17β-Estradiol Protects Neural Stem/Progenitor Cells Against Ketamine-Induced Injury Through Estrogen Receptor β Pathway

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Ketamine inhibits neural stem/progenitor cell (NSPC) proliferation and disrupts normal neurogenesis in the developing brain. 17β-Estradiol alleviates neurogenesis damage and enhances behavioral performance after ketamine administration. However, the receptor pathway of 17β-estradiol that protects NSPCs from ketamine-induced injury remains unknown. In the present study, we investigated the role of estrogen receptor α (ER-α) and estrogen receptor β (ER-β) in 17β-estradiol’s protection against ketamine-exposed NSPCs and explored its potential mechanism. The primary cultured NSPCs were identified by immunofluorescence and then treated with ketamine and varying doses of ER-α agonist 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) or ER-β agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) for 24 h. NSPC proliferation was analyzed by 5-bromo-2-deoxyuridine incorporation test. The expression of phosphorylated glycogen synthase kinase-3β (p-GSK-3β) was quantified by western blotting. It was found that treatment with different concentrations of PPT did not alter the inhibition of ketamine on NSPC proliferation. However, treatment with DPN attenuated the inhibition of ketamine on NSPC proliferation at 24 h after their exposure (P < 0.05). Furthermore, treatment with DPN increased p-GSK-3β expression in NSPCs exposed to ketamine. These findings indicated that ER-β mediates probably the protective effects of 17β-estradiol on ketamine-damaged NSPC proliferation and GSK-3β is involved in this process.

Keywords: ER-α, ER-β, 17β-estradiol, neural stem/progenitor cells, ketamine, GSK-3β

INTRODUCTION

Every year, millions of children over the world receive surgery or imaging examination under general anesthesia. Several non-clinical studies have indicated that early or repeated exposure to anesthetics leads to neurodegeneration in the developing brain, as well as cognitive impairment later in life (Jevtovic-Todorovic et al., 2003; Paule et al., 2011; Xiao et al., 2017; O’Farrell et al., 2018). Besides, retrospective clinical data reported that use of anesthetics during early life might be associated with late-onset learning disabilities (Block et al., 2012). The mechanism that
anesthetic exposure causes such changes remains incompletely understood. Early studies suggested anesthetic-induced neuronal apoptosis to be a possible mechanism of such changes, and more recent work indicates that anesthetics may disturb several critical processes in brain development (Meredith et al., 2014).

Neurons are organized into circuits for complex functions such as learning and cognition. To perform these circuits functioning, the correct populations of neurons must exist in anatomically appropriate locations, and they must communicate through precise connections established during critical periods of brain development. Neurogenesis, i.e., the production of new neurons from neural stem/progenitor cells (NSPCs), includes NSPC proliferation, differentiation, and migration. In mammals, most neurons are generated from NSPCs in the ventricular zone (VZ) and subventricular zone (SVZ) before the birth, but new neurons derived from NSPCs that locate primarily in two distinct areas of the brain, the SVZ and subgranular zone (SGZ), are continuously added to certain brain areas postnatally (Ming and Song, 2005; Alberto et al., 2017). Neurogenesis is vital for brain development and postnatal brain injury repair. Several studies report that anesthetics inhibit NSPC proliferation, disturb neuronal differentiation, and increase NSPC death (Paule et al., 2011; O’Farrell et al., 2018). These may imply protection of NSPCs to be a novel target for the treatment of anesthetic neurotoxicity.

