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17β-Estradiol Downregulated the Expression of TASK-1 Channels in Mouse Neuroblastoma N2A Cells

Xuran Hao · Xiaoqing Li · Xiantao Li

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Abstract TASK channels, an acid-sensitive subgroup of two pore domain K^+ (K2P) channels family, were widely expressed in a variety of neural tissues, and exhibited potent functions such as the regulation of membrane potential. The steroid hormone estrogen was able to interact with K^+ channels, including voltage-gated K^+ (Kv) and large conductance Ca^{2+}-activated (BK) K^+ channels, in different types of cells like cardiac myocytes and neurons. However, it is unclear about the effects of estrogen on TASK channels. In the present study, the expressions of two members of acid-sensitive TASK channels, TASK-1 and TASK-2, were detected in mouse neuroblastoma N2A cells by RT-PCR. Extracellular acidification (pH 6.4) weakly but statistically significantly inhibited the outward background current by 22.9 % at a holding potential of 0 mV, which inactive voltage-gated K^+ currents, suggesting that there existed the functional TASK channels in the membrane of N2A cells. Although these currents were not altered by the acute application of 100 nM 17β-estradiol, incubation with 10 nM 17β-estradiol for 48 h reduced the mRNA level of TASK-1 channels by 40.4 % without any effect on TASK-2 channels. The proliferation rates of N2A cells were also increased by treatment with 10 nM 17β-estradiol for 48 h. These data implied that N2A cells expressed functional TASK channels and chronic exposure to 17β-estradiol downregulated the expression of TASK-1 channels and improved cell proliferation. The effect of 17β-estradiol on TASK-1 channels might be an alternative mechanism for the neuroprotective action of 17β-estradiol.

X. Hao · X. Li · X. Li (✉)
Department of Biomedical Engineering, South-Central University for Nationalities, 182 Minyuan Road, Wuhan 430074, China
e-mail: lixiantao@yahoo.com

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Introduction

Two pore domain K^+ (K2P) channels, which contain four transmembrane segments and two pore domains, can produce the non-inactivating and voltage-independent background or leak K^+ currents; thus, these channels serve to regulate resting membrane potential and electrical excitability in various tissues (Lesage et al. 1996; Reyes et al. 1998). The mammalian K2P channels are classified into five main structural subgroups: (1) TWIK-1, TWIK-2, and KCNK7; (2) TASK-1, TASK-3, and TASK-5; (3) TREK-1, TREK-2, and TRAAK; (4) TASK-2, TALK-1, and TALK-2; (5) THIK-1 and THIK-2; and (6) TRESK (Enyedi and Czirjak 2010). TASK channels, an acid-sensitive subgroup of the K2P family, widely distribute in a variety of neural tissues such as cerebellum, thalamus, and pituitary gland (Medhurst et al. 2001). Several pathophysiological effects were demonstrated to be mediated by these channels, including the regulation of the action potential firing pattern in cerebellar granule neurons (Aller et al. 2005; Brickley et al. 2007) and the induction of cell death in hippocampal neurons (Lauritzen et al. 2003).

Estrogens are ovarian hormones and regulate lots of physiological functions, such as growth, differentiation, and homeostasis, in a variety of target tissues. Moreover, estrogens also involve the reduction of oxidative stress as well as improvement of neuronal survival and cognitive function (Green and Simpkins 2000; Prediger et al. 2004). Therefore, estrogen deficiency in postmenopausal women is associated with various diseases. The beneficial effects of estrogen replacement therapy may relate to the prevention of osteoporosis (Torgerson and Bell-Syer 2001), cardioprotection

(Barrett-Connor and Bush 1991; Sullivan et al. 1988), as well as the mitigation of dementia (Valen-Sendstad et al. 2010; Wroolie et al. 2011). Although a number of explanations for the effects of estrogen have been proposed, the actual underlying mechanisms of these effects have not been fully understood.

