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Research report

Deletion of *Numb/Numblike* in glutamatergic neurons leads to anxiety-like behavior in mice

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ABSTRACT

Endocytic adaptor protein Numb is the first identified cell fate determinant in *Drosophila melanogaster*. It has been implicated in Notch signaling pathway and regulation of neural stem cells proliferation in the central nervous system. Numb is also expressed in postmitotic neurons, in vitro studies showed that Numb is involved in neuronal morphologic development, such as neurite growth, axonal growth and spine development. However, in vivo functions of Numb in the postmitotic neurons are largely unknown. Here we show that deletion of *Numb/Numblike* in glutamatergic neurons causes anxiety-like behavior in mouse. In this study, we conditionally deleted *Numb* and its homologous gene *Numblike* in the glutamatergic neurons in dorsal forebrain, and thoroughly characterized the behavioral phenotypes of mutant mice. On a battery of tests for anxiety-like behavior, the conditional double knockout mice showed increased anxiety-like behavior on light/dark exploration and novel open field tests, but not on elevated zero maze tests. The conditional double knockout mice also displayed novelty induced hyperactivity in novel open field test. Control measures of general health, motor functions, startle response, sensorimotor gating, depression-related behaviors did not show differences between genotypes. Our present findings provide new insight into the indispensable functions of *Numb/Numblike* in the brain and behavior, and suggest that *Numb/Numblike* may play a role in mediating neuronal functions that underlie behaviors related to anxiety.

1. Introduction

Drosophila Numb was the first discovered cell fate determinant in the *Drosophila melanogaster* (Uemura et al., 1989). During the course of asymmetric cell division of sensory organ precursor cells, *Drosophila* Numb protein is selectively segregated into one daughter cell, through antagonizing Notch signaling, it establishes different cell fates in two daughter cells (Guo et al., 1996).

Drosophila Numb has two mammalian homologues, *Numb* and *Numblike* (Verdi et al., 1996; Zhong et al., 1996, 1997). In most cases, these two homologues have functional redundancies (Li et al., 2003; Nishimura et al., 2006; Petersen et al., 2002, 2004). Numb is a multiple-domain containing protein, having phosphorylated Tyrosine binding (PTB) domain at the N-terminal, and Proline rich region (PRR), DPF (Asp-Pro-Phe) and NPF (Asn-Pro-Phe) tripeptide motifs at the C-terminal. This structure pattern decides Numb's primary role as an endocytic adaptor protein. By recruiting different cargo molecules into clathrin dependent endocytic

Abbreviations: PTB, phosphorylated Tyrosine binding; PRR, proline rich region; CDKO, conditional double knockout; CTL, control; EZM, elevated zero maze; EPM, elevated plus maze; LDE, light/dark exploration; NOF, novel open field; PPI, prepulse inhibition; dB, decibel; TST, tail suspension test; FST, forced swim test.

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pathway, Numb is involved in multiple important cellular events, such as cell polarity maintenance and asymmetric cell division (Pece et al., 2011; Salcini et al., 1997; Santolini et al., 2000).

In the mammalian central nervous system (CNS), during the process of embryonic neurogenesis of neocortex, *Numb* and *Numblike* are required for the neural progenitor cell maintenance and asymmetric cell division (Bultje et al., 2009; Li et al., 2003; Liu et al., 2015; Petersen et al., 2002, 2004; Rasin et al., 2007; Shen et al., 2002). High and persistent expression of Numb protein in postmitotic neurons suggests its subsequent essential roles. In vitro works revealed that, during the processes of neuronal morphologic development, *Numb*/*Numblike* are required for neurite growth, axonal extension and dendritic spine morphogenesis (Nishimura et al., 2003, 2006; Sestan et al., 1999). In vivo studies also showed that, in sensory neurons, target deletion of *Numb* and *Numblike* causes severe reduction in axonal arborization (Huang et al., 2005). Besides, *Numb* and *Numblike* are implicated in BDNF-induced cerebellar granule cell precursors migration (Zhou et al., 2011). Intriguingly, in adult mouse brain lysate, Numb and Numblike can bind to β-amyloid precursor protein (APP) (Roncarati et al., 2002), and a study on brain specimen from postmortal AD patients showed that expression pattern of Numb protein isoforms are aberrant in parietal cortex of these patients (Chigurupati et al., 2011).

These studies showed that Numb and Numblike play important roles in neuronal development, migration, maturation and perhaps degeneration. However, in vivo functions of *Numb*/*Numblike* in the CNS, especially in the forebrain, the major brain region responsible for emotional and cognitive abilities, are largely unknown. Thus, this study is designed to investigate roles of *Numb*/*Numblike* in the glutamatergic system of dorsal forebrain in the mice. Here, we employed conditional knockout strategy, using Cre-Lox recombination system, to specifically delete *Numb*/*Numblike* in the glutamatergic neurons. We chose *NEX-Cre* transgenic mouse line, in this line, Cre recombinase expression is under the control of the promoter of a transcript factor gene *NEX* (Fig. S1A), which is exclusively expressed in glutamatergic neurons in brain regions mainly include neocortex, hippocampus and amygdala (Goebbels et al., 2006). Therefore, by crossing *floxed-Numb*/*Numblike* mice (Wilson et al., 2007; Zilian et al., 2001) with *NEX-Cre* mice, we can obtain the conditional double knockout (CDKO) mice (Fig. S1B, C). In these CDKO mice, *Numb*/*Numblike* are selectively deleted in the glutamatergic neurons of neocortex, hippocampus and amygdala.

