International Journal of Biochemistry and Cell Biology 116 (2019) 105620

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Diazepam enhances melanogenesis, melanocyte dendricity and melanosome transport via the PBR/cAMP/PKA pathway

Jinpeng Lv[a,b,c,*,1], Ying Fu[a,1], Rongyin Gao[d], Jiawen Li[a], Maofan Kang[a], Guoqiang Song[a], Changjun Yun[e]

[a] College of Pharmaceutical Engineering and Life Sciences, Changzhou University, Changzhou 213000, China
[b] Shanghai Institute of Pharmaceutical Industry, Shanghai 200000, China
[c] Yabang Medical Research Institute, Changzhou 213000, China
[d] Department of Pharmacy, The first people's Hospital of Changzhou, The third Affiliated Hospital of Soochow University, Changzhou 213000, China
[e] Changzhou Wujin People's Hospital, Changzhou 213000, China

ARTICLE INFO

Keywords:
Diazepam
Melanogenesis
Melanosome transport
PBR
cAMP/PKA

ABSTRACT

Diazepam is a medicament of the benzodiazepine family and it typically produces a sedative effect. Researchers have revealed that diazepam can induce melanogenesis and produce dendrite-like structures in B16 melanoma cells. However, the associated mechanisms of melanogenesis and phenotypic alterations have mostly remained unknown. In this study, we determined the effects of diazepam on melanogenesis, cellular phenotypic alterations, the location of melanosomes and the expression of relevant proteins in melanocytes using Masson–Fontana ammoniacal silver staining, scanning electron microscopy, immunocytochemistry and western blot analysis. Our results collectively indicated that diazepam had a pivotal role in melanocytes by enhancing melanin synthesis, melanocyte dendricity, melanosome trafficking, and capture at the dendrite tips. These functions might be attributed to the fact that diazepam activated the peripheral benzodiazepine receptor (PBR). This increased intracellular levels of cAMP, which stimulated the phosphorylation of cAMP response element-binding (CREB). As a result, this increased the tyrosinase, microphthalmia-associated transcription factor (MITF), Rab27a, Myosin Va, Rab17 and Cdc42 expression. This caused melanogenesis and melanosome transport. Therefore, our findings may provide a potential strategy for treating anti-hypopigmentation disorders.

1. Introduction

Skin melanin pigment plays a crucial role in human social interaction and protection against the harmful effects of ultraviolet radiation (Slominski et al., 2004). Cutaneous pigmentation is caused by a complex multi-step process that ultimately leads to the distribution of melanin in the various layers of the epidermis (Noguchi et al., 2014; Raposo and Marks, 2007). Melanin is produced in melanosomes, which are tissue-specific lysosome-related organelles in melanocytes. After melanosomes mature around the nuclei of melanocytes, they are removed from the perinuclear region along microtubules and are captured on actin filaments at the cell dendrites where they are transferred to adjacent keratinocytes (Beaumont et al., 2011).

During the past few decades, several key factors involved in pigmentation have been discovered. Tyrosinase (TYR), TYR-related protein 1 (TRP1) and TYR-related protein 2 (TRP-2) are the key melanogenic enzymes that participate in melanogenesis (Costin et al., 2005). Another key factor involved in melanogenesis is microphthalmia-associated transcription factor (MITF), which not only upregulates TYR, TRP-1 and TRP-2, but also regulates melanocyte proliferation and survival (Levy and Fisher, 2011; Vachtenheim and Borovanský, 2010). Kinesin superfamily proteins (KIFs) function as molecular motors involved in melanosome transport along microtubules (Hara et al., 2000; Hirokawa et al., 2009; Raposo and Marks, 2007). After being transferred to actin filaments, melanosomes are transported by the tripartite protein complex consisting of Rab27a-Myosin Va-Melanophilin (Ohbayashi and Fukuda, 2012). Furthermore, extension of dendrites is a fundamental requirement for the transfer of melanosomes (Ohbayashi and Fukuda, 2012). Melanocyte dendrites are the hormone-responsive structures containing actin and microtubules whose primary purpose is to transport melanosomes to the dendrite tip (Scott, 2002; Singh et al., 2010). Multiple Rab proteins (e.g. Cdc42 and Rab17) play a crucial role

* Corresponding author at: Changzhou University, No.1, Gehu Road, 213164, Changzhou, China.
E-mail address: lvjinpeng1988@126.com (J. Lv).
[1] These authors have contributed equally to this work and should be considered co-first authors.

https://doi.org/10.1016/j.biocel.2019.105620
Received 7 May 2019; Received in revised form 16 September 2019; Accepted 23 September 2019
Available online 24 September 2019
1357-2725/