Estrogen may act on targets either through two subtypes of nuclear estrogen receptors (ERs), ERα and ERβ, or directly exerts ''nongenomic'' effects on membrane proteins (Kelly and Levin 2001; Watson and Gametchu 2003). Previous works suggested that the K^+ channel was one of the targets of estrogen action in distinct tissues. 17β-Estradiol rapidly blocked voltage-gated K^+ channels (Kv) in rat medial preoptic neurons (Druzin et al. 2011) and human osteoblast-like MG63 cells (Li et al. 2013b). The incubation with 17β-estradiol augmented the expression of Ca^{2+}-activated K^+ channels in GT1-7 GnRH neuronal cell line (Nishimura et al. 2008). Acute application of 17β-estradiol increased the currents of large conductance Ca^{2+}-activated K^+ (BK) channels in human coronary artery smooth muscle cells (White et al. 2002) and the oocyte expression system (Valverde et al. 1999). In guinea pig ventricular myocytes, 17β-estradiol prolonged the action potential duration (APD) due to inhibitory effects on the IKr and IKs (Tanabe et al. 1999). Based on these reports, it seemed that estrogen could exhibit substantial physiological functions by modulating K^+ channels. Nevertheless, it remains obscure regarding the effects of estrogen on TASK channels.

The mouse neuroblastoma N2A cells were frequently employed as a model by numerous groups to perform the related studies. Both ERs, ERα and ERβ, were functionally expressed in these cells (Mendez and Garcia-Segura 2006). Recently, it has been reported that N2A cells abundantly expressed the subfamily members of Kv, including Kv1.1, Kv1.4, and Kv2.1 (Leung et al. 2011). To our knowledge, little is known about the expressions and functions of TASK channels in N2A cells. In current study, N2A cells also were taken as a model to explore the effects of estrogen on TASK channels. RT-PCR and patch-clamp experiments are conducted to clarify the presence of K2P channels in N2A cells first, and the possible effects of estrogen on these channels are established further.

Materials and Methods

Cell Culture

Mouse neuroblastoma N2A cells were grown at 37 °C and 5 % CO_2 in a humidified incubator with in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The medium was changed every 2 days, and the cultures were passed at 80 % confluence.

Cell Proliferation Assay

Cell proliferation was assessed using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-8) assay (Beyotime) according to the manufacture's instructions. After suspending N2A cells in DMEM medium without phenol red, the 100 μl of cell suspension (1,000 cells/well) was dispensed in a 96-well plate and cultured for 24 h; then 10 nM 17β-estradiol was added into each well and cells were grown for 24 or 48 h before the 10 μl WST-8 assay reagents were added. N2A cells were subsequently incubated at 37 °C for 1–4 h, and the absorbance at 450 nM was measured using a microplate reader.

PCR Analysis

Total RNA from N2A cells was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further treated with DNase I (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) was conducted in a 20 μl reaction mixture, including RNA (1 μg) and oligo-dT primer and M-MLV reverse transcriptase (Invitrogen). The forward and reverse PCR oligonucleotide primers selected to amplify cDNA are listed in Table 1. 1 μl cDNA was then amplified by polymerase chain reaction (PCR) in a 25 μl reaction mixture, containing 0.4 nM of each primer, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM of each dNTP and 0.625 units of Taq DNA polymerase. PCR was performed under the following conditions: 10 min at 95 °C for denaturation, then 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C for 35 circles, and 10 min at 72 °C for an additional extension. The PCR products were electrophoresed through a 1.5 % agarose gel, and amplified cDNA bands were visualized by 0.05 μg/ml GoldView (Geneshun Biotech Ltd., Guangzhou, China) and scanned by MultiImager (Bio-Rad, Philadelphia, PA, USA). After incubation with or without 17β-estradiol, the mRNA levels of TASK channels in N2A cells were determined by semiquantitative RT-PCR. The number of PCR cycles was selected to be within the range of the linear amplification for each transcript. A quantitative densitometry analysis was conducted using Quantity One software (Bio-Rad, Philadelphia, PA, USA). An invariant mRNA of GAPDH was taken as an internal control for analyzing the relative amount of PCR products of the TASK channels.

