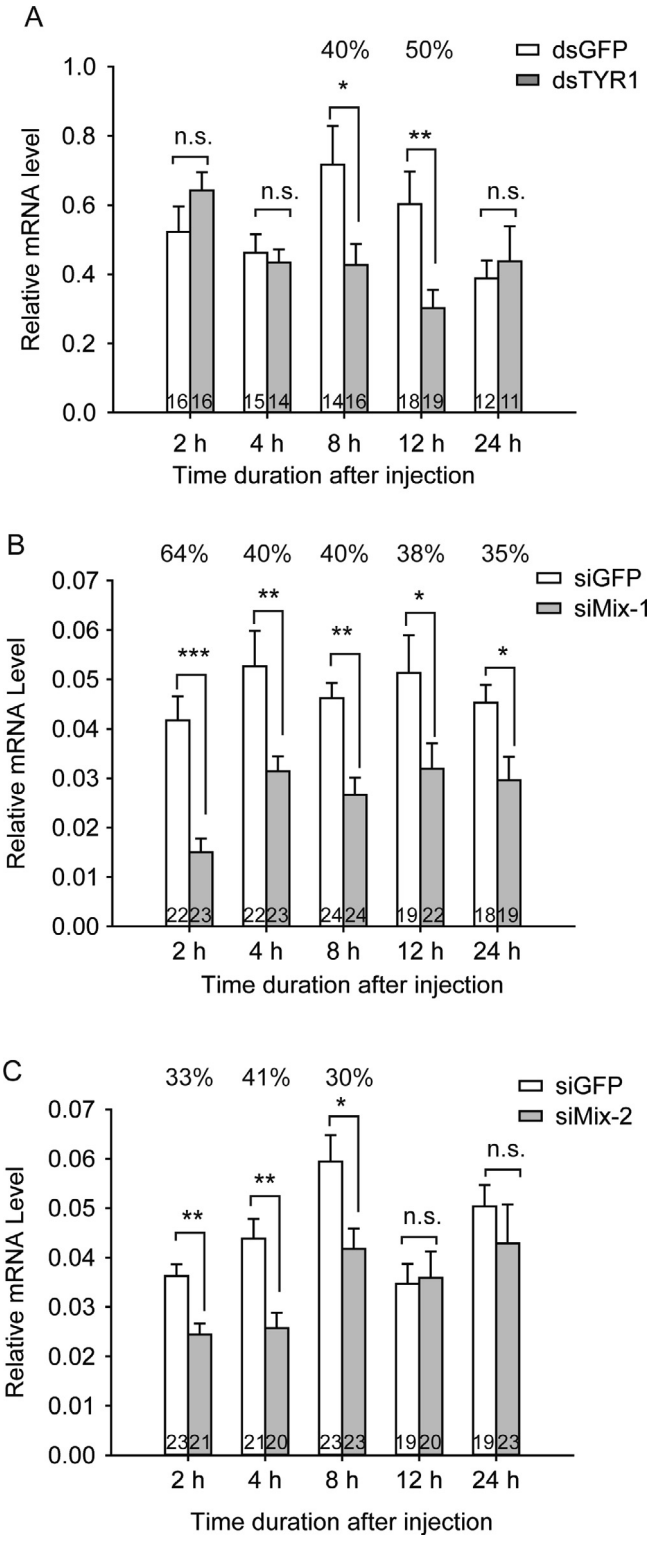

Fig. 1. Effects of dsRNA and siRNA on *tyr1* mRNA transcripts in honey bee brains. (A) The mRNA level of *tyr1* at 2 h, 4 h, 8 h and 12 h and 24 h after injection of (A) dsRNA, (B) siMix-1 (siRNA-1 + siRNA-3) or (C) siMix-2 (siRNA-2 + siRNA-4). The numbers of replicates are labeled in the bars. * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; n.s. indicates not significant.