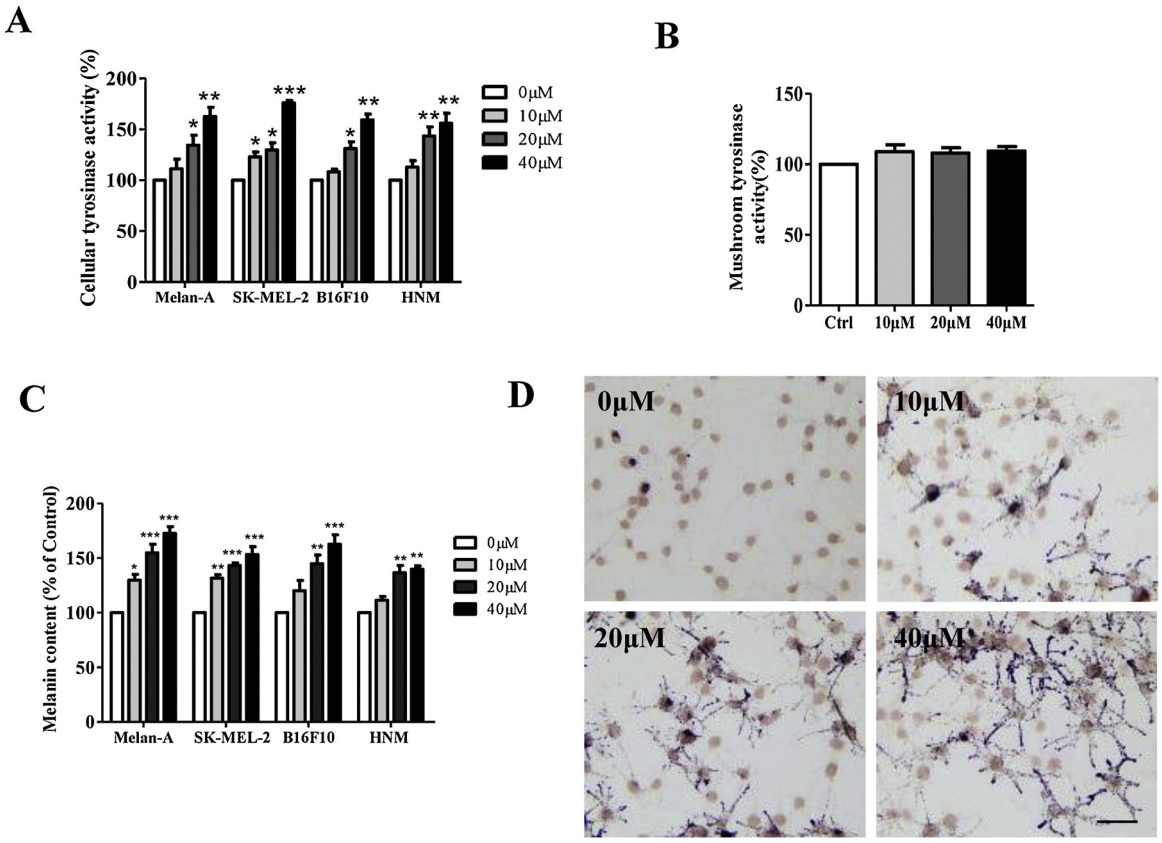

Fig. 1. Diazepam promoted tyrosinase activity and induced hyperpigmentation in melanocytes. (A) Cellular tyrosinase activity and (B) mushroom tyrosinase activity in a cell-free assay system were measured after diazepam-treated as described in Supplemental Material. (C) Melan-A, SK-MEL-2, B16F10 and HNM were treated with diazepam (10, 20, 40 μM) for 48 h, and melanin contents were measured. (D) SK-MEL-2 cells were treated with diazepam for 48 h, and were then stained with Masson–Fontana ammoniacal silver stain. Bar = 20 μm. Data are expressed as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated cells.

in dendrite formation of melanocytes (Beaumont et al., 2011; Luo, 2000). The second messenger cAMP activates protein kinase A (PKA), which phosphorylates the cAMP response element-binding protein (CREB), subsequently promoting the transcription of genes related to melanogenesis and dendrite formation, such as MITF and Cdc42 (Buscà and Ballotti, 2010; Saha et al., 2006; Scott and Leopardi, 2003).

Diazepam is a medicament of the benzodiazepine family that usually produces a calming effect by activating the central benzodiazepine receptor (CBR). It increases the frequency of opening of the chloride ion channel by promoting the binding of gamma aminobutyric acid (GABA) to $GABA_A$ receptors. In addition, previous studies suggested that diazepam induces melanogenesis and produces of dendrite-like structures in B16 melanoma cells (Matthew et al., 1981; Landau et al., 1998). However, the associated mechanisms of diazepam-induced melanogenesis and the phenotypic alterations have mostly remained unknown.

In this present study, we sought to understand how diazepam affects the pigmentation processes of melanocytes. We determined that diazepam-induced melanosome transport and promoted melanogenesis. Diazepam played these roles by activating the peripheral benzodiazepine receptor (PBR), not the central benzodiazepine receptor (CBR). Once activated, the PBR phosphorylated the CREB and elevated the expression of MITF, TYR, Rab27a, Myosin Va, Rab17 and Cdc42, resulting in melanogenesis and melanosome transport.

2. Materials and methods

2.1. Reagents

Diazepam was purchased from Sigma-Aldrich (MO,USA). Antibodies against TYR, TRP-1, TRP-2, MITF, p-PKA $_{cat}$ (phospho T197), PKA $_{cat}$, p-CREB and CREB were purchased from Abcam (Cambridge, UK). Antibodies against Myosin Va, KIF5b, Rab27a, Rab17, Cdc42, HMB45, p-PKA and PKA were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against p-p38 MAPK, p38 MAPK, p-ERK, ERK, p-JNK and JNK were obtained from Cell Signaling Technology (MA, USA). The PKA inhibitor H89, the BCA protein assay kit, the cAMP assay kit, the LDH release assay kit, antibodies against β-actin and Alexa Fluor 555-labeled Donkey Anti-Rabbit IgG (H + L) were purchased from Beyotime Biotechnology (Shanghai, China).

2.2. Cells and human skin culture

SK-MEL-2, Melan-A and B16F10 cells were obtained from the Cell Bank, Chinese Academy of Sciences. These cells were grown in DMEM medium (GIBCO, USA) supplemented with 10% fetal bovine serum (HyClone, USA) in a humidified atmosphere with 5% CO_2 at 37℃. Human epidermal melanocytes (HNM) were purchased from Sciencell Research Laboratories (CA, USA). The HNM were grown in Medium 254CF (GIBCO,USA) supplemented with Human Melanocyte Growth Supplement (GIBCO,USA) in a humidified atmosphere with 5% CO_2 at 37 ˚C.

The studies on human material were approved by the local ethics committee. Normal human skin was obtained from young male adult

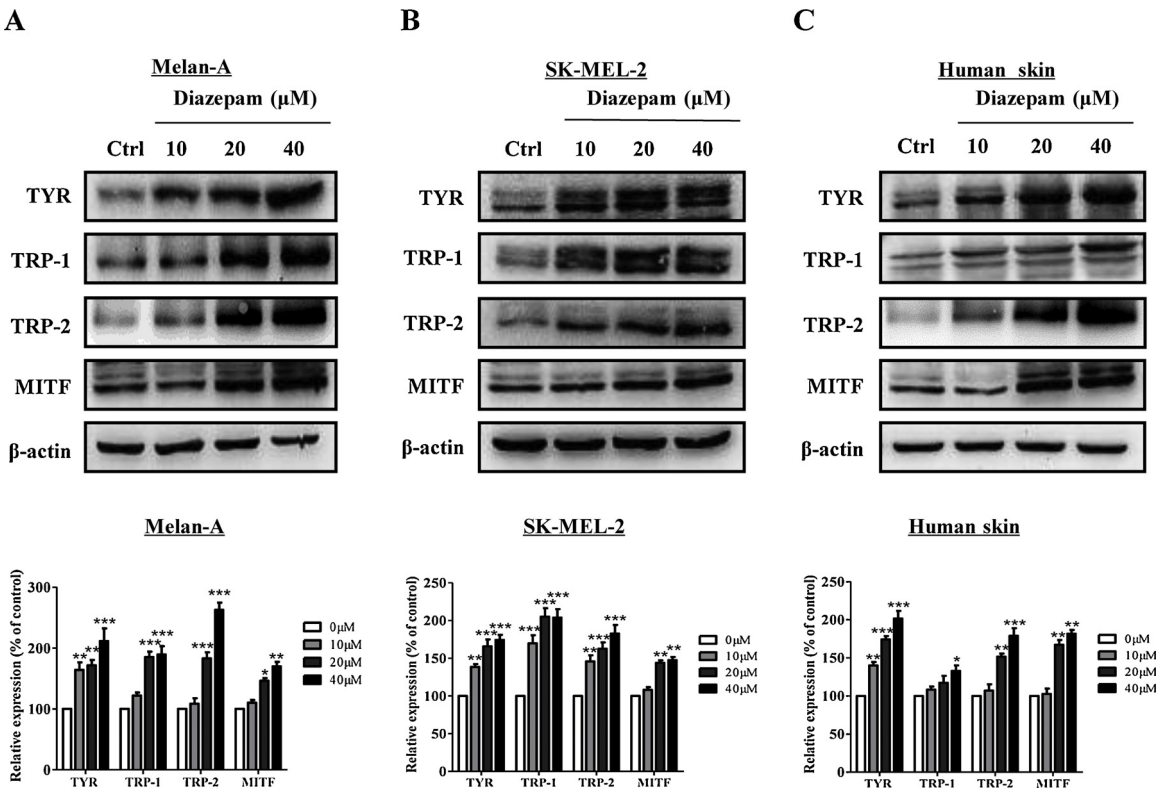

Fig. 2. Diazepam upregulated the expression levels of TYR, TRP-1, TRP-2 and MITF. (A) Melan-A cells, (B) SK-MEL-2 cells and (C) Human skin were treated with diazepam (10, 20, 40 μM) for 48 h and western blot was then applied to detect the protein levels of TYR, TRP-1, TRP-2 and MITF. Data are expressed as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated cells.