Electrophysiology

The effects of extracellular acidosis (pH 6.4) and 17β-estradiol on macroscopic currents in N2A cells were recorded using the whole cell patch-clamp technique. A data acquisition and analyses were performed by EPC-9 patch-clamp system

Table 1 Oligonucleotide sequences of primers used for RT-PCR

Gene	GI	Forward primer	Reverse primer	Length (bp)
TASK-1	11,093,517	TGGTGCTCATCGGTTTCGT	CAGCGCATGTGACTGGGTC	414
TASK-2	146,134,923	AGGAGTGAGCCTGAGGAA	AAGCCAATGGTGGAGATG	165
TASK-3	76,443,667	TAAAGCCGAAGAAGTCCG	CCCAGCACAGCGTAGAAC	228
TASK-5	71,892,415	CGCTTCTCCGCCGACGACTA	AGCAGCAGACACCGCACCAG	266
TREK-1	55,274,228	ACCTTCATAGCCCAGCAT	TTCCACCTTCAGTTCGTG	211
TREK-2	146,149,097	GACCTTACCCGAGGATGT	TGGCTGTGCTGGAGTTGT	119
TWIK-1	160,358,857	TTACTTCTGTTTCATCTCCCTG	GGTCTTCGTCCTTGTCTTTC	221
TWIK-2	170,295,854	TGTCACTGCTGCTCACCCA	GGAAACACGGCGGAAAGT	347
TRAAK	141,803,210	TCCTGCCCTCCTCTTTGC	CGCTCACTCTGCGTGTCTG	175
TRESK	118,131,054	GGAGAACCCTGAGTTGAAGA	CCTGGAACCGACTGTAAGC	344
TALK-1	251,823,954	AAAGGCAACTCCACCAATC	CGAAGTAGAAGCCCTCACG	367
THIK-2	40,445,392	TCAGCACCATCGGCTTCGG	GCCAGCGACACCTTGTTGGAC	388
KCNK7	51,944,954	AGCCATCTACTTCTGTTTCGG	CATCTTGATCTTCATCGGTTCT	242
GAPDH	193,423	AGGCCGGTGCTGAGTATGTC	TGCCTGCTTCACCACCTTCT	530

GAPDH glyceraldehyde-3-phosphate dehydrogenase, *GI* GenInfo identifier sequence ID

(HEKA Elektronik, Lambrecht, Germany). The bath solution contained (in mM): 75 Na-gluconate, 70 NaCl, 5 KCl, 1 $MgCl_2$, 5 HEPES, and 5 glucose, with pH adjusted to 7.4 using NaOH; the pipette solution contained (in mM): 140 KCl, 2 $MgCl_2$, 5 HEPES, 5 EGTA, 5 glucose, and 5 Na_2ATP, with pH adjusted to 7.3 using KOH. N2A cells were cultured on coverslips, and subsequently were transferred to a recording chamber. The pipettes pulled from borosilicate glass capillaries had a resistance of 3–5 MΩ. An Ag–AgCl wire connected to the bath solution via a salt bridge was used as the reference electrode. The liquid junction potential was around 1–3 mV under these conditions.

Analysis and Statistics

Results are described as mean ± SE and analyzed by paired Student's *t* test or one-way analysis of variance (ANOVA). A value of $P < 0.05$ was taken to indicate statistical significance.

Results

The mRNA Expressions of K2P Channels in Mouse Brain and N2A Cells

The K2P channels were background channels and set the resting membrane potential in much kind of tissues. To clarify the expression of these channels in neuronal cells, the specific primers were designed and RT-PCR was carried out at the beginning of this study (Table 1). Among 13 members of K2P channels already identified in mouse tissues, positive signals for TASK-1, TASK-2, TASK-3, TASK-5, TWIK-1, TREK-1, TREK-2, TRAAK, and KCNK7, but not TWIK-2, THIK-2, TALK-1, and TRESK, were observed after PCR amplification in the mouse brain (Fig. 1a), consistent with previous reports that brain tissues expressed several distinct subunits of K2P family (Enyedi and Czirjak 2010; Medhurst et al. 2001). The PCR product of GAPDH, a housekeeping gene, was also detected at the expected size (530 bp) and used to validate RT (Fig. 1a, right). In the samples of N2A cells, same primers also detected the mRNA transcripts of TASK-1, TASK-2, TWIK-1, and TREK-2, but not TASK-3, TASK-5, TREK-1, TRAAK, and KCNK7 (Fig. 1b). The different expressions of K2P channels mRNA between mouse brain and N2A cells may be attributed to more types of distinct tissues in the former.