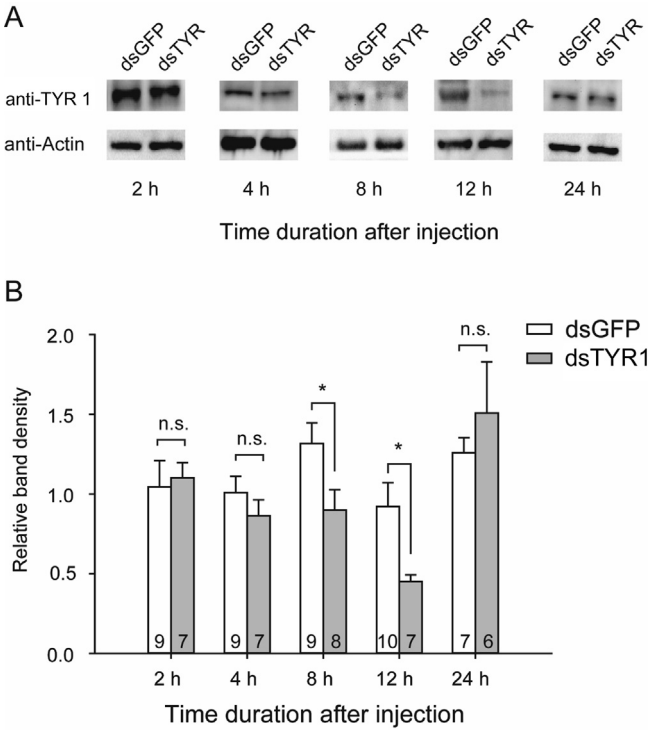

Fig. 2. Effects of dsRNA at protein level in the honey bee brain. (A) Randomly selected bands from western blots in each group. (B) The protein level of TYR1 at 2 h, 4 h, 8 h and 12 h and 24 h after injections of *ds*TYR1. Signs in the figure have the same statistical meanings as in Fig. 1.

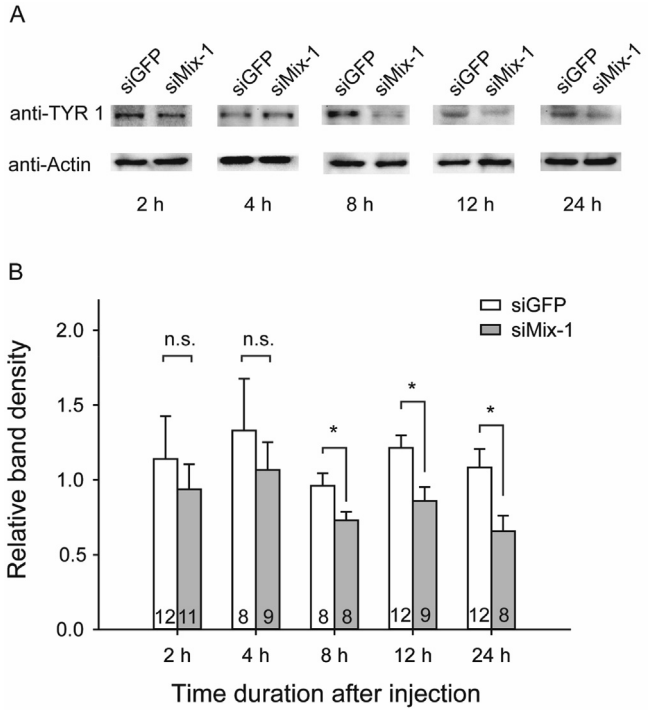

Fig. 3. Effects of siMix-1 at protein level in the honey bee brain. (A) Randomly selected bands from western blots. (B) Protein levels of TYR1 after injections of siMix-1 at 2 h, 4 h, 8 h and 12 h and 24 h. Signs in the figure have the same statistical meanings as in Fig. 1.

$t = 2.903$, $P = 0.012$for 8 h; $t = 0.616$, $P = 0.545$ for 12 h; $t = 0.963$, $P = 0.345$ for 24 h) (Fig. 4). Compared to the reduction in mRNA, there was a 6 h delay in the reduction of TYR1 proteins by both types of siRNA. And the duration of knockdown effect at the protein level was consistent with that of the mRNA.

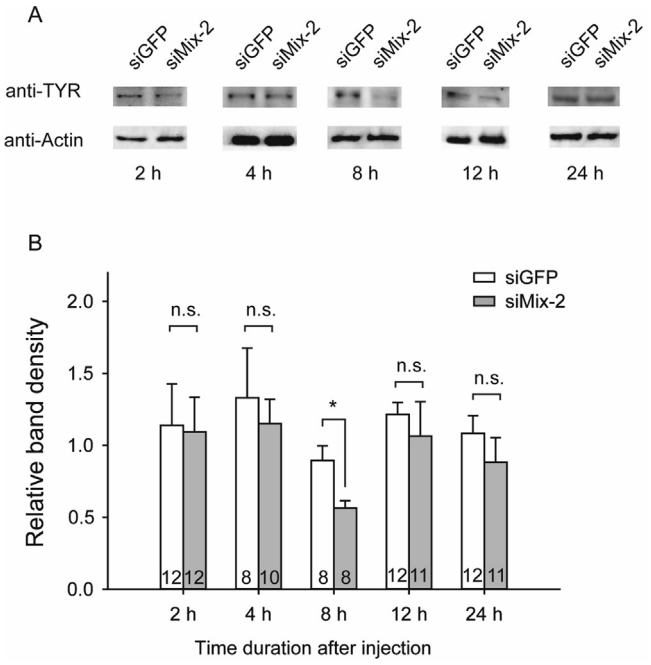

Fig. 4. Effects of siMix-2 at the protein level in the honey bee brain. (A) Randomly selected bands from western blots. (B) Protein levels of TYR1 after injections of siMix-2 at 2 h, 4 h, 8 h and 12 h and 24 h. Signs in the figure have the same statistical meanings as in Fig. 1.

