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miR-17-92 cluster regulates adult hippocampal neurogenesis, anxiety and depression

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Summary

Emerging evidence has shown that noncoding RNAs, particularly microRNAs (miRNAs), contribute to the pathogenesis of mood and anxiety disorders, although the molecular mechanisms are poorly understood. Here we show altered levels of miR-17-92 in adult hippocampal neural progenitors have a significant impact on neurogenesis and anxiety- and depression-related behaviors in mice. miR-17-92 deletion in adult neural progenitors decreases neurogenesis in the dentate gyrus, while its overexpression increases neurogenesis. miR-17-92 affects neurogenesis by regulating genes in the glucocorticoid pathway, especially serum- and glucocorticoid-inducible protein kinase-1 (Sgk1). miR-17-92 knockout mice show anxiety- and depression-like behaviors, whereas miR-17-92 overexpressing mice exhibit anxiolytic and antidepression-like behaviors. Furthermore, we show that miR-17-92 expression in the adult mouse hippocampus responds to chronic stress, and miR-17-92 rescues proliferation defects induced by corticosterone in

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Author Contributions

JJ, YK, and TS designed experiments. JJ did *in situ* hybridizations, analyzed mouse model phenotypes, performed tissue collection, immunostainings and cell counting for neurogenesis assays, and behavioral tests. SK did immunostainings and cell counting for all Sgk1 expression, treated mice with stress and antidepressant. XL performed HT-22 cell culture experiments. HZ performed RT-PCR analysis and luciferase experiments. JS performed the restraint stress test. CZ performed RNA-Seq analyses and GO analyses. The manuscript was written by JJ, YK, and TS. TS supervised the project.

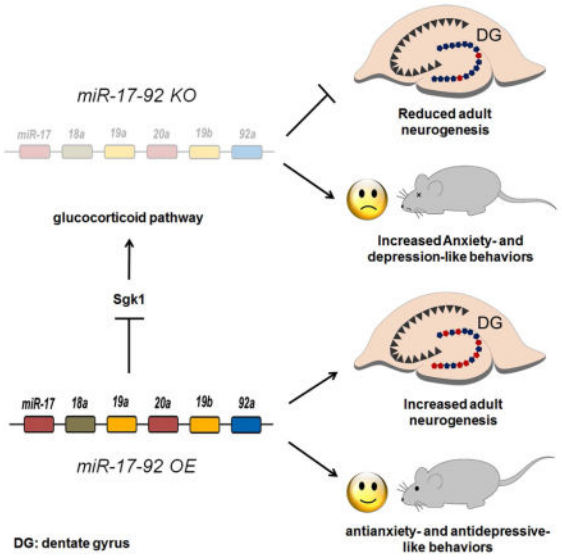
RNA sequencing (RNA-Seq) data

All RNA-Seq data from the adult hippocampus of wild type and miR-17-92 knockout mice were deposited at the GEO website. The GEO accession number is GSE83636.

hippocampal neural progenitors. Our study uncovers a crucial role for miR-17-92 in adult neural progenitors through regulation of neurogenesis and anxiety- and depression-like behaviors.

eTOC blurb

The molecular pathogenesis of anxiety and depression disorders is poorly understood. Jin et al. show that microRNA miR-17-92 plays a critical role in regulating adult hippocampal neurogenesis and anxiety- and depression-like behaviors by modifying expression of genes in the glucocorticoid pathway.



Keywords

miR-17-92; adult hippocampal neurogenesis; anxiety; depression; Sgk1

Introduction

Mood and anxiety disorders are known to be associated with many risk factors, such as environmental, genetic and epigenetic factors, making the investigation of pathophysiology of these disorders extremely challenging (Krishnan and Nestler, 2008). Antidepressants are used for the treatment of both depression and anxiety disorders, but only 30%–50% of patients show amelioration (Gaynes et al., 2008). Thus, it is essential to better understand the etiology of the disorders and to develop new targets for improved therapies.

The dentate gyrus (DG) in the hippocampus is one of the neurogenic zones in the adult brain. The DG is populated by neural stem cells and neural progenitors, and is continuously generating new neurons (Ming and Song, 2011; Zhao et al., 2008). The impact of adult neurogenesis in the function of hippocampus has drawn significant attentions (Deng et al., 2010; Jacobs et al., 2000; Kheirbek et al., 2012; Sahay and Hen, 2007). While some studies demonstrate the association of adult neurogenesis with depression and anxiety (Kheirbek et al., 2012; Sahay and Hen, 2007), some other reports show inconsistent outcomes (Petrik et

al., 2012). Even though the neurogenic theory of depression and anxiety appears controversial, it is still plausible and holds possibility for the adult hippocampal neurogenesis as a therapeutic means for mood and anxiety disorders (Eisch and Petrik, 2012; Hill et al., 2015; Miller and Hen, 2014). Thus, there is an unmet need for research to identify molecules that regulate both hippocampal neurogenesis and depression- and anxiety-like behaviors.