Ketamine, a non-competitive N-methyl-D-aspartate receptor antagonist, is usually used for pediatric anesthesia. Numerous animal experiments have indicated that multi-exposure to ketamine can inhibit NSPC proliferation and obstruct normal neurogenesis (Scallet et al., 2004; Lu et al., 2017), leading to long-term cognitive and memory defects (Paule et al., 2011). Because ketamine exerts long-term effects on the brain, discovering appropriate approaches or agents that could prevent or minimize its deleterious effects on NSPCs is essential. 17β-Estradiol is a principal female hormone. It has been reported that 17β-estradiol provides evident neuroprotection in many brain injury models in both sexes and relieves neurodegeneration induced by ketamine (Li et al., 2013, 2014). Our previous study showed that treatment with 17β-estradiol lessened neurogenesis damage and enhanced behavioral performance after ketamine administration in neonatal rats (Li et al., 2019). Neural stem cells (NSPCs) derived from embryonic and adult brains both express estrogen receptor α (ER-α) and ER-β (Brännvall et al., 2002; Isgor and Watson, 2005; Kishi et al., 2005). 17β-Estradiol promotes the proliferation and neuronal differentiation of embryonic NSPCs during brain development. Previously, we reported that 17β-estradiol enhanced post-stroke neurogenesis in adult rats (Zheng et al., 2013). It was found that 17β-estradiol also regulated the migration of embryonic neuroblasts via ERβ (Wang et al., 2003). However, the receptor subtype that mediates the protective effects of 17β-estradiol on ketamine-induced proliferation inhibition in NSPCs remains to be elucidated.

As a serine/threonine kinase, glycogen synthase kinase (GSK) 3β is the downstream molecule of PI3K/Akt, MAPK/ERK, Wnt, Notch, and other pathways. It can function independently or in combination with several signaling pathways and plays a pivotal role in multiple fundamental cellular functions in the developing brain, including neurogenesis and cell cycle (Hur and Zhou, 2010). Inhibition of glycogen synthase kinase 3 promoted adult hippocampal neurogenesis in vitro and in vivo (Morales-Garcia et al., 2012). Exposure of neonatal rats to ketamine decreased GSK-3β phosphorylation and inhibited the proliferation of NSPCs in the subventricular zone as well as induced neurotoxicity in neurons of the brains (Scallet et al., 2004; Bai et al., 2013; Liu et al., 2013; Huang et al., 2015). Conversely, activation of GSK-3β, namely, increasing its phosphorylation, attenuated ketamine-induced neurogenesis disorder and neuronal injury (Scallet et al., 2004; Lu et al., 2018). Interestingly, GSK-3β is also a downstream target of estradiol signaling (Shi et al., 2013; Wu et al., 2014). However, which subtype of ER mediated the response of GSK-3β to 17β-estradiol in NSPCs remains unclear. In this study, we tried to reveal what subtype of ER-mediated protective effects of 17β-estradiol on ketamine-induced proliferation inhibition in NSPCs. We also investigated the involvement of GSK-3β signaling pathway in this process.

MATERIALS AND METHODS

Cell Culture

Pregnant Sprague–Dawley rats were acquired from Laboratory Animal Center of Xi’an Jiaotong University. The experiment was in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the protocols were authorized by the Committee on Animal Care of Xi’an Jiaotong University. The minimal rats for essential requirement and appropriate steps were used in this study. Primary NSPCs were acquired from the cortex of rat brains at embryonic days 18–19 under sterile conditions. Briefly, the forebrain portions were isolated and placed in ice-cold Hank’s solution (CaCl2/Mg2+ free; Gibco, Carlsbad, CA, United States). Then, the tissues were dissociated and triturated mechanically by a fire-polished Pasteur pipette. After centrifugation, the cell pellets were re-suspended in serum-free DMEM/F12 medium (Gibco) supplemented with 2% B27 (Gibco), 20 ng/ml EGF (Gibco), 20 ng/ml bFGF (Gibco), and 100 U/ml of penicillin and streptomycin. After 7-day culture, the cells formed enough colonies on 100 mg/ml poly-L-lysine-coated coverslips and cultured in differentiation medium supplemented with 100 × N2 supplement, 100 × B27 supplement, and 1% fetal bovine serum (FBS; Gibco) in DMEM/F12 (without bFGF). After 7-day cultures, cells were fixed with 4% paraformaldehyde (PFA) for 30 min and harvested for immunohistochemical staining of a neuronal marker, β-tubulin III, or astrocyte marker, glial fibrillary acidic protein (GFAP).
Protocol
The cells were randomly assigned to the following five groups: control group, ketamine group, PPT plus ketamine group, DPN plus ketamine group, and DPN group. NSPCs in control group received an equal volume of phosphate-buffered saline (PBS) as PPT plus ketamine or DPN plus ketamine. NSPCs in ketamine group underwent 100 μM of ketamine exposure for 24 h. NSPCs in PPT plus ketamine group received 100 μM of ketamine and different concentrations of PPT (0, 0.1, 1.0, 10, 100 nmol/L) for 24 h. NSPCs in DPN plus ketamine group received 100 μM ketamine and different concentrations of DPN (0, 0.1, 1.0, 10, and 100 nmol/L) for 24 h. NSPCs in DPN group received 10 nmol/L of DPN for 24 h.

