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GRM7 Regulates Embryonic Neurogenesis via CREB and YAP

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SUMMARY

Metabotropic glutamate receptor 7 (GRM7) has recently been identified to be associated with brain developmental defects, such as attention deficit hyperactivity disorder (ADHD) and autism. However, the function of GRM7 during brain development remains largely unknown. Here, we used gain- and loss-of-function strategies to investigate the role of GRM7 in early cortical development. We demonstrate that *Grm7* knockdown increases neural progenitor cell (NPC) proliferation, decreases terminal mitosis and neuronal differentiation, and leads to abnormal neuronal morphology. GRM7 regulates the phosphorylation of cyclic AMP response element-binding protein (CREB) and the expression of Yes-associated protein (YAP) by directly interacting with CaM, which subsequently regulates the expression of *CyclinD1* and ultimately affects early cortical development. These defects in neurogenesis are ameliorated by *Grm7* overexpression, *Creb* knockdown, or *Yap* knockdown. Thus, our findings indicate that GRM7 signaling via CREB and YAP is necessary for neurogenesis in the brain.

INTRODUCTION

The complex structure of the mammalian cerebral cortex is derived from neuroepithelial (NE) cells in the neural tube (McConnell, 1995). NE cells give birth to multiple progenitor populations (Götz and Huttner, 2005; McConnell, 1995). There are two germinal zones in the embryonic neocortex: the ventricular zone (VZ) and the subventricular zone (SVZ) (Gal et al., 2006). Radial glial (RG) cells give rise to self-renewing cells and produce intermediate progenitor (IP) cells via asymmetrical division. IP cells subsequently divide into two neurons via symmetrical division (Götz and Huttner, 2005; McConnell, 1995; Rakic, 1995). During the process of progenitor cell transformation into mature neurons, the precise control of the timing of self-renewal, differentiation, neuronal migration, and neuronal maturation of neural progenitor cells (NPCs) is required (Xu et al., 2014). Therefore, it is not surprising that mistakes in this process of early cortical development lead to serious consequences, such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD).

Metabotropic glutamate receptor 7 (GRM7) is defined as an ASD- (Yang and Pan, 2013) and ADHD-related gene (Elia et al., 2012) and is exclusively expressed in the CNS (Bradley et al., 1996). Metabotropic glutamate receptors are potential targets for neuropsychiatric disorders (Dev, 2004) that modulate neurotransmitter release and neuronal excitability (Schlett, 2006). Metabotropic glutamate receptors are subdivided into groups I (GRM1 and GRM5), II (GRM2 and GRM3), and III (GRM4, GRM6, GRM7, and GRM8) on the basis of homology, intracellular messengers, and ligand selectivity (Schlett, 2006). Characteristic of all metabotropic glutamate receptors, the GRM7 protein is localized to the neuronal presynaptic membrane, and its protein sequence is highly conserved (Bradley et al., 1996). These findings suggest that GRM7 may play an important and irreplaceable role in the nervous system. However, its role in the process of cortical development is unclear.

During neurogenesis, cyclic AMP response element-binding protein (CREB) is involved in multiple aspects of neuronal development and plasticity, including cell survival, proliferation, and differentiation (Mantamadiotis et al., 2012). CREB is expressed throughout neurogenesis (Giachino et al., 2005), and a previous study has shown that neural proliferation defects result from the alteration of CREB activity during early development (Dworkin et al., 2007). Yes-associated protein (YAP) modulates organ size by regulating cell apoptosis and proliferation (Cai et al., 2010; Lian et al., 2010). YAP is expressed in mitotic neuronal progenitors, and it is downregulated during neuronal differentiation (Zhang et al., 2012). The phosphorylation of YAP at Ser127 results in a loss of function and the subsequent repression of downstream target genes, leading to premature neuronal differentiation (Cao et al., 2008). In the absence of inhibitory phosphorylation, YAP promotes cell proliferation and suppresses cell differentiation (Zhang et al., 2012). During neurogenesis, CYCLIND1 plays an important role in neural progenitor proliferation; when CYCLIND1 is constitutively activated, the proliferation of NPCs is increased (Das et al., 2010). To investigate the function of GRM7 in early cortical development, we downregulated its expression in neuronal progenitor cells of the cerebral ventricle of embryos via in utero electroporation (IUE). We determined that *Grm7* knockdown increases the proliferation of PAX6-positive RG cells, decreases the amplification of TBR2-positive IP cells, and

results in a reduction in the number of progenitor cells that differentiate into neurons. Furthermore, morphological maturation was seriously affected by the silencing of *Grm7*. We also demonstrated that *Creb* or *Yap* knockdown ameliorates the *Grm7* knockdown phenotype in vivo. Overall, our findings suggest that GRM7 regulates the phosphorylation of CREB and the expression of YAP in neuronal progenitor cells, affecting the expression of *CyclinD1*, which ultimately controls neuronal differentiation and maturation during cortical development.