MicroRNAs (miRNAs) normally silence protein-coding target genes and play crucial roles in many aspects of neural development and neurological disorders (Bian and Sun, 2011; Fineberg et al., 2009; Kosik, 2006; Qureshi and Mehler, 2012). Recent studies begin to reveal the contribution of miRNAs in the pathogenesis of mood and anxiety disorders (Issler et al., 2014; Lopez et al., 2014; Malan-Muller et al., 2013; Xu et al., 2012). However, little is known about specific miRNAs being involved in the regulation of adult hippocampal neurogenesis, and/or mood and anxiety behaviors.

The miR-17-92 cluster has been shown to be involved in embryonic brain development and tumorigenesis (Bian et al., 2013; Olive et al., 2010). In this study, we identified the miR-17-92 cluster highly expressed in the adult mouse hippocampus. We found that miR-17-92 regulates adult hippocampal neurogenesis and mood and anxiety-related behaviors in mice. We further established the regulatory role of miR-17-92 in modifying genes in the glucocorticoid pathway including Sgk1, and rescuing reduced proliferation, caused by corticosterone, in hippocampal progenitors. Our studies indicate that the miR-17-92 cluster is a pivotal regulator of adult hippocampal neurogenesis, and anxiety- and depression-like behaviors.

Results

Altering adult hippocampal expression of miR-17-92 in *miR-17-92 KO* and *OE* mice

Previous studies suggested a role of the miR-17-92 cluster in embryonic neural stem cell development and proliferation of cancer cells (Bian et al., 2013; Olive et al., 2010), its involvement in adult brain neurogenesis and function is unknown. Transcription of the miR-17-92 cluster generates 6 miRNAs that can be grouped into 4 subfamilies (Mendell, 2008), miR-17 (and -20a), miR-18a, miR-19a (and -19b) and miR-92a, based on their conserved sequences (Figure 1A). To examine expression patterns of miR-17-92, we performed *in situ* hybridization in 12 weeks old adult mouse brains using locked nucleic acid (LNA) probes for miR-17 (which also recognizes 20a), -19a (which also recognizes 19b) and -92a. All three subfamilies of the miRNA were expressed in the adult hippocampus and in the cortex (Figure 1B).

Because miR-17-92 has been shown to regulate proliferation in embryonic neural stem cells and cancer cells (Bian et al., 2013; Olive et al., 2010), we speculated that they might be involved in adult hippocampal neurogenesis and function. To examine the possible role for miR-17-92 in maintaining progenitor cells in adult hippocampus, we generated conditional *miR-17-92* knockout (*miR-17-92 KO*) mice and *miR-17-92* overexpressing (*miR-17-92 OE*) mice. We utilized a *Nestin-CreER* line (Kuo et al., 2006), in which the Cre recombinase is expressed in neural progenitors in the adult brain upon tamoxifen injection. The *Nestin-*

CreER mice were bred with floxed *miR-17-92* knockout mice (Ventura et al., 2008) or floxed *miR-17-92* overexpressing mice (Xiao et al., 2008), and tamoxifen was injected at the age of 5-weeks (Figures 1C, 1D, and S1A). To test altered miR-17-92 expression, real-time quantitative Reverse Transcription PCR (qRT-PCR) was performed. Expression levels of miR-17, -18, -19a and -92 were decreased in hippocampus of *miR-17-92 KO* mice, while they were increased in hippocampus of *miR-17-92 OE* mice, suggesting expected alteration of miR-17-92 in the adult hippocampus (Figures S2A and S2B).

miR-17-92 KO and *OE* mice showed normal brain morphology (Figure 1E). In addition, *miR-17-92 KO* mice displayed slightly reduced body weight but normal brain weight compared to wild type control littermates (Figure 1F). *miR-17-92 OE* mice were indistinguishable from controls in brain and body weights (Figure 1F). Furthermore, we measured the thickness of the cortical wall. It did not show difference among control, *miR-17-92 KO* and *OE* mice (Figures S2C-E). These results suggest that altered miR-17-92 expression in neural progenitors in the adult brain has minimal effects on brain and body growth.