Acid-Sensitive TASK Currents in N2A Cells

Although the mRNAs of TASK-1 and TASK-2 were defined in N2A cells by RT-PCR, it does not necessarily imply that there existed the functional TASK channels on the membrane of these cells. It is well-known that the basic electrical characteristic of TASK-like currents is inhibited by extracellular acidosis. At a holding potential of −80 mV, the outward Kv currents in N2A cells were elicited by depolarization to test potentials of +60 mV as reported before (Leung et al. 2011) (Fig. 2a). To exclude the effects of acidification on the outward Kv currents of N2A cells, a long-term (10 s) holding potential of 0 mV was performed, and consequently these Kv currents were almost completely abolished (our unpublished observation). The remaining outward currents were considered as voltage-independent background currents and measured in the following experiments (Fig. 2a). These remaining currents were weakly and significantly blocked by changing the pH value of bath

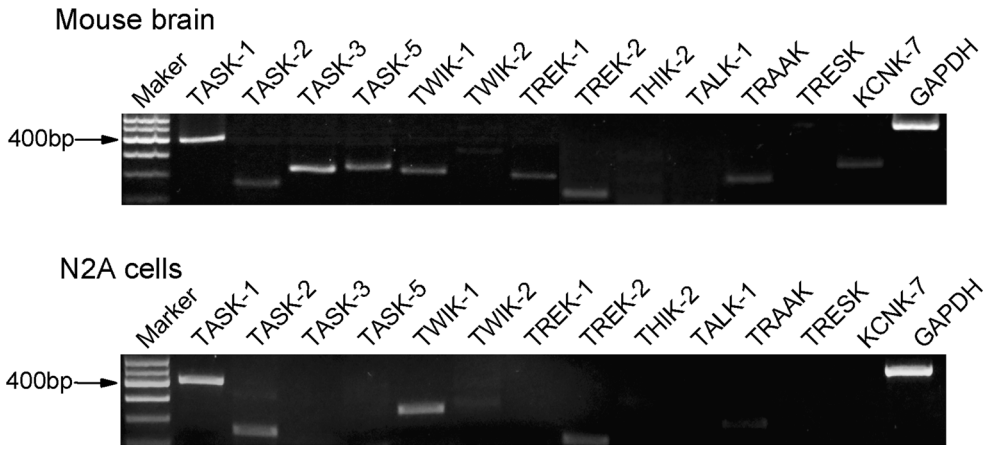

Fig. 1 The mRNA expression of K2P channels in mouse brain and N2A cells. Original gel at the *upper panel* showed that the expression of TASK-1, TASK-2, TASK-3, TASK-5, TWIK-1, TREK-1, TREK-2, TRAAK, and KCNK7 at expected length were detected in the mouse brain, but no signals appeared for TWIK-2, THIK-2, TALK-1, and TRESK. N2A cells at the *lower panel* displayed the expressions of TASK-1, TASK-2, TWIK-1, and TREK-2, corresponding to expected length. The PCR product of GAPDH (530 bp) in mouse brain and N2A cells was used as positive control. The *first lane* showed the DNA maker

solution from 7.4 to 6.4, suggesting that the N2A cells functionally expressed an acid-sensitive TASK-like current. The current–voltage curves (I–V) clearly displayed the inhibitory effects of extracellular acidosis (pH 6.4) on remaining outward currents at different potentials (Fig. 2b). At +60 mV, the bath solution with a pH value of 6.4 significantly declined currents by 22.9 % ($n = 5, P < 0.05$). I–V curves of acid-sensitive currents also were plotted as difference curves and reversed near the calculated K^+ equilibrium potential (−83 mV). The acid-sensitive TASK-like currents showed the outward rectification as described by previous studies (Decher et al. 2011; Duprat et al. 1997).