Immunohistochemistry and Nuclear Staining
The cultured cells were characterized by immunofluorescence analysis. For identification assessment, the harvested cells were placed in 0.3% Triton X-100 for 10 min. Then, they were treated with blocking buffer (2% goat serum) for 1 h and incubated with primary antibodies, including mouse monoclonal anti-nestin antibody (1:500; Millipore, MA, United States), rabbit polyclonal anti-GFAP antibody (1:1000; Sigma-Aldrich, St. Louis, MO, United States), and mouse monoclonal anti-β-tubulin III antibody (1:1000; Sigma-Aldrich) overnight at 4°C. The cells were next washed with PBS and incubated in the solution of secondary antibodies (fluorescein isothiocyanate or (tetramethylrhodamine isothiocyanate–conjugated IgG) for 2 h followed by DAPI for 10 min at room temperature. For cell proliferation analysis, the cells were seeded on cover slips precoated with 100 mg/ml poly-L-lysine and incubated with 5-bromo-2-deoxyuridine (BrdU) for the last 4 h (Chen et al., 2017; Qi et al., 2017). After fixing with 4% PFA, the cells were incubated with 2 N HCl for 30 min at 37°C to denature the DNA. After incubation with 0.1 mol/L boric acid (pH 8.5) for 10 min at room temperature and three washes with 0.1 M PBS, the slides were blocked with 2% goat serum and 0.3% Triton X-100 for 2 h at room temperature, then incubated with the rat monoclonal anti-BrdU antibody (1:200; Abcam, United Kingdom) and DAPI. The slides were rinsed with PBS and the images were captured by a laser confocal microscope (TSC SP2; Leica, Manheim, Germany). Five to seven randomly selected fields from each coverslip were imaged, and nestin-positive cells, β-tubulin III-positive cells, GFAP-positive cells, and BrdU-positive cells were counted (at least 200 cells per test case). Data were collected from three independent experiments.

Western Blot Analysis
After 24 h of treatment, cells were lysed, and Western blot analyses of p-GSK-3β protein levels were performed as described in our previous studies (Scallet et al., 2004). Briefly, an equal amount of cell lysate was resolved by sodium dodecyl sulfate–polyacrylamide gel, and separated proteins were transferred to nitrocellulose membranes. After incubation with blocking buffer for 2 h at room temperature, blots were incubated overnight at 4°C with primary antibodies, including mouse monoclonal anti-β-actin antibody (as a control, internal standard, 1:10,000) and rabbit polyclonal anti-phosphorylated GSK-3β antibody (p-GSK-3β, 1:1000; Cell Signaling Technology, United States). After washes, the blots were incubated with secondary antibody (1:5000; Santa Cruz, CA, United States) for 2 h at room temperature. After being enhanced by chemiluminescence (ECL), the signals were exposed to X-ray films. Each band in the Western blotting represented an independent experiment, and at least three independent experiments were conducted. Data were expressed as the ratio of target proteins to the corresponding controls.

Statistical Analysis
Nestin+/DAPI+ cells, β-tubulin III+/DAPI+ cells, GFAP+/DAPI+ cells, and BrdU+/DAPI+ cells were counted blindly and randomly in five to seven sections per well, six wells in each group. At least 200 cells were counted per section and the average number of cells in one section is around 330–350. The experiments were repeated at least three times. The density of positive cells was presented as the rate of positive cells. SigmaPlot 12.0 was applied for all statistical analyses. Data were evaluated for normality and equal variance. Student t test or one-way ANOVA followed by the post hoc Holm–Sidak test was used to compare different groups. A two-tailed probability value P < 0.05 was considered statistically significant.