Adult hippocampal neurogenesis is altered in *miR-17-92 KO* and *OE* mice

Even though brain weights were not changed in *miR-17-92 KO* and *OE* mice, altered miR-17-92 expression might affect neurogenesis in the adult hippocampus. We thus investigated generation of adult hippocampal neural progenitors and newborn neurons in *miR-17-92 KO* and *OE* mice. To detect proliferative neural progenitors, bromodeoxyuridine (BrdU) was administered in the 12 weeks old mice, 6 days before brain tissue collection (Figure S1A). The number of $BrdU^+$ cells was reduced in the dentate gyrus of the *miR-17-92 KO* hippocampus (Figures 2A and 2B), but increased in *OE* mice (Figures 2C and 2D). Moreover, the number of cells expressing Ki67, which indicates progenitors in the G1-, S-, G2-, and M-phases of the cell cycle, also was decreased in the *miR-17-92 KO* (Figures 2E and 2F) but increased in the *OE* hippocampus (Figures 2G and 2H). These results indicate that miR-17-92 function is required for maintaining proliferative neural progenitors in the adult hippocampus.

We also examined the number of Doublecortin (Dcx) expressing newborn neurons. Consistent with changes in the number of neural progenitors, the number of Dcx^+ cells was reduced in the DG of the *miR-17-92 KO* hippocampus (Figures 2I and 2J), but increased in *OE* mice (Figures 2K and 2L), suggesting that generation of newborn neurons is impaired. Moreover, the number of $BrdU^+$ or $NeuN^+/BrdU^+$ cells at 13 weeks old, which were labelled by BrdU injection at 7 weeks old to detect newborn neurons (Figure S1B), also was decreased in the *miR-17-92 KO* hippocampus (Figures S3A and S3B). Taken together, our results indicate that miR-17-92 is required to maintain proliferative neural progenitors and to generate new neurons in the adult hippocampus.

miR-17-92 KO and *OE* mice exhibit elevated anxiety- or anti-anxiety-like behaviors

Previous studies have shown that adult hippocampal neurogenesis is associated with mood and anxiety disorders (Eisch and Petrik, 2012; Miller and Hen, 2014). Because altered expression of *miR-17-92* caused changes in hippocampal neurogenesis, we examined

whether anxiety-related behaviors are affected in *miR-17-92 KO* and *OE* mice (Figure S1C). In the open field test, *miR-17-92 KO* mice and *OE* mice showed comparable locomotor activity as measured by total distance compared to their wild type controls (Figures S3C). However, *miR-17-92 KO* mice displayed markedly less frequency of stay, less time spent, and less distance moved in the center of the open field compared to wild type controls (Figure 3A). On the other hand, *miR-17-92 OE* mice exhibited opposing behaviors (Figure 3B). Moreover, in the elevated plus maze test, *miR-17-92 KO* mice showed significantly less duration and less distance travelled in open arms (Figure 3C), while *miR-17-92 OE* mice showed a significantly greater frequency of stay and longer total distance moved in open arms (Figure 3D). These results indicate that *miR-17-92* deletion or overexpression in mice results in elevated anxiety-like or antianxiety-like behaviors, respectively.

Altered expression of miR-17-92 in the hippocampus regulates depression-like behaviors

Adult hippocampal neurogenesis has been shown to be related to depression (Sahay and Hen, 2007), we also examined depression-like behaviors in *miR-17-92 KO* and *OE* mice. In the forced swim test, *miR-17-92 KO* mice displayed significantly increased immobility time (Figure 3E). In the tail-suspension test, the immobility time was also increased in *miR-17-92 KO* mice, suggesting that *miR-17-92 KO* mice exhibit elevated depression-like behaviors (Figure 3F). Moreover, in the sucrose preference test, *miR-17-92 KO* mice showed reduced intake of sucrose, suggesting anhedonia-like behavior (Figure 3G). Conversely, in both the forced swim and tail-suspension tests, *miR-17-92 OE* mice had greatly reduced immobility time, compared to their controls, suggesting an antidepression-like effect of miR-17-92 (Figures 3H and 3I). In sucrose preference test, *miR-17-92 OE* mice did not show detectable difference compared to controls (Figure 3J). These results altogether indicate that miR-17-92 deletion or overexpression in the adult neural progenitors results in increased or decreased anxiety- and depression-like behaviors.