17β-Estradiol Downregulated the Expression of TASK-1 but No Effect on TASK-2

To test the possible effects of estrogen on TASK channels in N2A cells, we first examined the action of acute application of 17β-estradiol (100 nM) on the remaining outward current in N2A cells, but no effect was observed ($n = 6$, $P > 0.05$). It is generally believed that the acute and long-term applications of estrogen may produce two kinds of responses, including "nongenomic" and "genomic" effects, respectively (Kelly and Levin 2001). Therefore, further experiment was designed to explore the long-term effects of estrogen on TASK channels. N2A cells were incubated with 10 nM 17β-estradiol for 24 and 48 h, and subsequently the RNA was extracted and analyzed by semiquantitative RT-PCR. As shown in Fig. 3a, it certainly revealed that there were relatively high levels of expression for TASK-1 compared with TASK-2 in N2A cells ($n = 6$, $P < 0.05$). More importantly, an incubation with 10 nM 17β-estradiol for 48 h significantly reduced the mRNA levels of TASK-1 by 40.4 % ($n = 7$, $P < 0.05$), but no effect was observed for 24 h incubation with identical concentration of 17β-estradiol (Fig. 3a, b). Same experiments were conducted to test the effects of 17β-estradiol on the expression of TASK-2 in N2A cells; statistic difference was not validated between control and testing group (Fig. 3a, b; $n = 5$, $P > 0.05$). These results suggested that the long-term but not acute treatment of estrogen can alter the mRNA expression of TASK-1 channels in N2A cells.

Estrogen Affected the Cell Proliferation

The present work revealed that the long-term (48 h) application of 17β-estradiol can downregulate the expression of TASK-1 channels in N2A cells, whereas the pathophysiological sense about this finding is unclear. Previous reports suggested that TASK channels participated in the development and proliferation of some kind of cells such as MG63 cell (Li et al. 2013a). Therefore, it is possible that estrogen may also involve the proliferation of N2A cells by modulating the expression of TASK-1 channels. To address this issue, additional experiments were designed to test the effects of 17β-estradiol on the proliferation of N2A cells. N2A cells were cultured in the medium with 10 nM 17β-estradiol for 24 and 48 h and subsequently cell proliferation was measured by WST-8 as described above (see "Materials and Methods" section). In contrast to the control, treatment with 10 nM 17β-estradiol for 48 h but not 24 h significantly improved the proliferation of N2A cells by 12.7 % (Fig. 4; $n = 5$, $P < 0.05$). Together these data, it implicated that estrogen maybe modulated the cell proliferation through one of its candidate target: TASK-l channels.

