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Activity-dependent expression of miR-132 regulates immediate-early gene induction during olfactory learning in the greater short-nosed fruit bat, *Cynopterus sphinx*

Murugan Mukilan [a], Durairaj Ragu Varman [a], Sivasubramaniam Sudhakar [b], Koilmani Emmanuvel Rajan [a,*]

[a] Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620024, India
[b] Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli 627012, India

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ABSTRACT

The activity-dependent expression of immediate-early genes (IEGs) and microRNA (miR)-132 has been implicated in synaptic plasticity and the formation of long-term memory (LTM). In the present study, we show that olfactory training induces the expression of IEGs (EGR-1, C-fos, C-jun) and miR-132 at similar time scale in olfactory bulb (OB) of *Cynopterus sphinx*. We examined the role of miR-132 in the OB using antisense oligodeoxynucleotide (AS-ODN) and demonstrated that a local infusion of AS-ODN in the OB 2 h prior to training impaired olfactory memory formation in *C. sphinx*. However, the infusion of AS-ODN post-training did not cause a deficit in memory formation. Furthermore, the inhibition of miR-132 reduced the olfactory training-induced expression of IEGs and post synaptic density protein-95 (PSD-95) in the OB. Additionally, we show that miR-132 regulates the activation of calcium/calmodulin-dependent protein kinase-II (CaMKII) and cAMP response element binding protein (CREB), possibly through miR-148a. These data suggest that olfactory training induces the expression of miR-132 and IEGs, which in turn activates post-synaptic proteins that regulate olfactory memory formation.

1. Introduction

Activity-dependent changes in gene expression have been implicated in synaptic plasticity and long-term potentiation (LTP) (Waltereit et al., 2001; Yuste & Bonhoeffer, 2001). LTP can be initiated by either an influx of Ca^{2+} or cyclic adenosine monophosphate (cAMP) (Frey, Huang, & Kandel, 1993; Malenka, Lancaster, & Zucker, 1992) followed by the activation of protein kinase A (PKA) and extracellular-signal-regulated kinase-1/2 (ERK1/2) (Abel et al., 1997; English & Sweatt, 1997). Studies in animal models have shown that behavioral training induces the expression of several protein kinases, including ERK-1/2 (Besnard, Laroche, & Caboche, 2014; Duvarci, Nader, & Le Doux, 2005; Thomas & Huganir, 2004). ERK 1/2 phosphorylates the transcriptional activator cAMP response element binding protein-1 (CREB-1) at Ser^{133} (Adams & Sweatt, 2002; Peng, Zhang, Zhang, Wang, & Ren, 2010; Thiels & Klann, 2001; Zhang, Okutani, Inoue, & Kaba, 2003), which in turn activates immediate-early genes (IEGs), including early growth response gene-1 (*Egr-1*) (Cheval et al., 2012; Ganesh, Bogdanowicz, Balamurugan, Ragu Varman, & Rajan, 2012; Guzowski, Setlow, Wagner, & McGaugh, 2001; Veyrac, Besnard, Caboche, Davis, & Laroche, 2014), *C-fos* (Charra, Datiche, Gigot, Schaal, & Coureaud, 2013; Chawla et al., 2013; Peng, Zhang, Ren, Zhang, & Wang, 2011), and *C-jun* (Reinecke, Herdegen, Eminel, Aldenhoff, & Schiffelholz, 2013). The expression of IEGs has been known to facilitates synaptic plasticity in the olfactory bulb (OB) (Brennan, Hancock, & Keverne, 1992; Charra et al., 2013; Ganesh et al., 2012; Guthrie, Anderson, Leon, & Gall, 1993; Veyrac, Wang, Baum, & Bakker, 2011).

Recently, several lines of evidence have suggested that CREB regulates synaptic plasticity by controlling the expression of IEGs and micro-RNAs (miRNAs) (Nudelman et al., 2010; Smalheiser et al., 2010; Vo et al., 2005). Increasing evidence has indicated that miRNAs could be involving in different biological processes, including synaptic plasticity and memory formation (Bredy, Lin, Wei, Baker-Andresen, & Mattick, 2011; Scott et al., 2012; Smalheiser & Lugli, 2009). It has been reported that the exposure to odors induces the expression of CREB-regulated miRNA-132 (Hansen, Sakamoto, Wayman, Impey, & Obrietan, 2010; Im & Kenny, 2012; Nudelman et al., 2010; Smalheiser et al., 2010), which can regulate the genes involved in synaptic plasticity and memory formation.

* Corresponding author. Fax: +91 431 2407045.
E-mail address: emmanuvel1972@yahoo.com (K.E. Rajan).

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The greater short-nosed fruit bat, *Cynopterus sphinx*, begins to visit fruit-bearing trees after sunset (Balasingh, Koilraj, & Kunz, 1995; Marimuthu, Rajan, Koilraj, Isaac, & Balasingh, 1998) and feeds on variety of fruits, flowers and leaves (Bhat, 1994). It has been stated that fruit-eating bats could have learned which fruits, flowers and leaves are palatable along with their locations by the volatile compounds originated from the food source (Hodgkison et al., 2007; Luft, Curio, & Tacud, 2003; Sánchez et al., 2006). Previously, we have shown that when the level of 5-HT was depleted in the OB, odor stimuli failed to activate the 5-HT_{1A} receptor, the phosphorylation of ERK-1/2 and CREB (Ganesh, Bogdanowicz, Haupt, Marimuthu, & Rajan, 2010), and IEGs (Ganesh et al., 2012). Further, we demonstrated that the infusion of *egr-1* AS-ODN inhibited the expression of *egr-1* and impaired olfactory memory (Ganesh et al., 2012). In the present study, we examined the roles of CREB-regulated different IEGs (*Egr-1, C-fos, C-jun*) and miR-132 in olfactory learning and memory formation.