The glucocorticoid pathway is affected in *miR-17-92 KO* hippocampus

miRNAs exert their functions by silencing target genes. To investigate miR-17-92 regulation mechanisms, we next quantified gene expression levels in the hippocampus of *miR-17-92 KO* and wild type mice using Illumina RNA-sequencing (RNA-Seq) to identify genes with altered expression when *miR-17-92* is knocked out (Figures 4A and S1D). We identified 504 genes significantly altered in the hippocampus of *miR-17-92 KO* mice (false discovery rate <0.1). Among them, 229 genes were upregulated, suggesting that they are usually directly or indirectly silenced by miR-17-92, and 78 out of 229 genes were predicted targets of miR-17-92 (Table S1). Gene ontology (GO) analyses of the 504 genes with altered expression in *miR-17-92 KO* hippocampus revealed that many of them regulate cell proliferation and differentiation (Figure S4A). This finding is consistent with the function associated with the 229 upregulated genes, including predicted targets of the miR-17-92 cluster (Figure S4B).

Moreover, using four target prediction tools (Targetscan, miRDB, DIANA-microT and TarBase), we identified 150 genes, which are either up- or down-regulated, as predicted targets of the miR-17-92 cluster (Table S1). Interestingly, a detailed GO analysis of the 150 predicted target genes revealed their involvement in glucocorticoid-related functions in the

absence of miR-17-92 (Figure 4B). Among them, the kinase Sgk1 (Lang et al., 2010) was found to have a significant upregulation in the *miR-17-92 KO* hippocampus (about 1.3 fold increase), and to be involved in 6 out of the top 10 predictable major biological functions that are associated with the 150 target genes (Figure 4B). Sgk1 is known as a downstream effector of the glucocorticoid receptor (GR) (Anacker et al., 2013). Functional interaction analysis of Sgk1 with 504 genes that showed altered expression in the *miR-17-92 KO* hippocampus further indicated that Sgk1, as a crucial modulator in the glucocorticoid pathway, interacts with genes directly involved in cell cycle control, developmental process and neurogenesis (Figure 4C). Our results indicate the regulatory effect of the miR-17-92 cluster on the glucocorticoid pathway.

The expression level of Sgk1 is specifically increased in *miR-17-92 KO* hippocampus

To further test whether Sgk1 is a target of the miR-17-92 cluster, we searched the *Sgk1* 3'untranslated region (3'UTR), where miRNAs typically bind to silence target gene expression. We found that *Sgk1* 3'UTR contains binding sites for miR-19a and miR-92a (Figure 5A). We cloned the *Sgk1* 3'UTR into a luciferase vector. When the *Sgk1* 3'UTR-luciferase was co-expressed with miR-19a or miR-92a, wild type but not their mutants significantly reduced the relative luciferase activity, suggesting that miR-19a and miR-92a have specific targeting effects on the *Sgk1* 3'UTR (Figure 5B).

We next examined the expression of Sgk1 in the DG of 13 weeks old wild type and *miR-17-92 KO* mice. Scattered $Sgk1^+$ cells were observed in the subgranular zone (SGZ) and the granule cell layer (GCL) in the DG of wild type and *miR-17-92 KO* hippocampus (Figure 5C). Interestingly, the number of $Sgk1^+$ cells was significantly increased in the SGZ in *miR-17-92 KO* mice (Figures 5D and 5E). Moreover, $Sgk1^+$ cells were also detected in the polymorphic layer (PML) (Scharfman and Myers, 2012), the area between the upper and lower GCLs (Figure S5A). The number of $Sgk1^+$ cells also was increased in the PML in *miR-17-92 KO* mice (Figures S5B and S5C). However, the number of cells expressing GR, an upstream gene of Sgk1 (Anacker et al., 2013), didn't show significant difference in the DG of control and *miR-17-92 KO* mice (Figures S5D and S5E). These results, consistent with RNA-seq data, indicate that *miR-17-92* knockout in neural progenitors results in elevated level of Sgk1 expression in the DG.

To test whether the number of $Sgk1^+$ cells also is increased in other brain regions in *miR-17-92 KO* mice, we examined Sgk1 expression in the amygdala, prefrontal cortex and hypothalamus. Sgk1 expression was detected in the amygdala, but the number of $Sgk1^+$ cells did not show detectable change between *WT* control and *miR-17-92 KO* mice (Figure 6A). In addition, Sgk1 expression was not detected in the prefrontal cortex and hypothalamus (Figures 6B and 6C). Moreover, the overall neurogenesis, labeled by $NeuN^+$ cells, also did not show significant changes in the amygdala, prefrontal cortex and hypothalamus (Figure 6). Our results suggest that elevated expression of Sgk1 in the hippocampus in *miR-17-92 KO* mice is brain region specific.

miR-17-92 rescues stress induced proliferation defect in hippocampal neural progenitors