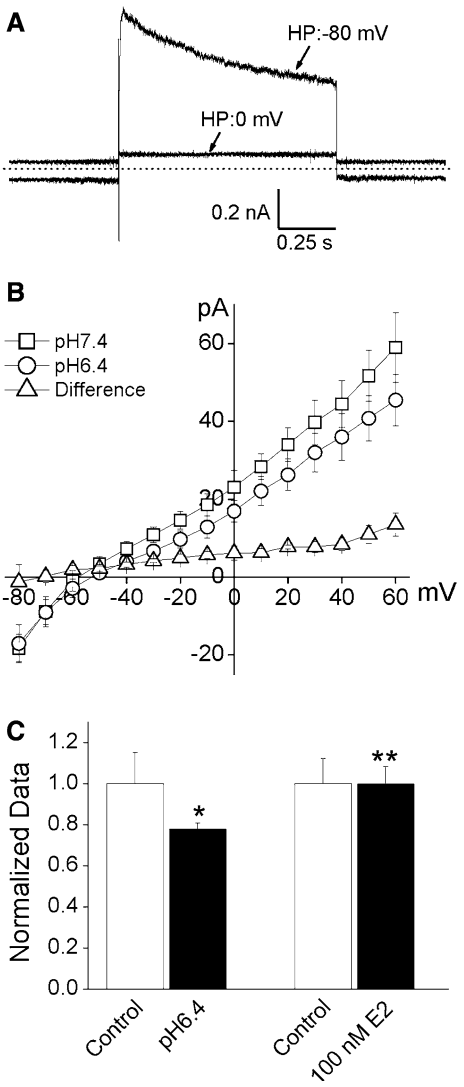

Fig. 2 Extracellular acidosis (pH 6.4) inhibited TASK-like outward currents in N2A cells. **a** The outward Kv currents were elicited by stepped up to +60 mV for 500 ms at a holding potential of −80 mV, and abolished by a holding potential of 0 mV. *Horizontal dot lines* depict zero current levels. **b** Current–voltage relations of outward background currents in the pH 7.4 (control, *white square*) and pH 6.4 (*white circle*) at a holding potential of 0 mV. I–V curves of acid-sensitive currents also were plotted as difference curves (*white up pointing triangle*). **c** The effects of extracellular acidosis ($n = 5$) and acute application of 100 nM 17β-estradiol ($n = 6$) on outward background current. The amplitudes of the outward background currents at pH 6.4 and 100 nM 17β-estradiol (E2) were normalized to control values, respectively. $^{*}P < 0.05$ and $^{**}P > 0.05$ compared with control

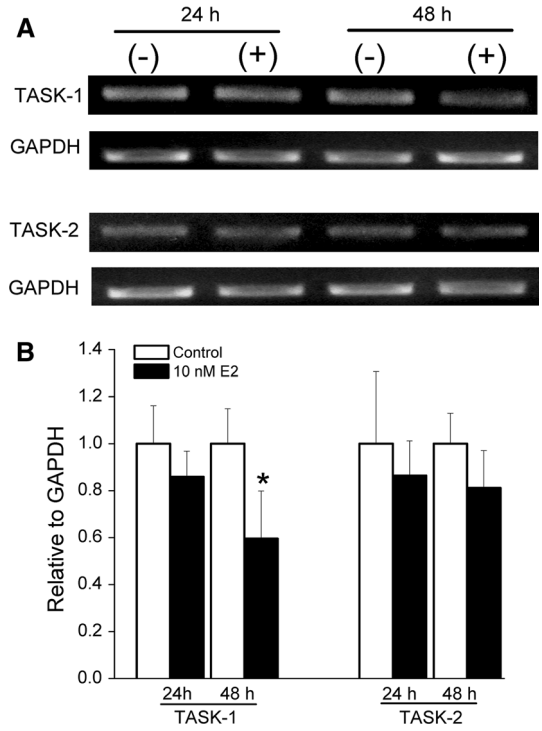

Fig. 3 The incubation with 17β-estradiol (E2) reduced the mRNA level of TASK-1 but not TASK-2. **a** An incubation with 10 nM 17β-estradiol for 48 h declined the expression of TASK-1 ($n = 7$), but not effects on TASK-2 ($n = 5$). GAPDH was used as internal control. **b** Statistic analysis of the results of semiquantitative RT-PCR. The expression levels of each TASK channels mRNA relative to the level of GAPDH mRNA in the same sample were normalized to control values. $^{*}P < 0.05$ compared with control

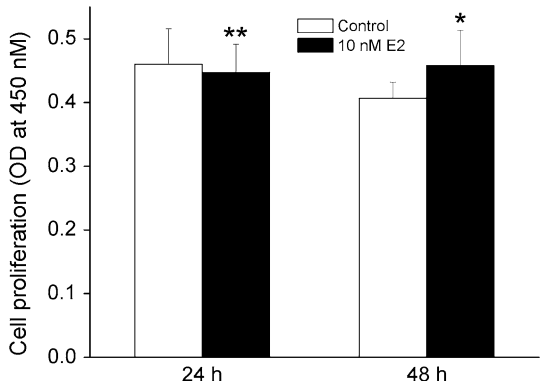

Fig. 4 Effects of incubation with 17β-estradiol (E2) on cell proliferation. The *graph* showed that incubation with 17β-estradiol for 48 h improved the proliferation rate of N2A cells ($n = 5$). The * and ** denoted $^{*}P < 0.05$ and $^{**}P > 0.05$ compared with control, respectively