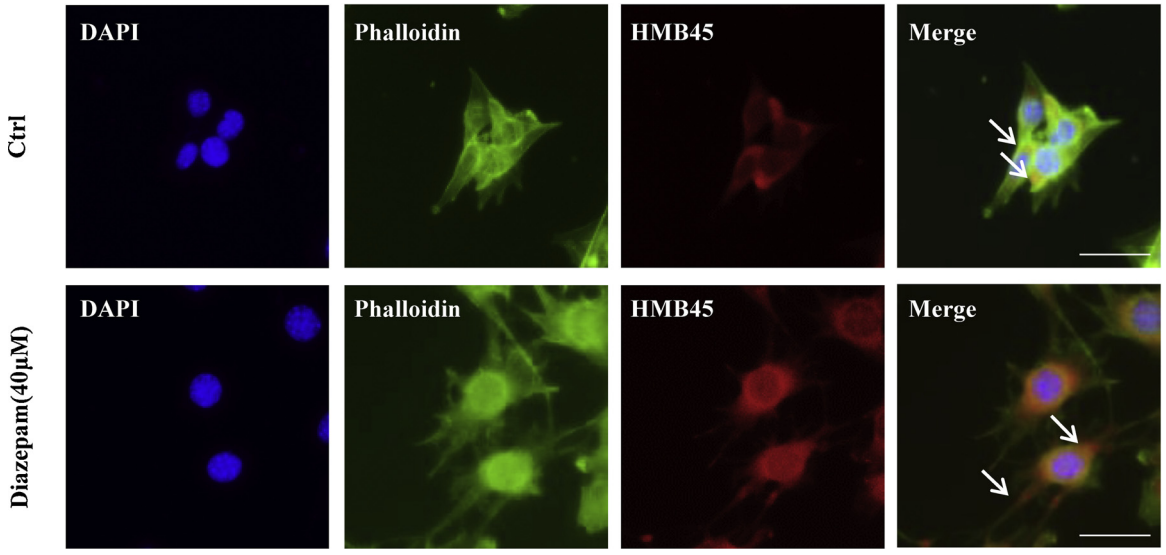

Fig. 3. SK-MEL Diazepam treatment: imaging of morphology and melanosome distribution. Immunostaining by the immunofluorescence method was performed 48 h after treatment with diazepam (40 μM). The arrowhead indicates accumulated melanosomes. Bar = 20 μm.

foreskins and the operation was followed standard protocols (Lu et al., 2007). The foreskins were cut into packets. Subsequently, the packets were carefully placed into William's E medium, supplemented with 10 μg/ml of insulin (Aladdin), 10 ng/ml of hydrocortisone (Aladdin) and 2 mmol/L of L-glutamine (Aladdin). The cultures were maintained a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Melanin assay

Total melanin in the cell pellet was dissolved in 100 μl of 1 mol/L NaOH (containing 10% DMSO) for 2 h at 80 °C, and the dissolved melanin was measured at 405 nm. Melanin content was calculated as a percent of the control (Zhou et al., 2014).

2.4. Masson–Fontana ammoniacal silver staining

According to the Fontana-Masson stain protocol (Gu et al., 2017), the cells were first fixed in formalin for 15 min and then washed three times in distilled water. Then these cells were incubated in Fontana ammoniacal silver solution overnight at room temperature. After

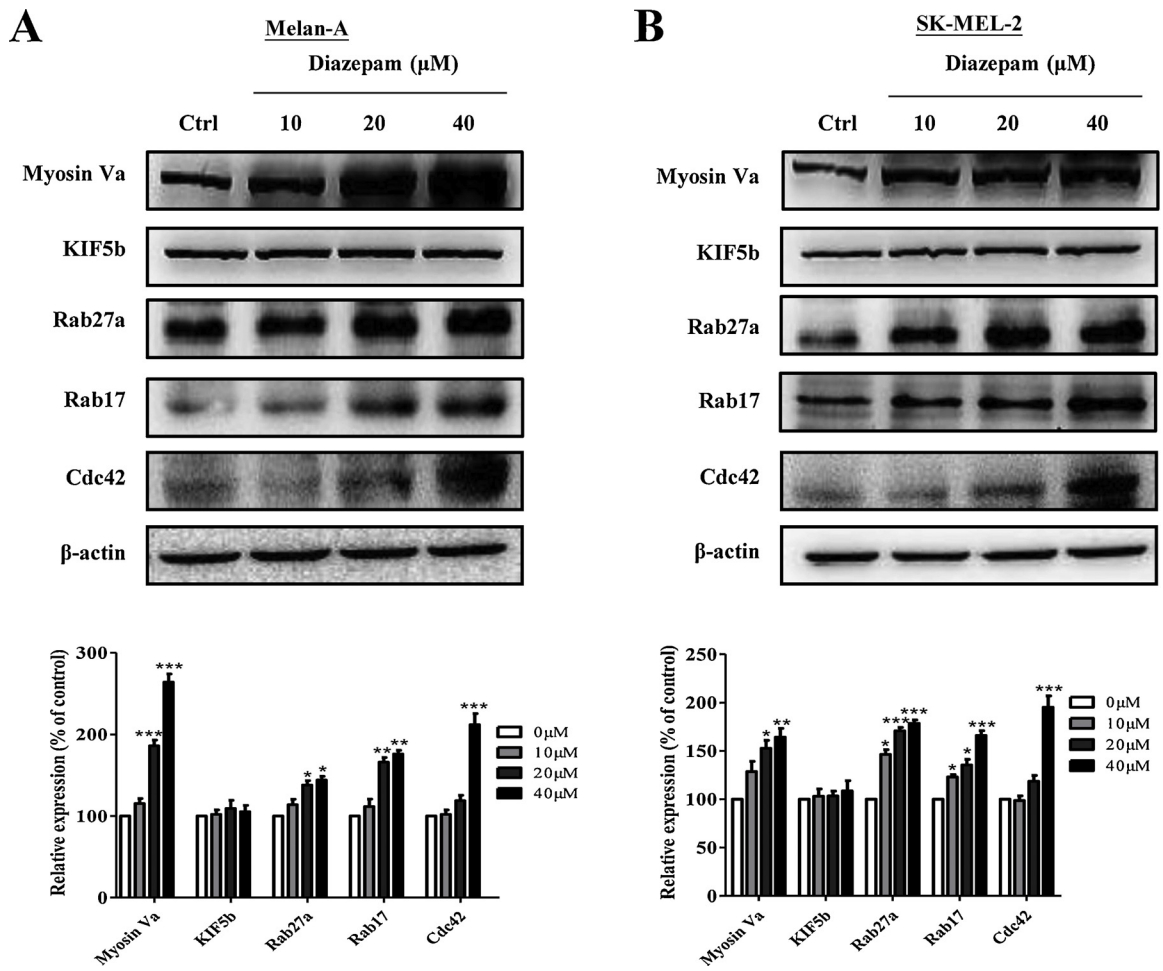

Fig. 4. Diazepam altered expression of proteins relating to melanosome transport in melanocytes. (A) Melan-A cells and (B) SK-MEL-2 cells were treated with diazepam (10, 20, 40 μM). After 48 h treatment, the expression levels of Myosin Va, KIF5b, Rab27a, Rab17 and Cdc42 were examined. The results were shown as relative values to the control. Data are expressed as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated cells.

rinsing three times in distilled water, the cells were incubated in Hypo solution for 3 min. Next, the cells were washed in distilled water and counterstained with neutral red stain for 5 min. Finally, the cells were washed in distilled water, dehydrated in absolute alcohol, and mounted for observations.