A large body of studies has shown that chronic stress alters the release of adrenal glucocorticoid hormones and in turn affects hippocampal neurogenesis, and has a detrimental influence on learning and memory and mood behaviors (Joels, 2008; McEwen, 2001; Saaltink and Vreugdenhil, 2014; Schoenfeld and Gould, 2012). Because we found that miR-17-92 regulates the glucocorticoid pathway, we next examined whether chronic stress also has a direct effect on miR-17-92 expression in the hippocampus. 8 weeks old mice were subjected to restraint stress for 2 weeks, while being administered an antidepressant (antidep) fluoxetine, or vehicle as a control (Seo et al., 2012). Consistent with previous reports (Anacker et al., 2013; Saaltink and Vreugdenhil, 2014), expression levels of *GR* and *Sgk1* were increased due to stress and rescued by antidepressant treatment (Figure S6A). On the contrary, chronic stress significantly reduced expression levels of *miR-17*, *-18*, *-19a* and *-92a* in the hippocampus, compared to their controls, and the reduction was prevented by antidepressant treatment (Figure S6B). These results suggest that hippocampal expression of miR-17-92 is responsive to chronic stress and antidepressant.

To further test the effect of miR-17-92 on proliferation of hippocampal neural progenitors under stress treatment, we used HT-22 cells, which are immortalized mouse hippocampal neurons (Davis and Maher, 1994). HT-22 cells were treated with corticosterone (CORT) and the vehicle control (Malviya et al., 2013), and were then transfected with the pCAGIG vector containing a green fluorescent protein (GFP) reporter gene, and pulsed with BrdU for 2 hours. CORT-treatment significantly reduced $BrdU^+/GFP^+$ cells, compared to the vehicle control (Figures S6C and S6D). Furthermore, miR-19a and miR-92a precursors were cloned into the pCAGIG vector and transfected into CORT-treated HT-22 cells (Bian et al., 2013). Compared to those transfected with the empty pCAGIG vector, $BrdU^+/GFP^+$ cells were significantly increased upon miR-19a and miR-92a transfection (Figures S6C and S6D). These results suggest that miR-19a and miR-92a in the miR-17-92 cluster rescues proliferation defect in hippocampal neural progenitors caused by corticosterone.

miR-17-92 mediates antidepressant- and stress-regulated adult hippocampal neurogenesis *in vivo*

We found that *miR-17-92 KO* and *OE* mice show altered hippocampal neurogenesis. We wanted to examine whether there is a direct causal relationship between miR-17-92 expression and hippocampal neurogenesis defects. Previous studies have shown that antidepressant treatment has a positive impact on adult hippocampal neurogenesis (Boldrini et al., 2009; Malberg et al., 2000). We then tested whether antidepressant-regulated neurogenesis is mediated by miR-17-92. Wild type control and *miR-17-92 KO* mice were administered the antidepressant fluoxetine for 2 weeks. Consistent with previous reports, elevated neurogenesis was observed in control and *miR-17-92 KO* hippocampus (Figures 7A–C). However, the degree of increased neurogenesis was significantly restrained by miR-17-92 knockout (Figure 7D).

Furthermore, studies have shown that chronic stress has a negative effect on hippocampal neurogenesis (Joels, 2008; McEwen, 2001; Saaltink and Vreugdenhil, 2014; Schoenfeld and Gould, 2012). We next examined whether overexpression of miR-17-92 protects stress-

induced decrease of adult hippocampal neurogenesis. Wild type control and *miR-17-92 OE* mice at 8 weeks old were subjected to restraint stress for 2 weeks (Seo et al., 2012). While stress caused a reduction in neurogenesis in the control hippocampus, its effect on *miR-17-92 OE* hippocampus was subtle, suggesting that overexpression of miR-17-92 rescues stress induced neurogenesis defect (Figures 7E–H).

Sgk1 expression displays opposite change compared to hippocampal neurogenesis upon antidepressant and stress treatment

If miR-17-92-mediated adult hippocampal neurogenesis is partly regulated through their target gene Sgk1, changes in Sgk1 expression and neurogenesis should be opposite. We examined Sgk1 expression in the hippocampus of wild type control and *miR-17-92 KO* mice that are administered the antidepressant fluoxetine. In the control hippocampus treated with antidepressant, the number of $Sgk1^+$ cells was not altered compared to untreated (Figure S7A). Moreover, while antidepressant treatment resulted in an increased neurogenesis (Figures 7A–D), it caused decreased number of $Sgk1^+$ cells in *miR-17-92 KO* hippocampus, compared to untreated *miR-17-92 KO* (Figure S7A).