RESULTS

GRM7 Is Expressed in Neural Progenitor Cells

During cortical development, GRM7 displays a specific temporal and spatial expression pattern. To determine whether the GRM7 protein is expressed in brain tissue during different developmental periods, we performed western blot to analyze its expression pattern. Our results showed that the GRM7 protein expressed was already detectable at embryonic day 12.5 (E12.5), gradually increased until E15.5, and then dramatically decreased between E15.5 and E18.5 (Figure 1A). This finding suggests that GRM7 may play an important role in early embryonic cortical development. Neocortical sections of E12.5, E15.5, and E18.5 mouse brains were collected and immunostained for GRM7, and the results showed that its expression pattern was dynamic in the VZ/SVZ. At E12.5, in which the cerebral cortex is primarily composed of NPCs, we determined that the GRM7 protein was expressed throughout the cortex. At E15.5, GRM7 protein was highly expressed in the VZ/SVZ. However, at E18.5, which is near the end of the embryonic neurogenesis period, the expression of GRM7 in the cerebral cortical plate was higher than that in the VZ/SVZ (Figure 1B). In the embryonic cerebral cortex, we determined that the GRM7 protein colocalized with NESTIN- and PAX6-positive neuronal progenitor cells residing in the VZ/SVZ at E15.5 (Figures 1C and 1D). Moreover, we determined that GRM7 colocalized with NESTIN- and PAX6-positive cultured neuronal progenitor cells, which were isolated from E12.5 mouse brains and cultured in proliferative medium for 24 hr (Figures S1A and S1B). Additionally, based on in situ hybridization, *Grm7* was expressed in the VZ/SVZ of the neocortex (Figure S1C). For further experiments, we generated two *Grm7*-specific short hairpin RNA (shRNA) (*Grm7*shRNA-1 and *Grm7*shRNA-2) plasmids and a *Grm7* overexpression plasmid to efficiently silence or overexpress GRM7 expression, respectively, in embryonic NPCs (Figures 1E and 1F).

Grm7 knockdown decreased the immunostaining for GRM7 in E16.5 brain sections following the electroporation of the *Grm7*shRNA-1 or *Grm7*shRNA-2 plasmid into the embryonic brains at E13.5 (Figures 1G and 1H). A similar result was obtained in cultured NPCs transfected with these knockdown plasmids in vitro (Figure S1D).

Overall, the specific temporal and spatial expression pattern of GRM7 suggests that GRM7 may be involved in the regulation of the proliferation and differentiation of NPCs during early cortical development.

GRM7 Regulates Neural Progenitor Proliferation in the Brain

To study the functional role of GRM7 in neurogenesis, we investigated NPCs proliferation in vivo based on its expression in neural progenitor cells via IUE. The control or *Grm7* shRNA plasmid was electroporated into E13.5 mouse brains, which were collected 72 hr later for phenotypic analysis. Analysis of the distribution of GFP-positive cells revealed that *Grm7* knockdown caused an augmentation of GFP-positive cells in the VZ/SVZ and a corresponding reduction in GFP-positive cells in the cortical plate (CP). In the intermediate zone (IZ), the GFP-positive cell number did not change (Figures 2A and 2B).

To further explore the effects of GRM7 on NPC proliferation, we injected pregnant dams with bromodeoxyuridine (BrdU) 2 hr prior to collecting the electroporated brains. The knockdown of *Grm7* led to an increase in GFP and BrdU double-positive neural progenitor cells (Figures 2C and 2D) and in the mitotic index in the VZ/SVZ (Figures S2A and S2B). We employed an in vitro culture system to assess the function of GRM7 in neuronal proliferation. Neural progenitor cells were isolated from E12.5 mouse brains and cultured for 1 day; then, the cells were infected with either control or *Grm7* shRNA lentivirus. The infected cells were cultured for 3 days under proliferative conditions. Then, 5-ethynyl-2′-deoxyuridine (EdU) was added to the culture medium for 2 hr. The quantification of GFP and EdU double-positive cells revealed that the knockdown of *Grm7* in vitro increased neuronal proliferation compared with the control treatment (Figure S2C).

To investigate the specific cell groups of proliferative progenitor cells in the VZ/SVZ, we selected two types of neural progenitor cell markers: PAX6 and TBR2. During the process of early brain development, PAX6-positive RG cells divide asymmetrically to self-renew and generate TBR2-positive IP cells or neurons. Subsequently, IP cells divide symmetrically to produce two neurons (Götz and Huttner, 2005). Our results showed that *Grm7* knockdown significantly increased the proportion of PAX6-positive RG cells (Figures 2E and 2F) at the expense of TBR2-positive IP cells (Figures 2G and 2H). We also determined that *Grm7* knockdown had no effect on the apoptosis of neural progenitor cells (Figure S3A). Further analysis showed that the proportion of GFP-BrdU-PAX6-positive cells was increased (Figures S3B and S3C) but that the proportion