2. Materials and methods

2.1. Animals

Wild, free-ranging short-nosed fruit bats, *C. sphinx*, were captured with a mist net in a guava orchard located 2 km away from Bharathidasan University campus, Tiruchirappalli, Tamil Nadu, India (10°16′N; 78°15′E). The mist net (9 m × 2 m; Avinet-Dryden, USA) was placed near the regular flight path of bats 1 h before sunset (Kunz & Brock, 1972) and was set 2 m above ground level (Nathan et al., 2001). The morphometric details of the bats were recorded, and the animals were tagged with plastic neck collars with light reflective colored tape (Rajan & Marimuthu, 1999) before they were transported to the animal house facility. The bats were maintained in a free flight chamber (2.2 × 1.3 × 2.1 m) with a temperature of 30 °C (±3 °C), relative humidity of 85 ± 3%, and a light and dark cycle of 12:12 h. During the dark phase, fruits such as *Carica papaya* (Papaya), *Achras sapota* (Sapotta) and *Psidium guajava* (guava) along with water were provided *ad libitum*. Animals were allowed to habituate themselves to the animal facility for seven days before the experiments were conducted. Care was taken to minimize pain and number of animals used. All experimental protocols were approved by Bharathidasan University Wild Animal Ethics Committee (03/AS/BUWAEC/2008) and complied with the current laws of India.

2.2. Experimental setup

The learning and memory abilities of the bats were tested in a double flight chamber, in which a free-flight chamber served as a roosting chamber while an experimental chamber (2.1 m × 2.4 m × 2.4 m) was used to conduct the behavioral experiments. A window connected the free-flight chamber and the experimental chamber, which enabled the bats to be transferred without disturbance. During each behavioral test, only one bat was permitted in the experimental chamber at one time, and the test was conducted under a red light (0.09 ± 0.02 lx). The activity of each bat was recorded on a computerized activity monitor (Electronic Engineering Corporation Inc., Chennai, India), which incorporated an infra-red (IR) receiver–transmitter and a mass sensitive platform. Two platforms were kept in the experimental room at a distance of 1.8 m: one for control fruit and another for novel fruit. The locations of the food tray and the platform were changed randomly every day to prevent spatial learning; however, the distance between the perch and the food tray (2.4 m) and the distance between the platforms (1.8 m) was always maintained.

2.3. Infusion procedure

The antisense oligodeoxynucleotide (AS-ODN)/nonsense oligodeoxynucleotide (NS-ODN) (50 pmol) or phosphate buffer saline (PBS, 30 μl) alone was infused with a Hamilton syringe that was placed into position for 1 min, which was followed by a 5-min infusion through the guide cannula into the olfactory bulb. The syringes were left in place for an additional 2 min to allow for diffusion. The placement of the cannula and the diffusion procedures were followed as reported previously (Ganesh et al., 2010). The injection time and experimental time point was chosen based on the relative peak expression of miR-132 from the time of olfactory stimulus.

2.3.1. Pre-training infusions

To examine the role of miR-132 in olfactory learning and memory, bats were divided into three groups ($n = 6$, for each group): PBS group (con), AS-ODN group (antisense-miR-132) and NS-ODN group (non-sense-miR-132). The control group received phosphate buffered saline (PBS); the AS-ODN group received antisense miRNA-132 (5′-CGACCATGGCTGTAGACTGTTAC-3); and the NS-ODN group received nonsense miRNA-132 (5′-GTTCCYACAG TAACAATC-3′). Training began 2 h after the infusion of the PBS/AS-ODN/NS-ODN.

2.3.2. Post-training infusions

To confirm the role of miR-132 in olfactory learning, bats were divided into similar PBS/AS-ODN/NS-ODN groups ($n = 6$, for each group) and were trained to a novel odor. Twenty-four hours after the training session, the control group received PBS, the AS-ODN group received anti-sense miRNA-132, and the NS-ODN group received non-sense miRNA-132 into the OB.

2.4. Behavioral analysis

2.4.1. Training

Pieces of peeled apple (50 g) mixed with freshly prepared cinnamon powder (0.8% wt/wt) (a food combination that is not available in the bats' natural habitat) (Ratcliffe & Ter Hofstede, 2005) was used as a novel odor. Pieces of apple without cinnamon powder were provided as a control to determine whether the bats were attracted to the cinnamon odor. Before the training session, fruits such as *A. sapota*, *C. papaya*, and *P. guajava* were provided to all bats in the free-flight room to avoid food deprivation and to maintain their energy supply. The bat's responses to the novel odor during training and testing were classified as out-flies [a short flight from the perch (which indicated that the bat was active)], attempts [a bat approaching the food tray (novel odor), landing on the platform and returning without picking up the chopped fruit] and feeding bouts [a bat landing directly on the food tray and picking up a chopped fruit] as reported previously (Ganesh et al., 2010, 2012).

2.4.2. Retention test

Bats were allowed to stay in the free-flight room after training and were fed with fruits such as *C. papaya*, *A. sapota*, and *P. guajava* and water. The memory test was conducted for the pre- and post-training groups in a single session, 24 h and 48 h after training, respectively. The retention test was conducted following the same procedures as for training.

2.5. Sample preparation

To examine the expression patterns of miRNAs and other genes, bats were infused with PBS ($n = 3$) or AS-ODN ($n = 3$), or NS-ODN ($n = 3$) and trained to a novel odor. After training, the bats were euthanized at different time points (0 min, 30 min, 60 min,