Furthermore, while chronic stress caused a decreased neurogenesis in the control hippocampus (Figures 7E–H), it caused an increase in the number of $Sgk1^+$ cells (Figure S7B). Interestingly, in *miR-17-92 OE* mice treated with stress, miR-17-92 overexpression prevented a reduction in neurogenesis (Figures 7E–H), it caused a decrease in the number of $Sgk1^+$ cells, which further suggests a silencing effect of miR-17-92 on Sgk1 expression (Figure S7B). Moreover, miR-17-92 knockout caused a dramatic increase in the number of $Sgk1^+$ cells in the hippocampus of *miR-17-92 KO* mice (Figure S7B). These results indicate miR-17-92 targeting effect on Sgk1 *in vivo.*

Because increased neurogenesis and decreased Sgk1 expression were detected in the hippocampus of *miR-17-92 KO* mice treated with antidepressant, we further examined the behavioral outcome. The forced swim test and tail suspension test were performed in *miR-17-92 KO* mice treated with vehicle and antidepressant. *miR-17-92 KO* mice treated with antidepressant displayed reduced immobility time in both tests, compared to those treated with vehicle, suggesting that antidepressant treatment has a rescue effect on depression-like behaviors in *miR-17-92 KO* mice (Figures S7C and S7D).

Discussion

Identification of specific molecules that regulate anxiety- and depression-like behaviors would contribute to our better understanding of pathophysiology of mood and anxiety disorders. In this study, we have shown that miRNAs in the miR-17-92 cluster are highly expressed in the adult mouse hippocampus. Importantly, deletion or overexpression of miR-17-92 in adult neural progenitors causes decreased or increased hippocampal neurogenesis, resulting in elevated anxiety- and depression-like behaviors, or anxiolytic and antidepression-like behaviors, respectively. Our results suggest a molecular mechanism by which miRNAs are associated with adult hippocampal neurogenesis and mood and depression-like behaviors.

Adult hippocampal neurogenesis requires multiple steps including proliferation of neural progenitors, differentiation to neurons and survival of newborn neurons (Ming and Song, 2011; Zhao et al., 2008). Our results have shown that miR-17-92 plays a role in the maintenance of proliferative neural progenitors and the generation of newborn neurons. The role for miR-17-92 in the proliferation of neural progenitors in adult hippocampus is consistent with its demonstrated function in the maintenance of embryonic neural stem cells and the proliferation of various cancer cells (Bian et al., 2013; Olive et al., 2010). We have found that miR-17-92 knockout causes a reduction of antidepressant-induced increase in neurogenesis, while miR-17-92 overexpression is resistant to stress-induced reductions in neurogenesis, indicating a direct role of miR-17-92 in regulating hippocampal neurogenesis. Moreover, here we have identified that the expression levels of multiple genes, known to be involved in cell proliferation and differentiation, are altered in the hippocampus of *miR-17-92 KO* mice. Particularly, the alterations in the level of multiple genes that are implicated in glucocorticoid-mediated pathways are also observed in the hippocampus of *miR-17-92 KO* mice. Since glucocorticoid and its signaling are known to affect adult hippocampal neurogenesis (Joels, 2007), altered expression of target genes for miR-17-92 in glucocorticoid pathways may be responsible for impaired adult neurogenesis in *miR-17-92 KO* mice. Future studies should address which target genes for miR-17-92 contribute to which specific steps of the adult neurogenesis.

Recent studies have suggested an involvement of miRNAs in the regulation of anxiety- and depression-like behaviors. miR-16 was found to play a role in the actions of SSRI (selective serotonin reuptake inhibitor) by targeting serotonin transporter in serotonergic and noradrenergic neurons (Baudry et al., 2010). miR-135 was shown to target serotonin transporter and serotonin receptor-1a in the serotonergic neurons, and regulate anxiety- and depression-like behaviors and behavioral response to antidepressant treatment (Issler et al., 2014). miR-18 might negatively regulate the glucocorticoid pathway (Shimizu et al., 2015; Vreugdenhil et al., 2009). Glucocorticoid is associated with stress-induced anxiety and mood behaviors (McEwen, 2001; Pace and Miller, 2009). Previously, the increased level of Sgk1 was observed in peripheral blood of depressed patients (Anacker et al., 2013). In this study, we have identified Sgk1 as a direct target of miR-19a and miR-92a in the miR-17-92 cluster *in vitro* and *in vivo.* The increased Sgk1 expression, as a result of *miR-17-92* deletion, was observed in the SGZ of the DG but not in other brain regions. It is possible that the effects of *miR-17-92* deletion in hippocampal neurogenesis and mouse behaviors are, at least in part, mediated by increased Sgk1.