2.5. Western blot analysisw

The extracted total proteins (40 μg) were separated on 6%, 10%, or 15% SDS polyacrylamide gels using a vertical electrophoresis system (Bio-Rad, USA) and then transferred onto a nitrocellulose filter (NC) membrane using an electroblotting device (Bio-Rad, USA). The membranes were blocked for 90 min at room temperature with 2.5% BSA in TBST and then incubated with appropriate antibodies overnight at 4 °C (Yoshizaki et al., 2017). Subsequently, the blots were incubated with peroxidase-conjugated secondary antibodies for 60 min at room temperature and visualized by enhanced chemiluminescence using a multifunctional chemiluminescence imaging system. The results of the western blot analysis are representative of at least three repeated experiments.

2.6. Immunocytochemistry

SK-MEL-2 cells were grown on glass coverslips in DMEM medium (GIBCO, USA) supplemented with 10% fetal bovine serum (HyClone, USA) in a humidified atmosphere with 5% CO_2 at 37 °C. About 48 h after the diazepam treatment, the cells were immunostained with anti-HMB according to the standard immunofluorescence protocol. The nuclei were stained with DAPI and Rhodamine Phalloidin (fluorescent F-actin probe) to label actin (Noguchi et al., 2014). Images were obtained using an Olympus BX41 fluorescent microscope (Tokyo, Japan).

2.7. Statistical analysis

All data are expressed as mean ± standard error. The statistical analysis was performed using one-way ANOVA, followed by Turkey's post-hoc test for multiple comparisons. Significant differences were accepted when $P < 0.05$.

3. Results

3.1. Diazepam regulated the pigmentation process

Before investigating the effects of diazepam on melanogenesis, the MTT assay and LDH measurements were performed to examine whether diazepam was toxic to the melanocytes (Lu et al., 2007; Zhou et al., 2014). As shown in Figs. S1, S2, diazepam was not toxic to the melanocytes or human skin organ cultures at the concentrations of 10–40 μM after 48 h. TYR activity is critical in melanogenesis. The effect of diazepam on cellular TYR activity was measured by L-DOPA oxidation. As shown in Fig. 1A, treatment with diazepam at dosages of 10–40 μM resulted in a dose-dependent increase in TYR activity in the Melan-A, SK-MEL-2, B16F10 and HNM cells. Furthermore, mushroom TYR activity assay was carried out to investigate the direct effects of

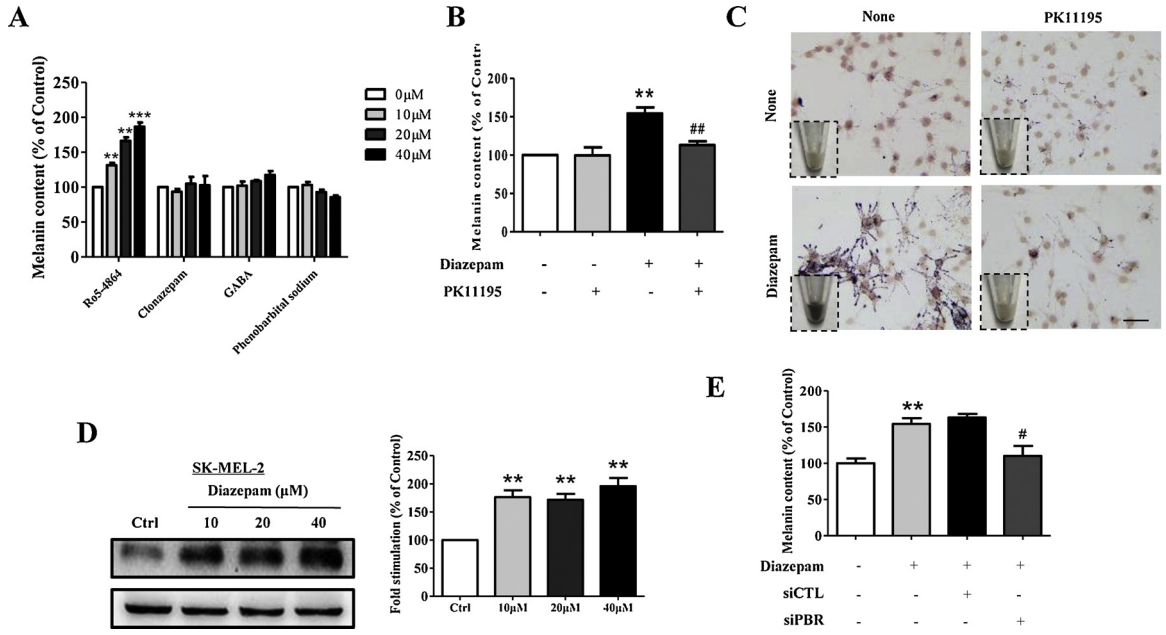

Fig. 5. The effects of the PBR on diazepam-induced pigmentation. (A) SK-MEL-2 cells were treated with Ro5-4864, clonazepam, GABA or phenobarbital sodium for 48 h and melanin contents were measured. (B and C) SK-MEL-2 was pretreated or not with 10 μM PK11195 for 1 h before diazepam was applied for 48 h at 40 μM. Melanin contents were measured and SK-MEL-2 cells were stained with Masson–Fontana ammoniacal silver stain. Bar = 20 μm. (D) SK-MEL-2 cells were treated with diazepam (40 μM) for 48 h and western blot was then applied to detect the protein levels of PBR. (E) SK-MEL-2 cells were transfected with siPBR using transfection reagent for 24 h. Subsequently, SK-MEL-2 cells were treated with diazepam (40 μM) for 48 h, and melanin contents were measured. Data are expressed as the mean ± SD (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated cells. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus diazepam-treated cells.

diazepam on TYR activity. As shown in Fig. 1B, diazepam had no direct effect on the enzymatic activities of TYR. In melanin content assay, diazepam significantly induced melanogenesis in Melan-A, SK-MEL-2, B16F10 and HNM (Fig. 1C). Consistently, diazepam increased hyperpigmentation in SK-MEL-2 cells and the human skin organ culture, as shown by Masson–Fontana ammoniacal silver staining (Figs. 1D and S3). Western blot analysis suggested that diazepam increased the expression levels of TYR, TRP-1,TRP-2 and MITF in Melan-A cells, SK-MEL-2 cells and human skin organ culture (Fig. 2A–C).

3.2. Diazepam increased the density of dendrites and filopodia

Diazepam induced the production of dendrite-like structures in B16/C3 melanoma cells (Matthew et al., 1981; Landau et al., 1998). Thus, to examine the effect of diazepam on the number of dendrites in other melanocytes, SK-MEL-2 cells were stained with the fluorescent F-actin probe Rhodamine Phalloidin. The number of dendrites increased after the culture in the medium containing 40 μM diazepam for 48 h (Fig. 3).