These CDKO mice are viable, fertile, and indistinguishable from their control (CTL) littermates. To evaluate behavioral consequence of *Numb*/*Numblike* deletions, here we performed a behavioral phenotyping to the CDKO mice. First, to avoid artifacts due to general health problems, we measured physical indexes such as neurological reflexes, sensory abilities, and motor functions, and the CDKO mice are normal in most of these indexes. Next, to assess anxiety-like behavior, a battery of tests, such as elevated zero maze, light/-dark exploration, and novel open field, were employed. Depression-related behavior was also assessed using forced swim test and tail suspension test. Other behaviors such as motor learning, startle response and sensorimotor gating were also tested as regular strategies.

2. Results

2.1. Selective and efficient elimination of Numb in the neocortex, amygdala and hippocampus in CDKO mice (Fig. 1)

The deletion efficiency was evaluated by Western blotting. The protein level of Numb markedly reduced in the brain lysate of cortex or hippocampus, as compared with which of CTL littermates, indicating sufficient deletion efficiency (Fig. 1A). Note that Numb is widely expressed in different cell types in the CNS (Nishimura et al., 2006; Zhou et al., 2011), the residual protein levels of Numb in the brain lysate of CDKO mice were largely due to its existence in other undisturbed cell types, such as interneuron and glia cells. Next, to confirm the expression pattern of *NEX-Cre*, we crossed CDKO mice with *LacZ* reporter mouse line (Fig. S1D). The LacZ staining of brain section of *Nex-Cre;Numb$^{F/F}$;Numblike$^{F/F}$;LacZ* mice clearly showed that the expression of *NEX-Cre* was properly restricted in the dorsal forebrain regions, including cortex, hippocampus and amygdala (Fig. 1B), which was consistent with previous report (Goebbels et al., 2006). Furthermore, immunofluorescent staining was applied to determine the expression pattern of Numb protein in various brain tissues, such as neocortex, hippocampus, amygdala, striatum and cerebellum (Fig. 1C). Numb shows wide expression across forebrain and cerebellum in the brain of CTL mice, and its expression level is especially high in neocortex, pyramidal and granular cell layers of hippocampus, amygdala, striatum, and Purkinje cell layers of cerebellum. However, in brain sections of CDKO mice, Numb expression reduces markedly in neocortex, hippocampus, and amygdala, but is not altered in striatum and cerebellum (Fig. 1C), demonstrating that *NEX-Cre* mediated knock out effectively reduces Numb expression in the neocortex, hippocampus and amygdala, but does no affects which in striatum and cerebellum.

Thus, taken together, these results showed that *Numb* was selectively and efficiently deleted in the neocortex, hippocampus and amygdala in our CDKO mice, rendering them suitable for following behavioral research.

2.2. No obvious cortical developmental deficits are found in CDKO mice (Fig. S2)

Considering *NEX-Cre* mediated deletion of *Numb/Numblike* occurs shortly after newborn neurons establish their neuronal identity, and starts from around embryonic day 11.5 in the brain, when first wave of newborn neurons emerges, we wondered whether this deletion would cause abnormal migration or morphogenesis in these knock out neurons, which would ultimately leads to cortical developmental defects. So we compared cortical structures between two genotypes from early postnatal to adult mice. Using DAPI staining of brain sections, we found that, at each early postnatal stage tested (P0.5, P10.5, and P15.5), global cortical structures are comparable between two genotypes (Fig. S2A). At adult stage (P120), Nissl staining of brain sections also showed that cortical structures appear grossly normal in CDKO mice brain, as compared with CTL littermates (Fig. S2B). Cortical layer 5 pyramidal neurons are the major excitatory output glutamatergic neurons in the neocortex, to see whether organizational structures of this layer are normal in the cortex of CDKO mice brain, we crossed mice of both genotypes with *Thy1-EGFP* transgenic mice (Feng et al., 2000), in which neocortical layer 5 pyramidal neurons are specifically illuminated with EGFP. We found that the structural patterns of cortical layer 5 are rather similar between two genotypes (Fig. S2C). To see whether neuronal morphology is normal in glutamatergic neurons of CDKO mice, we performed Golgi staining in adult mouse brains of both genotypes. Hippocampal CA1 pyramidal neurons were selected for imaging and morphological analysis. Dendritic morphology is grossly normal in CDKO mice (Fig. S3A), and total dendritic lengths of these pyramidal neurons are comparable in CDKO mice (Fig. S3B). Besides, dendritic density is also similar between CTL and CDKO mice (Fig. S3C, D).

Taken together, these results suggested that deletion of *Numb/Numblike* in glutamatergic neurons in the dorsal forebrain does