Furthermore, although we observed a correlation between the altered hippocampal neurogenesis and behavioral phenotypes in *miR-17-92 KO* or *OE* mice, the connection of adult hippocampal neurogenesis to anxiety- and depression-like behaviors remains controversial (Petrik et al., 2012; Samuels and Hen, 2011; Snyder et al., 2011). Nevertheless, because miRNAs are useful tools for the treatment of mood and anxiety disorders (O'Connor et al., 2012), the causal relationship observed in this study between altered levels of miR-17-92 and anxiety- and depression-like behaviors suggests that the miR-17-92 cluster has potential as a therapeutic target for these disorders.

Experimental procedures

Transgenic mouse lines

To delete the miR-17-92 cluster in the adult mouse brain, floxed *miR-17-92* transgenic mice (Ventura et al., 2008) (requested from the lab of Dr. Tyler Jacks, MIT, USA) were bred with the tamoxifen-inducible *Nestin-Cre* line, called *Nestin-CreER*^{tm} mice (Kuo et al., 2006) (requested from the lab of Dr. Yuh-Nung Jan, UCSF, USA), to generate *miR-17-92*$^{flox/flox}$:*Nestin-CreER*. *miR-17-92* knockout (*KO*) mice were generated after the injection of tamoxifen. To overexpress the miR-17-92 cluster in the adult brain, we employed transgenic mice with a floxed stop codon in front of the *miR-17-92* transgene (Xiao et al., 2008). Once bred with the *Nestin-CreER* line, the overexpression of the miR-17-92 cluster was induced by tamoxifen, called here *miR-17-92* overexpressing (*OE*) mice. Wild type mice used in this project were mice that carry homozygote floxed alleles but are Cre-negative. Tamoxifen was administered all genotyping mice to avoid tamoxifen drug effect.

Transgenic animals were maintained at the facility of Weill Cornell Medical College. Animal use was overseen by the Animal Facility and approved by the IACUC at the Weill Cornell Medical College. Experiments with chronic restraint stress were performed at The Rockefeller University, and the experimental procedure was approved by The Rockefeller University Institutional Animal Care and Use Committee, and was in accordance with guidelines of the National Institutes of Health.

RNA sequencing (RNA-Seq)

The adult hippocampi of the 13 weeks old mice were dissected from three wild-type and three *miR-17-92 KO* mice, each of them from a different litter. Total RNA was extracted and purified using the RNeasy mini kit with optional on-column DNAse digestion (Qiagen). Whole RNA sequencing was performed at the Weill Cornell Genomics Core Facility using an IlluminaHiseq 1000.

Statistics

All data were shown as means ± standard error of the mean (s.e.m.). Two group comparisons were done by two-tailed, unpaired Student's *t*-test. The influence of drug and stress treatment were assessed using two-way ANOVA with Bonferroni's *posthoc* tests.

Full Experimental procedures and associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- miR-17-92 knockout and overexpression alter adult hippocampal neurogenesis.
- Expression levels of miR-17-92 affect mood and anxiety-like behaviors in mice.
- miR-17-92 regulates genes in the glucocorticoid pathway and targets Sgk1.
- miR-17-92 rescues reduced hippocampal proliferation caused by corticosterone.