Discussions

K2P channels are widely distributed in neuronal and non-neuronal tissues, as well as regarded as background or leak K^{+}-selective conductance due to opening at the resting membrane potential (Bayliss and Barrett 2008). We designed specific primers according to reported mRNA sequences and conduct RT-PCR to screen the expression of these channels in N2A cells. The PCR products of the predicted size for TASK-1, TASK-2, TWIK-1, and TREK-2 were amplified from total RNA templates of N2A cells, consistent with previous finding that neural tissues

expressed many kinds of K2P channels (Bayliss and Barrett 2008; Enyedi and Czirjak 2010). Among these K2P channels expressed in N2A cells, TASK-1 and TASK-2 belong to the acid-sensitive TASK subfamily and inhibited by extracellular acidosis with half-inhibition at pH 7.3 (Duprat et al. 1997) and pH 7.8 (Reyes et al. 1998), respectively. The other two members of TASK channels, TASK-3 and TASK-5, have not been verified in N2A cells. Extracellular acidosis (pH 6.4) significantly inhibited the voltage-independent outward K^+ currents (see "Results" section), suggesting that the TASK-like channels was functionally expressed in N2A cells.

Although previous works showed that 17β-estradiol rapidly blocked other K^+ channels, such as Kv in cardiac myocytes (Moller and Netzer 2006), neurons (Druzin et al. 2011; Fatehi et al. 2005), and osteoblast-like MG63 cells (Li et al. 2013b), as well as activated BK channels in smooth muscle cell (White et al. 2002), acute application of 100 nM 17β-estradiol has no effect on the voltage-independent outward currents in N2A cells. Interestingly, the incubation with 10 nM 17β-estradiol for 48 h lowered the mRNA level of TASK-1 but not TASK-2 in N2A cells. Generally, estrogen may interact with targets through two subtypes of nuclear ERs, ERα and ERβ, and trigger a signal cascade with 10–20 min delays (Carrer et al. 2003; Kelly and Levin 2001). Moreover, Mendez et al. reported that both ERs were functionally expressed in N2A cell (Mendez and Garcia-Segura 2006). Therefore, our results suggested that long-term exposure to 17β-estradiol may exert a slow effect of "genomic" on TASK-1 channels. Similarly, Nishimura et al. reported that chronic exposure to 17β-estradiol for 72 h upregulated the expressions of BK channels in GT1-7 GnRH neuronal cell line (Nishimura et al. 2008).

Normally, the concentrations of estrogen are less than 10 nM in the plasma of mammals (Naftolin et al. 1990); however, the level of this sex steroid in the woman can reach to 100 nM at the end of the pregnancy (Runnebaum and Raube 1987). Therefore, it is possible that estradiol at the physiological level is able to modulate the expression of TASK-1 channels in N2A cells. Sawada et al. found that 17β-estradiol provided antiapoptotic and neuroprotective effect on in nigral dopaminergic neural cultures (Sawada et al. 2000). Previous finding also indicated that cytoplasmic K^+ efflux facilitated apoptosis (Burg et al. 2006). In agreement with these notions, the decline of K^+ efflux due to the downregulation of expression of TASK-1 channels may decrease apoptosis and contributed to the neuroprotective effect of estrogen. Further, the extracellular acidosis was capable of preventing the neuronal death of cultured rat granule neurons through the inhibition of TASK channels (Lauritzen et al. 2003). Our data reveal that the incubation with 17β-estradiol for 48 h augmented the proliferation of N2A cells, and these effects could occur possibly through one of the targets of estrogen action: TASK-1 channels; thus, it could be an alternative explanation for the neuroprotective effects of estrogen replacement therapy.

Estrogen may act on not only TASK-1 channels but also other types of ion channels such as BK channels, which so far has not been identified in N2A cells, to increase the proliferation of N2A cells. As a result, a limitation of this study is that TASK-1 channels were not the only candidate target of estrogen action. Moreover, further experiments also need to be conducted to elucidate the actual mechanism of the effects of estrogen on TASK-1 channels.

Collectively, the expressions of several distinct K2P channels have been clarified by RT-PCR in N2A cells. The recording of acid-sensitive outward currents by patch-clamp technique confirmed that N2A cells expressed functional TASK channels. Long-term application of 17β-estradiol downregulated the mRNA level of TASK-1 channels and improved the proliferation of N2A cells, although no effect was observed at acute exposure to 17β-estradiol. These data provided an insight for the mechanism of neuroprotective effects of estrogen.

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