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Trek2a regulates *gnrh3* expression under control of melatonin receptor Mt1 and α_2-adrenoceptor

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ABSTRACT

Gonadotrophin-releasing hormone (GnRH) expression is associated with the two-pore domain potassium ion (K^+) channel-related K^+ (TREK) channel *trek2a* expression and melatonin levels. We aimed to investigate correlation of *trek2a* expression with *gnrh3* expression, and regulatory mechanisms of *trek2a* expression by the melatonin receptor Mt1 and α_2-adrenoceptor which are regulated by melatonin. *trek2a* specific siRNA, Mt1 antagonist luzindole and α_2-adrenoceptor antagonist prazosin were administered into the adult zebrafish brain and gene expressions were examined by real-time PCR. *trek2a* specific siRNA administration significantly reduced expression levels of *trek2a*, *gnrh3* and *mt1*. Luzindole administration suppressed *trek2a* and *gnrh3* expressions. Prazosin administration reduced *trek2a* and *gnrh3* expressions. It is suggested that Trek2a regulates *gnrh3* expression under the control of Mt1 and α_2-adrenoceptor.

1. Introduction

Melatonin plays a pivotal role in fish reproduction [1,2]. Especially, melatonin affects expressions of kisspeptin and gonadotrophin-releasing hormone (GnRH) [3,4]. Melatonin treatment increases *kiss2* gene expression in zebrafish [1] as well as melatonin regulates *kiss* and *gnrh* expressions in sea bass [5]. Meanwhile, plasma melatonin level is affected by environmental cues, such as ambient light and temperature [6–8], especially plasma melatonin level is high under dark conditions such as night time [9]. In addition, plasma melatonin level is also affected by ambient temperature [10–12]. Since reproduction is affected by environmental cues, such as light and temperature [13–15], melatonin is suggested to play a crucial role as a transducer of environmental cues to regulate reproduction in the brain. In fact, constant darkness and high temperature which cause high melatonin levels affect *kiss2* and *gnrh3* expressions in zebrafish (Loganathan et al., under review). *kiss2* and *gnrh3* expressions are increased under constant darkness, on the other hand, *gnrh3* expression is decreased under high temperature.

The two-pore domain potassium ion (K^+) channel-related K^+ (TREK) channel, which is a member of K^+ channels consists of Trek1, Trek2 and TRAAK in vertebrates [16–19]. In zebrafish, four TREK isoforms, *trek1a*, *trek1b*, *trek2a* and *trek2b* have been estimated [19]. We have recently identified the full length sequence of *trek2a* in zebrafish [20]. Trek2a suggests to be involved in the regulation of reproduction [20], furthermore, *trek2a* expression is increased under constant darkness (Loganathan et al., under review). Therefore, Trek2a could be involved in the regulation of *kiss2* and *gnrh3* expressions under the control of melatonin.

Since Trek2a possesses no melatonin binding site, Trek2a is regulated by melatonin through other molecules with melatonin binding sites. Melatonin binds to melatonin receptors, Mt1, Mt2 and Mel1C to initiate a downstream signaling cascade [4,21,22]. *mt1* expression is decreased under constant darkness and high temperature (Loganathan et al., under review), therefore, *trek2a* expression could be regulated through the signaling cascade of Mt1.

Meanwhile, *trek2a* expression is not affected by high temperature, although *mt1* expression is decreased by high temperature (Loganathan et al., under review). This suggests that another molecule is involved in the regulation of *trek2a* expression. One candidate family is the adrenoceptor, which is classified into three groups, α_1-, α_2-, and β-adrenoceptor, and expressed in a wide range of species such as human, rodents and zebrafish [23,24]. In addition to being both light- [25,26] and temperature-sensitive [27], the adrenoceptor is involved in modulating reproductive function via the regulation of GnRH neuronal excitability [28]. Crucially, the α_2-adrenoceptor has a melatonin binding site in cuckoo wrasse

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[29,30]. These suggest that Tre2a regulates kiss2 and gnrh3 expressions under the control of Mt1 and α_2-adrenoceptor. In this study, we aimed to investigate the involvement of Trek2a in the regulation of *kiss2* and *gnrh3* gene expressions and the regulatory mechanisms of *trek2a* expression by Mt1 and α_2-adrenoceptor.

We first evaluated the involvement of Trek2a in the regulation of *kiss2* and *gnrh3* gene expressions by knocking-down *trek2a* expression with *trek2a* specific siRNA. We further chemically inhibited melatonin receptors and α_2-adrenoceptors via the use of selected antagonists which are luzindole [31–33] and prazosin [23] respectively in the brains of adult male zebrafish.

2. Materials and methods

2.1. Animal care and maintenance

Sexually matured adult male zebrafish (*Danio rerio*) were obtained from a local aquarium shop and maintained in freshwater aquaria at 27 ± 0.5 °C on a controlled natural photo regimen (14-h light, 10-h dark cycle), and fed a standard zebrafish diet twice daily (Ziegler Bros, PA, USA). The fish were maintained and used in accordance with the Guidelines of the Animal Ethics Committee of Monash University (Approval no. MARP/2015/145).