RESULTS
ER-β but Not ER-α Mediated the Protection of 17β-Estradiol on Ketamine-Damaged NSPC Proliferation
To reveal what subtype of ER mediated the protective effects of 17β-estradiol on ketamine-induced proliferation inhibition in NSPCs, we first cultured and identified cells in vitro. The experimental design is shown as Figure 1. The cells were adhering to the bottom of 24-well culture plates pre-coated by 10 mg/ml poly-L-ornionic hydrobromide and 10 μg/ml Laminin at 24 h after seeding (Figure 2A). NSPC marker nestin staining was performed 72 h after adherent culture for identification of characteristics of the cultured cells. It was shown that the majority of cells were nestin-positive (94.8 ± 2.5%, Figure 2B). The marker for neurons (β-tubulin III) or astrocytes (GFAP) were further used to determine the differentiative capacity of the adherent cultured cells, it was showed that they could differentiate into β-tubulin III-positive cells (21.9 ± 2.0%, Figure 2C) and GFAP-positive cells (60.1 ± 2.9%, Figure 2C). These findings suggest that the cultured cells were NSPCs (Figure 2N).

Our previous study has shown that ketamine inhibited the proliferation of NSPCs and pretreatment with 17β-estradiol decreased the effects of ketamine on NSPC proliferation. To determine whether ER-α mediates this effect, NSPCs were treated...
with 100 µmol/L of ketamine and 0, 0.1, 1, 10, and 100 nmol/L of ER-α agonist PPT for 24 h, respectively. It was found that treatment of NSPCs with different concentrations of PPT did not alter the number of BrdU-positive cells after ketamine exposure (Figures 2D–H, \( F(4,70) = 5.35, P > 0.05 \)). These results suggested that ER-α did not mediate the protective effects of 17β-estradiol on ketamine-damaged NSPC proliferation.

Compared with the control group, treatment of NSPCs with ketamine decreased the number of BrdU-positive cells (Figure 3C). To determine whether ER-β mediates protective effects of 17β-estradiol on ketamine-damaged NSPC proliferation, NSPCs were co-treated with 100 µmol/L of ketamine and 0, 0.1, 1, 10, and 100 nmol/L of ER-β agonist DPN for 24 h, respectively. It was found that treatment of NSPCs with 0, 0.1, and 1 nmol/L of DPN did not alter the number of BrdU-positive cells after ketamine exposure [Figure 2I–K, \( F(4,70) = 5.32, P > 0.05 \)]. However, treatment of NSPCs with 10 or 100 nmol/L of DPN increased the number of BrdU-positive cells after 24 h of ketamine exposure, respectively [Figures 2L,M,P, \( F(4,70) = 63.25, P < 0.01 \)]. These results indicated that ER-β mediated the protective effects of 17β-estradiol on ketamine-damaged NSPC proliferation. Based on that both 10 and 100 nmol/L of DPN increased cell proliferation significantly after exposure to 100 µmol/L of ketamine, we chose 10 nmol/L of DPN for the next experiment.

**ER-β Agonist Attenuated Ketamine-Induced NSPC Proliferation Damage Time Dependent**

To determine the time effects of ER-β agonist DPN attenuating ketamine-induced NSPC proliferation damage, BrdU immunofluorescence staining was performed at 6, 12, and 24 h after different treatment, respectively. It was found that there were no significant differences in the number of BrdU-positive cells at 6 and 12 h after treatment of NSPCs with ketamine for 24 h compared with the control [Figure 3A, \( F(3,56) = 7.27, P > 0.05 \); Figure 3B, \( F(3,56) = 6.86, P > 0.05 \)]. However, treatment of NSPCs with DPN (10 nmol/L) or DPN plus ketamine for 24 h did not change the number of BrdU-positive cells compared with the control [Figure 3C, \( F(3,56) = 50.63, P < 0.01 \)]. Compared with ketamine treatment,
co-treatment with DPN (10 nmol/L) for 24 h increased significantly the number of BrdU-positive cells [Figure 3C, $F(3,56) = 30.45, P < 0.01$].