3.3. Diazepam regulated the intracellular distribution of melanosomes

To examine whether diazepam affects melanosome transport, we stimulated SK-MEL-2 cells with diazepam and measured the location of melanosome. Immunofluorescence analysis suggested that melanosomes clearly accumulated around the nuclei of SK-MEL-2 cells, whereas melanosomes were diffusely distributed in the dendrites and around the nuclei in the cells treated with diazepam (Fig. 3), which is consistent with the results observed by the amine silver staining analysis (Fig. 1D).

3.4. Diazepam regulated the expression levels of Myosin Va, Rab27a, Rab17 and Cdc42, but not KIF5b

To understand how diazepam regulates melanosome transport, we examined several key proteins related to melanosome transport. KIF5b, one of the kinesin superfamily proteins, which involved in melanosome transport alone microtubules (Hara et al., 2000). Myosin Va and Rab27a are the key regulators of actin-based melanosome transport (Ohbayashi and Fukuda, 2012). Furthermore, Rab17 and Cdc42 stimulate the formation of filopodia and mediate melanosome transfer in melanocytes (Beaumont et al., 2011; Hirokawa et al., 2009). Western blot analysis suggested that diazepam increased the expression levels of Myosin Va, Rab27a, Rab17 and Cdc42 in Melan-A cells and SK-MEL-2 cells. Unfortunately, the expression level of KIF5b remained unchanged compared to the control group (Fig. 4).

3.5. Effects of the PBR on diazepam-induced pigmentation

To investigate the effects of the PBR on diazepam-induced pigmentation, we examined the effect of the agonist or antagonist of PBR and CBR on melanin synthesis. As shown in Fig. 5A, selective CBR agonist clonazepam, $GABA_A$ receptor agonist GABA and phenobarbital sodium did not promote melanin synthesis. However, selective PBR agonist Ro5-4864 significantly increased melanogenesis. As shown in Fig. 5B and C, the PBR antagonist PK11195 attenuated diazepam-induced melanogenesis in SK-MEL-2 cells. Next, we examined the expression of PBR. As shown in Fig. 5D, diazepam increased the expression of PBR in SK-MEL-2 cells. Finally, the critical role of PBR in diazepam-mediated melanogenesis was confirmed by applying siRNAs to the PBR. As shown in Fig. 5E, knockdown of PBR reversed diazepam-induced melanogenesis.

3.6. Diazepam activated the PBR, subsequently increasing intracellular levels of cAMP, and stimulated the phosphorylation of PKA_{cat} and CREB

To further understand the molecular mechanisms of melanin regulation by diazepam, we examined the signaling pathway related to the melanogenesis and melanosome transport. As shown in Fig. 6A, the phosphorylation of PKA_{cat} and CREB significantly enhanced by diazepam in SK-MEL-2 cells, whereas no effect was observed in the phosphorylation of p38 MAPK, ERK or JNK. Consistently, Ro5-4864, the

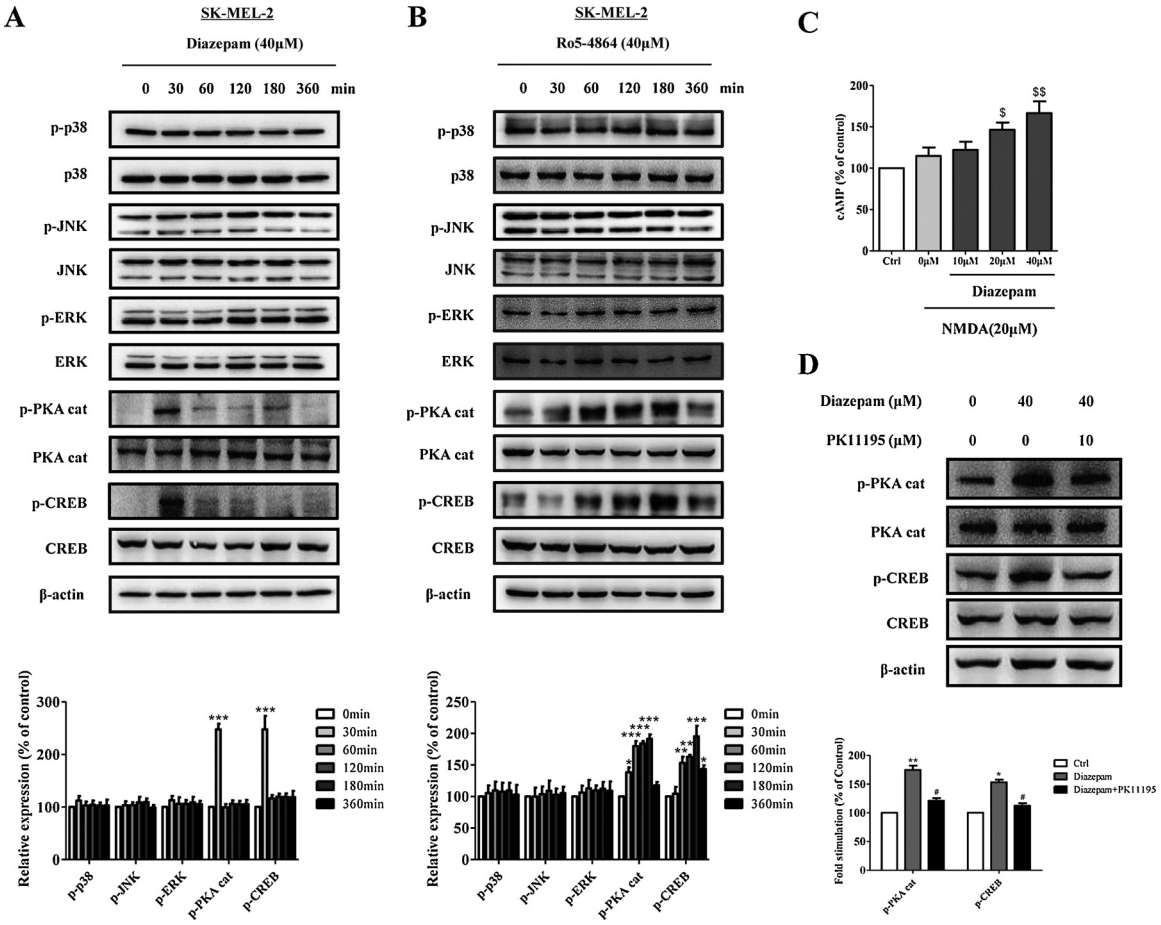

Fig. 6. The effects of diazepam and Ro5-4864 on the activity of MAPK and PKA signaling pathways in melanocytes. (A and B) SK-MEL-2 cells were treated with diazepam (40 μM) or Ro5-4864 (40 μM) for the indicated time period (0–360 min), and the phosphorylation of the p38 MAPK, ERK, JNK, PKA and CREB were measured by western blot. (C) SK-MEL-2 cells were treated with diazepam (10, 20, 40 μM) for 10 min and stimulated with NMDA (20 μM) for measurable cAMP signal. (D) SK-MEL-2 was pretreated or not with 10 μM PK11195 for 30 min before diazepam (40 μM) was applied for 30 min and the phosphorylation of PKA and CREB were measured by western blot. Data are expressed as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated cells. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ versus NMDA-treated cells. $^{\#}p < 0.05$ versus diazepam-treated cells.

selective agonist of PBR, also had an enhanced effect on phosphorylation of PKA $_{cat}$ and CREB (Fig. 6B). As shown in Fig. 6C, cAMP levels increased significantly after the diazepam treatment at concentrations of 20 and 40 μM for 10 min. In addition, the diazepam-induced increase of p-PKA $_{cat}$ and p-CREB expression were inhibited by PK11195 (Fig. 6D).