2.2. Trek2a-specific siRNA administration

To analyse the effects of knock-down of *trek2a* gene expression on reproductive function, a similar protocol was used as described [34] with slight modifications. Briefly, custom-designed *in vivo* siRNA (Ambion, TX, USA), *trek2a* specific siRNA (trek2a siRNA; sense: 5′ CAAUGUUCGUCUAGAAUAtt 3′, antisense: 5′ UAAUUCUAGACGAACAUUtt 3′) and green fluorescence protein (GFP) specific siRNA (GFP siRNA; sense: 5′GCAUCAAGGUGAACUUCAAtt 3′, antisense: 5′ UUGAAGUUCACCUUGAUGCtt 3′) were designed. GFP siRNA was used as a negative control. Sixty pmol/μL of trek2a or GFP siRNA was mixed with the same volume of 10% glucose and 0.06 volume of Turbofect (Thermo Scientific, MA, USA) and incubated for 20 min at room temperature. Sexually matured males were anesthetized in a 0.01% solution of benzocaine (Sigma-Aldrich, MO, USA) and, 1.0 μl of the mixture described above was intracranially administered at 10:00 a.m. using a heat-pulled micropipette attached to a microinjector (IM-9B; Narishige Co. Tokyo, Japan). The heat-pulled borosilicate glass micropipettes (1.5 mm outer diameter; Narishige Co.) were prepared with the help of a P-2000 laser pipette puller (Sutter Instrument Co. CA, USA). After the injection, the fish were returned to the aquarium for recovery. After 24, 48 or 72 h maintenance, the fish were anesthetized, killed and the brains were dissected under a stereoscopic microscope (Nikon, Tokyo, Japan). Experimental groups were as follows: 24 h post-injection: GFP siRNA (n = 13), trek2a siRNA (n = 14); 48 h post-injection: GFP siRNA (n = 10), trek2a siRNA (n = 8); 72 h post-injection: GFP siRNA (n = 7), trek2a siRNA (n = 9).

2.3. Melatonin, luzindole and prazosin administration

2.3.1. Dose-response study

Three different doses were used in this experiment, 10 fmole, 100 fmole and 1000 fmole. Sexually matured males were anesthetized in a 0.01% solution of benzocaine (Sigma-Aldrich) and 1 μl of either melatonin (Sigma-Aldrich), luzindole (Tocris Bioscience, MN, USA) or prazosin hydrochloride (Sigma-Aldrich) solution was administrated at 10:00 a.m. into the cranial cavity. The same volume of water was administered as vehicle. At 2 h after injection, the fish were anesthetized, killed and the brains were dissected under a stereoscopic microscope (Nikon). Experimental groups were divided as follows: vehicle (n = 16); melatonin 10 fmole (n = 18), melatonin 100 fmole (n = 18), melatonin 1000 fmole (n = 18); luzindole 10 fmole (n = 16), luzindole 100 fmole (n = 16), luzindole 1000 fmole (n = 18); prazosin hydrochloride 10 fmole (n = 18), prazosin hydrochloride 100 fmole (n = 17), prazosin hydrochloride 1000 fmole (n = 16).

2.3.2. Time-course study

Based on the results of the dose-response study, a single concentration was chosen for a time-course analysis. The single dose of melatonin, luzindole or prazosin hydrochloride was injected into the cranial cavity at 10:00 a.m. and the fish were allowed to return to the home tank for either 24 or 48 h, after which the fish were killed as described above. Experimental groups were divided as follows: 24 h post-injection: vehicle (n = 9), melatonin (n = 10), luzindole (n = 10), prazosin hydrochloride (n = 9); 48 h post injection: vehicle (n = 8), melatonin (n = 8), luzindole (n = 9), prazosin hydrochloride (n = 9).

2.4. RNA isolation and cDNA preparation

Total RNA from the brain of adult zebrafish was isolated with TRIzol (Invitrogen, CA, USA) according to the manufacturer's instruction. Briefly, brain samples were homogenized in a microcentrifuge tube containing 400 μl of TRIzol and incubated at room temperature for 5 min 80 μl of chloroform was added, the tube contents mixed by shaking for 15 s and incubated for 3 min at room temperature. The suspension was centrifuged at 12,000 g at 4 °C for 15 min. The colourless aqueous upper phase was transferred into a new microcentrifuge tube and 190 μl of isopropyl alcohol was added. The contents were then incubated at room temperature for 10 min, followed by centrifugation at 12,000 g at 4 °C for 15 min. Supernatant was removed and the pellet rinsed with 75% ethanol. The supernatant was removed again and the air-dried pellet was dissolved in 20 μl RNase free water. Quantity and quality were determined with a Nanodrop Spectrophotometer ND-1000 UV/Vis (Nanodrop Technologies Inc. DE, USA). Total RNA with an absorbance ratio (*A*260/*A*280) between 1.8 and 2.0 was used for cDNA synthesis.

High Capacity Reverse Transcription Kit (Applied Biosystems, CA, USA) was used to reverse transcribe total RNA into cDNA. Reverse transcription reactions were performed in 20 μl reactions containing 500 ng of total RNA, 2.0 μl 10x RT buffer, 0.8 μl 25x dNTP (100 mM), 2.0 μl 10x random primer, 1.0 μl Multiscribe Reverse Transcriptase and 1.0 μl RNase Inhibitor. The reaction mixture was incubated in a thermocycler under the following conditions: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min.

2.5. Gene expression analysis

Real-time PCR primers were designed based on sequences available on PubMed for melatonin receptors *mt1*, *mel1c*, *gnrh3* and *kiss2*. Primer sequences are shown in Supplementary Table 1.

PCR reactions were prepared in volumes of 10 μl, each containing 1/20 volume of reverse transcribed cDNA, 2.5 μM forward and reverse primers and 1x POWER SYBR Master Mix (Applied Biosystems) using ABI 7500 Real-time PCR System (Applied Biosystems). Reaction conditions were as follows: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min for 40 cycles. The levels of each mRNA were normalized to *18s* RNA. Each sample is represented as mRNA levels relative to vehicle or GFP siRNA.

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