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Lin28b and Sox2 regulate anesthesia-induced neural degeneration in neural stem cell derived neurons

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ABSTRACT

Ketamine, a commonly used anesthetic compound, may cause neurotoxicity in immature or developing brains. In this study, we indented to understand the molecular expression and functional role of Lin-28 Homolog B (lin28b) in regulating ketamine-induced neurotoxicity in neural stem cells (NSCs)-differentiated neurons. NSCs from embryonic rat brains were cultured *in vitro*, and induced toward neuronal differentiation. NSCs-differentiated neurons were treated with various concentrations of ketamine for 24 h to evaluate the concentration-dependent effect of ketamine on endogenous lin28b mRNA level. QRT-PCR showed that lin28b was downregulated by ketamine in NSCs-differentiated neurons, in concentration-dependent manner. Neurons were then transfected with adenovirus to ectopically upregulate lin28b. We found that ketamine-induced apoptosis and neurite retraction in NSCs-differentiated neurons were significantly reduced by adenovirus-mediated lin28b upregulation. Expression of sex determining region Y box 2 (Sox2) mRNA was examined in ketamine-injured and lin28b-upregulated NSCs-differentiated neurons. It was found Sox2 was downregulated by ketamine, and overexpressed by lin28b upregulation. Finally, Sox2 was downregulated by siRNA in NSCs-differentiated neurons. And we discovered that Lin28b-upregulation-associated neural protection was severely hampered by Sox2 downregulation in ketamine-injured neurons. Thus, Lin28b and Sox2 are important molecular components in ketamine-induced neurotoxicity.

1. Introduction

Strong evidence demonstrates that, through both human and animal studies, excessive or prolonged application of anesthetic compound, ketamine, may induce severe and permanent neurological injuries in immature or developing brains (Dong and Anand, 2013; Wang and Slikker, 2008; Zou et al., 2009). Several molecular signaling pathways had been proposed to be the underlying contributors to ketamine-induced neurodegeneration or neurotoxicity in cortical neurons, such as ketamine-induced upregulation of N-methyl-D-aspartate (NMDA) receptors (Slikker et al., 2007; Wang et al., 2005), increase in neuronal nitric oxide synthase (Wang et al., 2008), or impairment of neurotrophic factor homeostasis (Obradovic et al., 2017). However, the exact events of ketamine-induced molecular cascades, including upregulation/downregulation of survival/apoptotic transcriptional factors in human or animal brains, are largely unknown.

Due to ethic issues, studies directly focusing on ketamine-induced neurological malfunctions in human brains are practically impossible. Also, *in vivo* studies using animal models are often time-consuming, or cost inefficient to pursue. Alternatively, neural stem cells (NSCs) originated from mammalian embryonic brains may be considered as an ideal model to study anesthetics-induced neurotoxicity in central nerve system, as NSCs-differentiated neurons *in vitro* bear many similar traits with mature cortical neurons *in vivo*, and it is relatively quick to generate, as well as easy to maintain large population of NSCs-differentiated neurons *in vitro* (Bai et al., 2013; Krug et al., 2013).

Gene of Lin-28 Homolog B (lin28b) belongs to the heterochronic gene family of lin-28 (including lin28a and lin28b) (Ambros and Horvitz, 1984). Lin28 has been implicated to play important roles, by associating with epigenetic regulator let-7, in various processes of cell development, such as transformation, transition or metabolism (Iliopoulos et al., 2009; West et al., 2009; Zhu et al., 2011). Unlike lin28a, lin28b adapts different mechanism to regulate let-7, thus suggesting distinctly different biological functions in modulating cell development (Piskounova et al., 2011). In regarding to neurotoxic injury, lin28b was demonstrated to be regulated by beta-catenin to activate Wnt signaling pathway and promote the proliferation of retinal Müller glial cells (Yao et al., 2016). However, little is known whether Lin28b may be involved in the regulatory process of ketamine-, or any other anesthetics-induced neurotoxicity among neuronal populations.