3.7. Effects of the inhibitor of PKA on diazepam-induced pigmentation and the expression of several key proteins relating to melanosome transport in melanocytes

As the PKA signaling pathway activated in in SK-MEL-2 cells, we examined whether the PKA signaling pathway involved in diazepam-induced pigmentation and melanosome transport in melanocytes. As shown in Fig. 7, the PKA inhibitor H89 attenuated diazepam or Ro5-4864 -induced melanogenesis in SK-MEL-2 cells. Furthermore, the improvement of MITF, TYR, Myosin Va, Rab27a, Rab17 and Cdc42 expression by diazepam or Ro5-4864 were reversed by the presence of H89, revealing the same involvement of PKA signaling in diazepam-induced melanogenesis and melanosome transport in melanocytes (Fig. 8).

4. Discussion

Melanin plays an important role in the protection of human from the deleterious effects of UV radiation (Slominski et al., 2004) and regulates epidermal homeostasis, suggesting a key role in the regulation of cellular metabolism (Slominski et al., 2004, 2015). Compounds that enhance pigmentation hold the potential to reduce UV-induced skin damage and carcinogenesis (Slominski et al., 2018). In the current study, we demonstrated that diazepam induced melanogenesis in Melan-A, SK-MEL-2, B16F10 and HNM cells (Fig.1C and D), confirming previous reports (Matthew et al., 1981; Landau et al., 1998). Consistently, diazepam increased hyperpigmentation in the human skin organ culture (Fig. S3). Although diazepam had no direct inhibitory effect on the activity of TYR, diazepam increased the expression levels of TYR, TRP-1,and TRP-2, which reflected that diazepam promoted melanogenesis by increasing the expression of these three key melanin synthesis enzymes (Figs. 1A, B and 2).

In contrast to the melanogenesis, melanosome transport is equally important for the pigmentation. Masson–Fontana ammoniacal silver staining and immunofluorescence analysis showed that diazepam increased the number of dendrites and promoted melanosome localization at the cell periphery (Figs. 1D and 3). As shown in Fig. 4, diazepam increased the expression of two key proteins (Cdc42 and Rab17) to form dendrites in both Melan-A and SK-MEL-2 cells. Previous studies suggested that KIF5b played a key role in the outward transport of melanosomes along microtubules. Following the transfer of melanosomes from microtubules to the actin filaments, Myosin Va regulates their movement and then anchors them to the cell periphery by Rab27a

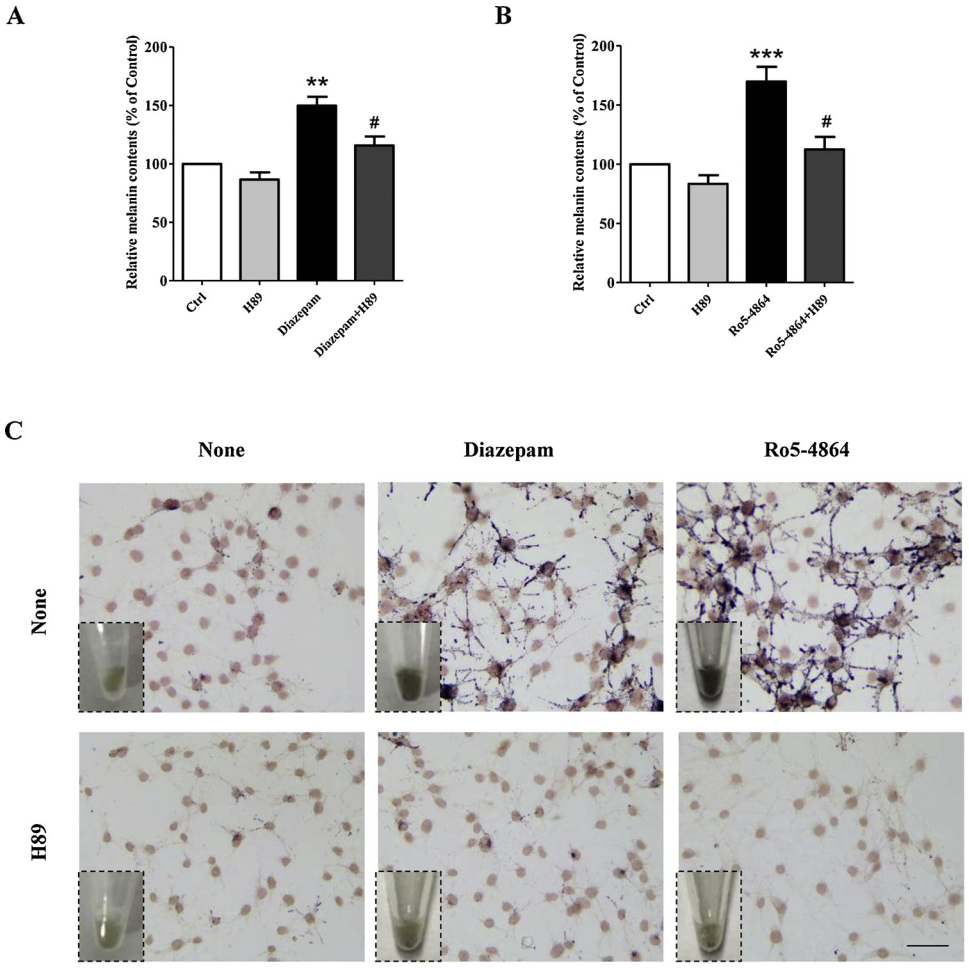

Fig. 7. The effects of H89 (the inhibitor of PKA) on diazepam or Ro5-4864 induced melanin contents in SK-MEL-2 cells. SK-MEL-2 was pretreated or not with 10 μM H89 for 1 h before diazepam or Ro5-4864 was applied for 48 h at 40 μM. (A and B) Melanin contents were measured and (C) SK-MEL-2 cells were stained with Masson–Fontana ammoniacal silver stain. Bar = 20 μm. Data are expressed as the mean ± SD (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated cells. $^{\#}p < 0.05$ versus diazepam or Ro5-4864 treated cells.

(Chang et al., 2012; Koike et al., 2018). As shown in Fig. 4, diazepam increased the protein expression levels of Rab27a and Myosin Va in both Melan-A and SK-MEL-2 cells. In contrast, the expression level of KIF5b was almost unchanged compared to the control group. These results indicated that diazepam promoted actin-based melanosome transport and localization at cell periphery by increasing the expression of Rab27a and myosin Va. However, whether diazepam affects movement of the melanosome along microtubules requires further study.