**GSK-3β Pathway Participated in the Protective Effects of ER-β Agonist on Ketamine-Injured NSPCs**

To examine whether GSK-3β pathway participated in the protective effect of ER-β agonist DPN on ketamine-exposed NSPCs, Western blotting was performed to assess the levels of p-GSK-3β in different groups (Figure 4A). It was found that ketamine decreased the protein levels of p-GSK-3β [Figure 4A, $F(3,32) = 70.65, P < 0.01$] in NSPCs and treatment with ER-β agonist DPN significantly attenuated the effects of ketamine on p-GSK-3β expressions [Figure 4A, $F(3,32) = 38.75, P < 0.01$]. However, there was no difference in the protein levels of p-GSK-3β between the control and DPN group [Figure 4B, $F(3,32) = 7.67, P > 0.05$]. These findings indicated that GSK-3β was involved in DPN-elicited protection on ketamine-exposed NSPCs.
**DISCUSSION**

The present study demonstrated that treatment with ER-β agonist DPN but not ER-α agonist PPT attenuated the inhibition of NSPC proliferation after ketamine exposure. Furthermore, treatment with DPN increased p-GSK-3β protein levels in NSPCs exposed to ketamine. These findings indicated that ER-β probably mediates the protective effects of 17β-estradiol on ketamine-damaged NSPC proliferation and GSK-3β is involved in this process.

As a non-competitive N-methyl-D-aspartate receptor antagonist commonly used for anesthesia, ketamine has been proven to induce neurotoxicity in the developing brain, which may result in long-term neurocognitive dysfunction in adulthood (Scallet et al., 2004; Paule et al., 2011). Many studies have been performed to prevent the neurotoxicity of ketamine (Turner et al., 2012; Chaki, 2017). We previously found that treatment with 17β-estradiol lessened neurogenesis damage and enhanced behavioral performance in neonatal rats after ketamine administration (Li et al., 2019). However, we could not define which subtype of the estrogen receptors mediated the protection of 17β-estradiol in vivo. To clarify this question and further explore the detailed signaling mechanism, we applied selective ER-α and ER-β agonist in this *in vitro* study as both receptors are widely expressed on neurons and glial cells during early brain development (Wilson and Westberry, 2009). Our data showed that ER-α agonist PPT did not alter the proliferation of NSPCs after ketamine exposure, whereas ER-β agonist DPN increased the proliferation of ketamine-exposed NSPCs in a dose- and time-dependent manner, indicating that the neuroprotection of 17β-estradiol on ketamine-induced proliferation inhibition in NSPCs was mainly mediated by ER-β. This could be attributed to that ER-β is more highly expressed in NSPCs compared with ER-α. In the developing brain, ER-β expression increased during the development of NSPCs, whereas ER-α level decreased (Wang et al., 2007). Moreover, lack of ER-β would increase the risk of neurodegeneration, abnormal behavior, and cognitive dysfunction, whereas stimulating ER-β could increase NSPC proliferation and promote neuronal differentiation (Brännvall et al., 2002; Varshney et al., 2017). In this study, we did not observe that 10 nmol/L DPN increased the number of BrdU-positive cells compared with the control group. This might be due to that we used EGF in NSPC culture and 10 nM 17β-estradiol decreased NSPC proliferation in the presence EGF (Brännvall et al., 2002). Whether the effect of 10 nmol/L DPN on NSPC proliferation was equivalent to that of 10 nM 17β-estradiol is unknown. In addition, the difference in stage of neural development and time of drug treatment might also contribute to this inconsistency. Interestingly, we observed that ER-β agonist
which is consistent with the former reports and our previous studies (Scallet et al., 2004; Lu et al., 2018). It has also been demonstrated that potential toxicity of ketamine is both dose and time dependent. The concentration of ketamine used in this study was clinically relevant (37.8–108.4 μM, Domino et al., 1982), but exposure time was much longer than clinically indicated more than 3 h or repeated exposure. This indicates that in vivo, other mechanisms such as neural circuits and neurogenesis microenvironment may be involved in ketamine’s neurotoxicity (Mansouri et al., 2017). Further studies need to be done to explore this hypothesis. Interestingly, ketamine plays a neuroprotective role under some pathological conditions. Ketamine reduced the death of excitotoxic neurons during cerebral ischemia and traumatic brain injury (Pukasundvall et al., 2000; Cheung and Yew, 2019). Ketamine inhibited the activity of macrophages and the production of pro-inflammatory cytokines (Chang et al., 2016). Pain and surgery-related psychological stress induce harmful neurobiological effects on immature brain, which is related to brain development damage and long-term behavioral consequences (Brummelte et al., 2012; Michaelsson et al., 2019). Ketamine can reduce neuronal activation and cell death in cortex and subcortical area of neonatal rats caused by inflammatory pain (Liu et al., 2012). This inconformity may be contributed to different pathophysiological states. Further studies need to be performed to explore the effects of ketamine and operation on NSPCs and neurogenesis.