4. Discussion

In this study, we investigated the efficiency of *tyr1* gene knockdown by both dsRNA and siRNAs in honey bee brain at both mRNA and protein levels. We determined the optimal time window for studying the functions of TYR1 in the honey bee brain using the RNAi with both types of molecules.

We compared the efficacies of different RNAi molecules and show they all work, albeit on different time scales. The initial effects of siRNAs are earlier than that of dsRNA. siMix-1 and siMix-2 affect *tyr1* mRNA expression from 2 h, whereas dsRNA decreased *tyr1* mRNA level from 8 h. Moreover, there was also variation between the same types of molecules. The effect of siMix-1 lasted at least for 24 h, and was longer than that of siMix-2.

SiRNA is the product of dsRNA that is processed by Dicer, and siRNA is the direct mediator for RNAi. Because the preprocessing of dsRNA was skipped by use of siRNA, it was reasonable to expect that the effect of siRNA would occur earlier than with use of dsRNA. In addition, the durations of the gene knockdown from three types of RNAi molecules were different at both mRNA and protein level: siMix-1 had the longest duration for the knockdown, dsRNA was in the middle and siMix-2 had the shortest. In general, the knockdown effect varies in different organisms, in different tissues and for different targeted genes (Mittal, 2004), and it depends on the specific construction of siRNA, delivery method and dose of the RNAi molecules (Mittal, 2004). Studies also showed that a large variability in knockdown effect by both dsRNA (Loy et al., 2012; Pridgeon et al., 2008) and siRNA (Krautz-Peterson et al., 2007) can result from targeting different regions of the gene. This may be the reason for variability that we observed in the knockdown efficiency among three types of RNAi molecules, since the dsRNA targeted the CDS, and each siMix targeted both CDS and 3′UTR. Furthermore, studies have suggested that dsRNA had higher efficiency than siRNA, but combinations of two interfering siRNA could have enhanced knockdown effect (Dell'Oca et al., 2014; Wang et al., 2013a) because it

may increase the accessibility of siRNAs to target mRNA (Dell'Oca et al., 2014; Krautz-Peterson et al., 2007). This may explain why we observed that siMix-1 had the highest efficiency among the three types of RNAi.

The change in the protein level by RNAi is directly correlated to the effect on phenotypes. However, the reduction of mRNA transcripts caused by dsRNA and siRNA does not necessarily lead to a reduction of the protein level. The reason is that different proteins have different turnover rates and a slow turnover can weaken or mask the knockdown effect causing no change in phenotype (Wu et al., 2004). So it is necessary to quantify the effect on protein levels to increase confidence that the phenotypic effect can be due to the specific knockdown of the gene expression. In this study, we only observed a 6-h delay of protein reduction of TYR1 induced by siMix-1 and siMix-2 compared to its mRNA, implying that TYR1 turnover is relatively fast (up to 6 h). Biogenic amines have very rapid turnover rate (Berry, 2004; Pei et al., 2016). And their receptors play critical roles in a tissue specific manner. On the other hand, there is no delay in the reduction of TYR1 proteins by dsRNA. Multiple siRNAs would be produced after dsRNA splicing, and these siRNAs probably targeted different regions of tyr1 mRNA. Thus, the effect on the protein level can be immediately observed when mRNA significantly decreased.

Overall, we found considerable variability in gene knockdown by dsRNA and siRNAs for TYR1 in honey bee brain. Therefore, use of any RNAi method for studying phenotypes should be coupled with a precise quantification of the amount and time course of mRNA and protein knockdown. The efficiency and duration of dsRNA and siRNAs on tyr1 mRNA and protein level are all different. For phenotyping studies, 8 h is the best time point if using dsRNA or siRNAs, and siMix-1 is the best choice for testing phenotype at different time points. This kind of analysis will be necessary particularly when knockdowns need to occur over long time course, which might be achievable with other delivery methods. The effect we observed may be influenced by the use of injection of small amounts into the brain, rather than injection into the hemolymph (Van Wielendaele et al., 2013) or feeding (Christiaens et al., 2014). Injection is necessary for studies of the brain because the neural sheath prevents entry of si- and ds-RNA molecules. Nevertheless, careful consideration of the regions of a target gene and combinations of interfering siRNAs are important to achieve a high efficiency of RNAi. This factors all can be a key to a success of gene functional studies in the future.

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