There are two kinds of benzodiazepine receptors in our body, namely the central benzodiazepine receptor (CBR) and the peripheral benzodiazepine receptor (PBR). The CBR is located in the central nervous system and is coupled with $GABA_A$ receptors and chloride ion channels. The PBR has been identified in various peripheral tissues and it is not coupled with $GABA_A$ receptors (Lacapère and Papadopoulos, 2003). Diazepam is active at both the CBR and PBR. Therefore, further detailed studies are needed to reveal the mechanism by which diazepam regulates pigmentation. Clonazepam (CBR selective agonist), GABA ($GABA_A$ receptor agonist) and phenobarbital sodium ($GABA_A$ receptor agonist) did not promote melanin synthesis. However, Ro5-4864, the selective agonist of PBR, significantly promoted melanogenesis (Fig. 5A). The PBR antagonist PK11195 markedly blocked diazepam-induced melanogenesis (Fig. 5B and C). In addition, knockdown of PBR reversed diazepam-induced melanogenesis (Fig. 5E). These results suggested that diazepam promoted pigmentation by targeting the PBR, but not the CBR.

To identify the potential pathway known to play a role in diazepam-induced melanogenesis and melanosome transport, we first demonstrated that diazepam increased intracellular levels of cAMP and the phosphorylation of PKA $_{cat}$ and CREB in SK-MEL-2 cells, whereas no effect was observed in phosphorylation of p38 MAPK, JNK or ERK (Fig. 6A and C). Consequently, Ro5-4864 also activated PKA $_{cat}$ and CREB in SK-MEL-2 cells (Fig. 6B). Moreover, PK11195 markedly blocked the diazepam-induced increase in p-PKA $_{cat}$ and p-CREB expression in melanocytes (Fig. 6D). Given that the cAMP/PKA signaling pathway was activated in melanocytes, whether it was involved in the diazepam-induced melanogenesis and the up-regulation of myosin Va, TYR, MITF, Rab27a, Rab17 and Cdc42 expression were then assessed. The PKA inhibitor H89 markedly attenuated diazepam or Ro5-4864 induced melanogenesis (Fig. 7) and increased Myosin Va, TYR, MITF, Rab27a, Rab17 and Cdc 42 expression in melanocytes (Fig. 8). Our data indicated that diazepam promoted pigmentation by activating the PBR. Once it was activated, the PBR stimulated the phosphorylation of CREB, resulting in melanogenesis and melanosome transport. These are contradictory with the previous studies in which diazepam stimulated phosphorylation of CREB by inhibiting phosphodiesterase type 4 (PDE4) (Vargas et al., 2001). This difference may result from the different expression levels of the PBR and PDE4 between tissues. Further comprehensive studies are needed to disclose the function of the PBR in melanocytes.

In conclusion, we showed that diazepam enhanced the melanogenesis by increasing the expression of MITF and TYR. Our data also demonstrated that diazepam directly promoted actin-based

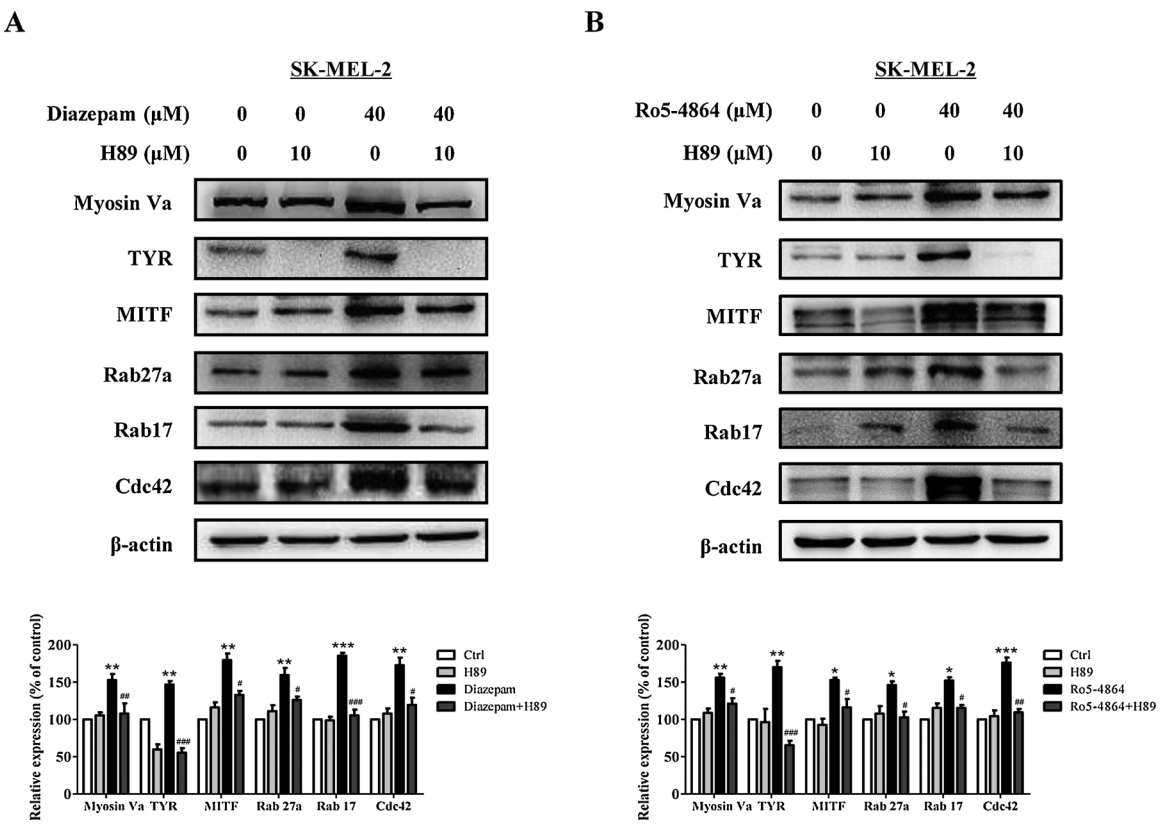

Fig. 8. Effect of the inhibitor of PKA (H89) on diazepam-induced protein expressions relating to melanogenesis and melanosome transport in SK-MEL-2 cells. SK-MEL-2 was pretreated or not with 10 μM H89 for 1 h before diazepam or Ro5-4864 was applied for 48 h at 40 μM. Western blotting for the proteins expression relating to melanogenesis and melanosome transport were measured. Data are expressed as the mean ± SD (n = 3). $^{*}p < 0.05$,$^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated cells. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus diazepam-treated cells.

melanosome transport and localization at cell periphery by increasing the expression of Rab27a and myosin Va. In addition, diazepam promoted the formation of dendrites in melanocytes by increasing the expression of Rab17 and Cdc42. Furthermore, our data indicated the involvement of PBR/cAMP/PKA signaling in diazepam-induced melanogenesis and melanosome transport. Taken together, our results indicated that diazepam had a pivotal role in melanocytes by enhancing melanin synthesis, melanocyte dendricity and melanosome trafficking at the dendrite tips. Therefore, our findings may provide a potential candidate or lead compound to protect human from UV-induced skin damage and carcinogenesis.

Funding

This work was supported by the Fund of Changzhou Sci&Tech Program (Grant No. CJ20180007).

Declaration of competing interest

The authors have declared no conflicting interests.

Acknowledgments

No.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105620.

References

Beaumont, K.A., Hamilton, N.A., Moores, M.T., Brown, D.L., Ohbayashi, N., Cairncross, O., Cook, A.L., Smith, A.G., Misaki, R., Fukuda, M., Taguchi, T., Sturm, R.A., Stow, J.L., 2011. The recycling endosome protein Rab17 regulates melanocytic filopodia formation and melanosome trafficking. Traffic 12, 627–643. https://doi.org/10.1111/j.1600-0854.2011.01172.x.