As GSK-3β is the downstream molecule of several signaling pathways closely associated with cell biology such as cell cycle, proliferation, survival, and apoptosis (Kaidanovich-Beilin and Woodgett, 2011) and it also played an important role in estradiol actions (Shi et al., 2013; Wu et al., 2014), we analyzed its protein level in ER-β-induced attenuation of neurogenesis damage by ketamine in vitro. Our data showed that ketamine decreased protein levels of p-GSK-3β in NSPCs, which is consistent with our former study in vivo (Scallet et al., 2004; Lu et al., 2018), while co-treatment with ER-β agonist DPN significantly attenuated the effect of ketamine on p-GSK-3β protein levels, suggesting that GSK-3β was involved in DPN-elicited protection on ketamine-exposed NSPCs. This was also consistent with an in vivo study that 17β-estradiol increased the protein levels of p-GSK-3β in C57BL/6J mice (Kararigas et al., 2014). These findings indicated that p-GSK-3β is an important molecule in estrogen-elicited protection against ketamine-induced damage on neurogenesis. Further studies should be done to find how p-GSK-3β protect neurogenesis.

Our study has some limitations. First, we did not observe the effects of ketamine on NSPC differentiation and migration of progeny cells as well as DPN’s protection in this study. It is well known that neurogenesis includes three stages: (1) cell proliferation to generate new cells; (2) newly generated cells to move for their final destination; (3) neuronal differentiation. The accuracy of one stage is dependent on NSPCs and their subsequent behavior (Joan and Terry, 2010). To evaluate 17β-estradiol protecting NSPCs against ketamine-induced injury
completely, further experiments are needed to verify the effects of DPN on ketamine-induced changes of NSPC differentiation and cell migration. Second, we only used ER-α agonist PPT or ER-β agonist DPN to investigate receptor subtype involved in 17β-estradiol’s protection on ketamine-exposed NSPCs. For a more convincing conclusion, the group that is exposed to ketamine and treated with 17β-estradiol and ER-β antagonist should be designed in this study. Further experiments are needed to observe the changes of NSPC proliferation and GSK-3β expression after ketamine exposure and 17β-estradiol combined with ER-β antagonist treatment. Third, we did not perform an experiment to modulate GSK-3β pathway. Thus, the possibility that other signal pathways could be responsible for mediation of DPN’s protection against ketamine-induced proliferation damage cannot be excluded. Further study should be done to exclude the role of other molecular cascades.

CONCLUSION

The present study showed that ER-β may play a role in neuroprotective effects of 17β-estradiol on NSPCs against ketamine-induced proliferation inhibition, and that GSK-3β may be involved in this process. These findings are important for potential use of 17β-estradiol and avoiding its side effects in prevention of anesthetic neurotoxicity.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

WL designed and performed the cell culture experiments, analyzed data, and co-wrote the manuscript. PL and YL performed the immunohistochemistry and nuclear staining experiments. HW co-wrote and edited the manuscript. XN, JX, and KW performed Western blot analysis. HZ, RL, ZQ, NW, PJ, YZ, and SZ analyzed the data. HL, XC, and YL co-wrote and edited the manuscript. PZ co-designed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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