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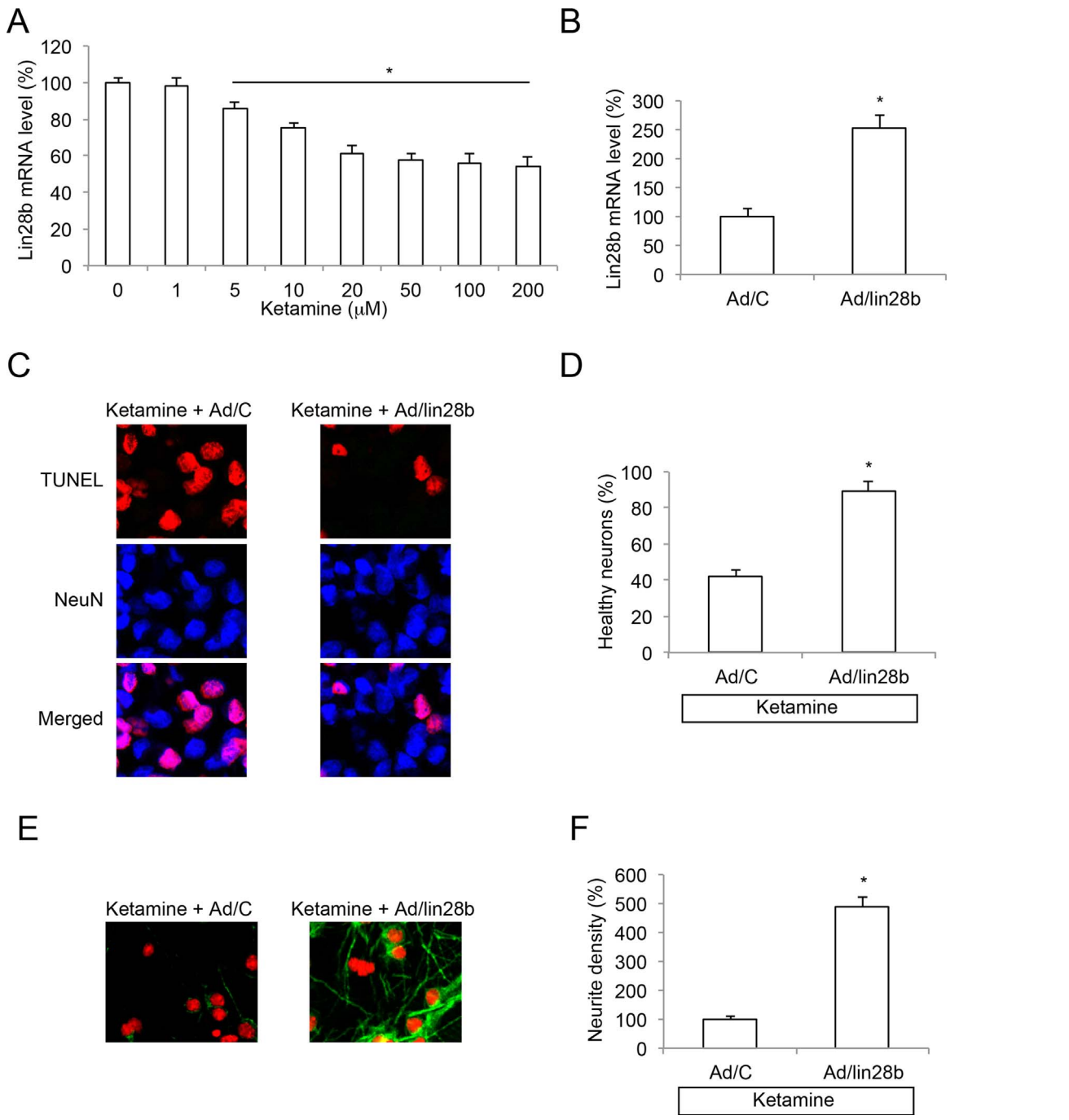

Fig. 1. Expression and function of lin28b during the process of ketamine-induced neurotoxicity in neural stem cells differentiated neurons. (A) Neural stem cells (NSCs) were extracted from rat embryonic brain and induced toward neuronal fate *in vitro*. After 3 days of differentiation, various concentrations of ketamine (0, 1, 5, 10, 20, 50, 100 and 200 μM) were added into culture for 24 h. QRT-PCR was applied to measure lin28b mRNA levels in NSCs- differentiated neurons corresponding to ketamine treatment (* $P < 0.05$, n = 11). **(B)** NSCs- differentiated neurons were infected with an adenovirus, Ad/lin28b, to upregulate their endogenous lin28b mRNA levels. Control neurons were infected with a control adenovirus, Ad/C. QRT-PCR was then applied to measure lin28b mRNA levels in infected neurons (* $P < 0.05$, n = 8). **(C)** After adenoviral transfection, NSCs-differentiated neurons were treated with 20 μM ketamine for 24 h. A TUNEL assay was then applied. A fluorescence-conjugated NeuN antibody was applied as neural marker to identify neurons in the culture. Representative fluorescent images were shown for TUNEL (Red) and NeuN (Blue) stainings in neurons pre-transfected with Ad/C and those pre-transfected with Ad/lin28b. **(D)** For NSCs- differentiated neurons in (C), the percentages of healthy neurons (TUNEL-negative & NeuN-positive) were compared between neurons pre-transfected with Ad/C and neurons pre-transfected with Ad/lin28b (* $P < 0.05$, n = 8). **(E)** After adenoviral transfection, NSCs- differentiated neurons were treated with 20 μM ketamine for 24 h, and then maintained in regular differentiation medium for additional 7 days. A neurite density assay was applied. Representative fluorescent images were shown for Tuj1 (Green) and NeuN (Red) stainings in neurons pre-transfected with Ad/C and neurons pre-transfected with Ad/lin28b. **(F)** For NSCs- differentiated neurons in (E), relative neurite densities were compared between neurons pre-transfected with Ad/C and neurons pre-transfected with Ad/lin28b (* $P < 0.05$, n = 8).

In this study, we extracted NSCs from rat embryonic brains and culture them *in vitro*. As they were induced toward neuronal lineage, ketamine was applied in the culture to induce neurotoxicity in NSCs-differentiated neurons. Then, we utilized qRT-PCR to characterize the mRNA expression of lin28b in NSCs-differentiated neurons in response to ketamine treatment. Functionally, we used adenovirus to overexpress lin28b in NSCs-differentiated neurons, and then used apoptosis and neurite densities assays to evaluate the effect of lin28b upregulation on ketamine-induced neural injury in virally transfected neurons. In addition, we also evaluated mRNA expression of sex determining region Y