Buscà, R., Ballotti, R., 2010. Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigm. Cell. Melanoma. R 13, 60–69. https://doi.org/10.1034/j.1600-0749.2000.130203.x.

Chang, H., Choi, H., Joo, K.M., Kim, D., Lee, T.R., 2012. Manassantin B inhibits melanosome transport in melanocytes by disrupting the melanophilin-myosin Va interaction. Pigm. Cell Melanoma. R 25, 765–772. https://doi.org/10.1111/pcmr.12002.

Costin, G.E., Valencia, J.C., Wakamatsu, K., Ito, S., Solano, F., Milac, A.L., Vieira, W.D., Yamaguchi, Y., Rouzaud, F., Petrescu, A.J., Lamoreux, M.L., Hearing, V.J., 2005. Mutations in dopachrome tautomerase (Dct) affect eumelanin/pheomelanin synthesis, but do not affect intracellular trafficking of the mutant protein. Biochem. J. 391, 249–259. https://doi.org/10.1042/bj20042070.

Gu, Z., Li, Y., Li, H., 2017. Use of condensed nanofat combined with fat grafts to treat atrophic scars. JAMA. Facial. Plast. Su. 20, E1–E8. https://doi.org/10.1001/jamafacial.2017.1329.

Hara, M., Yaar, M., Byers, H.R., Goukassian, D., Fine, R.E., Gonsalves, J., Gilchrest, B.A., 2000. Kinesin Participates in Melanosomal Movement along Melanocyte Dendrites. J. Invest. Dermatol. 114, 438–443. https://doi.org/10.1046/j.1523-1747.2000.00894.x.

Hirokawa, N., Noda, Y., Tanaka, Y., 2009. Kinesin superfamily motor proteins and intracellular transport. Nat. Rev. Mol. Cell. Bio. 10, 682–696. https://doi.org/10.1038/nrm2774.

Koike, S., Yamasaki, K., Yamauchi, T., Inoue, M., Shimada-Ohmori, R., Tsuchiyama, K., Aiba, S., 2018. Toll-like receptor 2 and 3 enhance melanogenesis and melanosome transport in human melanocytes. Pigm. Cell. Melanoma. R 31, 570–584. https://doi.org/10.1111/pcmr.12703.

Lacapère, J.J., Papadopoulos, V., 2003. Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. Steroids 68, 569–585. https://doi.org/10.1016/S0039-128X(03)00101-6.

Landau, M., Weizman, A., Zorefshani, E., Wasseman, L., Landau, O., Gavish, M., Brenner, S., Nordenberg, J., 1998. Antiproliferative and differentiating effects of benzodiazepine receptor ligands on B16 melanoma cells. Biochem. Pharmacol. 56, 1029. https://doi.org/10.1016/S0006-2952(98)00149-X.

Levy, C., Fisher, D.E., 2011. Dual roles of lineage restricted transcription factors.

Transcription 2, 19–22. https://doi.org/10.4161/trns.2.1.13650.
Lu, Z., Hasse, S., Bodo, E., Rose, C., Funk, W., Paus, R., 2007. Towards the development of a simplified long-term organ culture method for human scalp skin and its appendages under serum-free conditions. Exp. Dermatol. 16, 37–44. https://doi.org/10.1111/j.1600-0625.2006.00510.x.
Luo, L., 2000. Rho GTPases in neuronal morphogenesis. Nat. Rev. Neurosci. 1, 173–180. https://doi.org/10.1038/35044547.
Matthew, E., Laskin, J.D., Zimmerman, E.A., Weinstein, I.B., Hsu, K.C., Engelhardt, D.L., 1981. Benzodiazepines have high-affinity binding sites and induce melanogenesis in B16/C3 melanoma cells. P. Natl. Acad. Sci. USA. 78, 3935–3939. https://doi.org/10.1073/pnas.78.6.3935.
Noguchi, S., Kumazaki, M., Yasui, Y., Mori, T., Yamada, N., Akao, Y., 2014. MicroRNA-203 Regulates Melanosome Transport and Tyrosinase Expression in Melanoma Cells by Targeting Kinesin Superfamily Protein 5b. J. Invest. Dermatol. 134, 461–469. https://doi.org/10.1038/jid.2013.310.
Ohbayashi, N., Fukuda, M., 2012. Role of Rab family GTPases and their effectors in melanosomal logistics. J. Biochem. 151, 343–351. https://doi.org/10.1093/jb/mvs009.
Raposo, G., Marks, M.S., 2007. Melanosomes-dark organelles enlighten endosomal membrane transport. Nat. Rev. Mol. Cell. Bio. 8, 786–797. https://doi.org/10.1038/nrm2258.
Saha, B., Singh, S.K., Sarkar, C., Bera, R., Ratha, J., Tobin, D.J., Bhadra, R., 2006. Activation of the Mitf promoter by lipid-stimulated activation of p38-stress signalling to CREB. Pigm. Cell. Res. 19, 595–605. https://doi.org/10.1111/j.1600-0749.2006.00348.x.
Scott, G., 2002. Rac and rho: the story behind melanocyte dendrite formation. Pigm. Cell. Res. 15, 322–330. https://doi.org/10.1034/j.1600-0749.2002.02056.x.
Scott, G., Leopardi, S., 2003. The cAMP signaling pathway has opposing effects on Rac and Rho in B16F10 Cells: implications for dendrite formation in melanocytic cells. Pigm. Cell. Res. 16, 139–148. https://doi.org/10.1034/j.1600-0749.2003.00022.x.
Singh, S.K., Kurfurst, R.C., Schnebert, S., Perrier, E., Tobin, D.J., 2010. Melanin transfer in human skin cells is mediated by filopodia-a model for homotypic and heterotypic lysosome-related organelle transfer. FASEB J. 24, 3756–3769. https://doi.org/10.1096/fj.10-159046.
Slominski, A., Tobin, D.J., Shibahara, S., Wortsman, J., 2004. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol. Rev. 84, 1155–1228. https://doi.org/10.1152/physrev.00044.2003.
Slominski, A.T., Zmijewski, M.A., Plonka, P.M., Szaflarski, J.P., Paus, R., 2018. How ultraviolet light touches the brain and endocrine system through skin, and why. Endocrinology 159, 1992–2007. https://doi.org/10.1210/en.2017-03230.
Slominski, R., Zmijewski, M., Slominski, A.T., 2015. On the role of melanin pigment in melanoma. Exp. Dermatol. 24, 258–259. https://doi.org/10.1111/exd.12618.
Vachtenheim, J., Borovanský, J., 2010. "Transcription physiology" of pigment formation in melanocytes: central role of MITF. Exp. Dermatol. 19, 617–627. https://doi.org/10.1111/j.1600-0625.2009.01053.x.
Vargas, M.L., Abella, C., Hernandez, J., 2001. Diazepam increases the hypothalamic-pituitary-adrenocortical (HPA) axis activity by a cyclic AMP-dependent mechanism. Brit. J. Pharmacol. 133, 1355–1361. https://doi.org/10.1038/sj.bjp.0704201.
Zhou, J., Song, J., Ping, F., Shang, J., 2014. Enhancement of the p38 MAPK and PKA signaling pathways is associated with the pro-melanogenic activity of Interleukin 33 in primary melanocytes. J. Dermatol. Sci. 73, 110–116. https://doi.org/10.1016/j.jdermsci.2013.09.005.