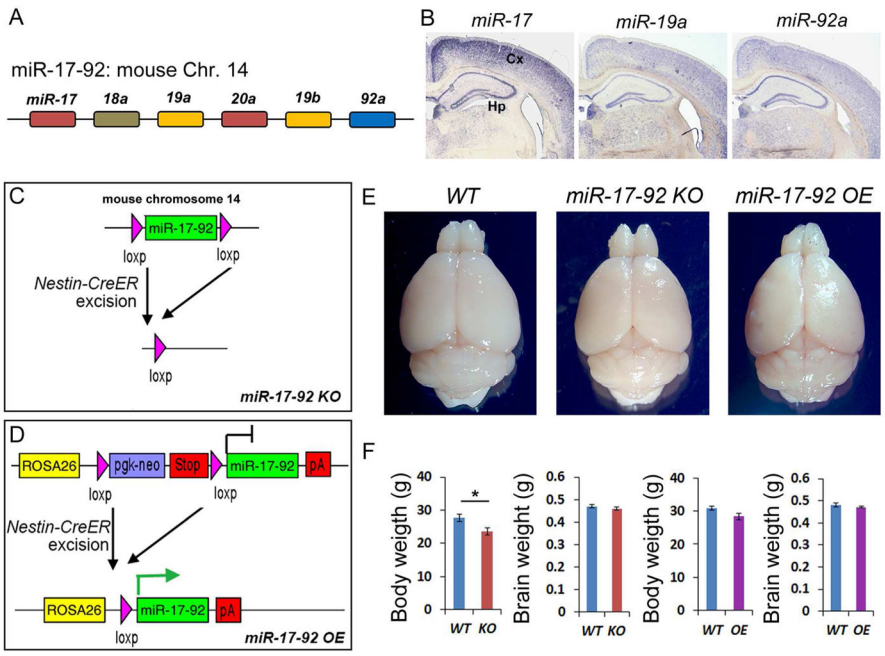

Figure 1. Altering adult hippocampal expression of miR-17-92 in *miR-17-92 KO* and *OE* mice

(A) Schematic genomic organization of miRNAs in the miR-17-92 cluster on mouse chromosome 14. The color code represents miRNAs with the conserved seed sequence.

(B) *miR-17* (*miR-20a*), *miR-19a* (*miR-19b*) and *miR-92a* were expressed in the 12 weeks old adult mouse hippocampus (Hp) and cortex (Cx).

(C and D) Generation of *miR-17-92* knockout (*KO*) and *miR-17-92* overexpressing (*OE*) mice using the *Nestin-CreER* line.

(E) The whole brain images of wild type (*WT*), *miR-17-92 KO*, and *miR-17-92 OE* mice at the age of 13 weeks old.

(F) The body weight of *miR-17-92 KO* was slightly reduced, and the body weight of *miR-17-92 OE* mice was not changed, compared to *WT* controls. Brain weights of *miR-17-92 KO* and *miR-17-92 OE* mice were indistinguishable from *WT* controls. Values plotted were means ± s.e.m. n=6 mice per group. *: $p < 0.05$. A two-tailed, unpaired Student's t-test was used for comparisons. See also Figures S1 and S2.

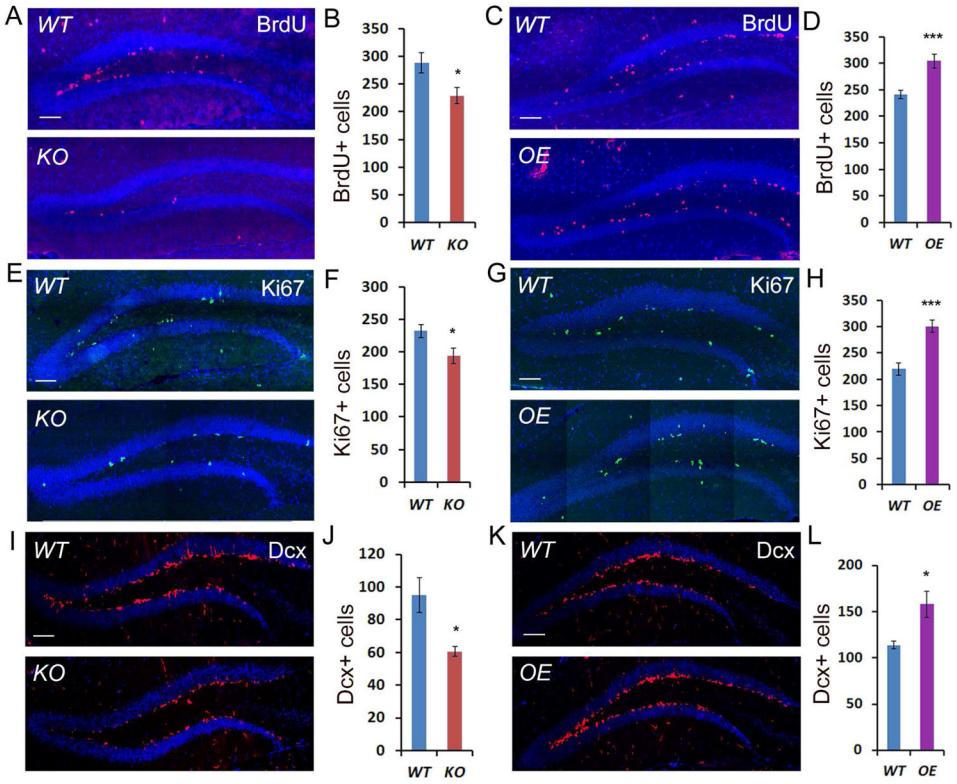

Figure 2. Deletion or overexpression of *miR-17-92* in adult neural progenitors causes altered hippocampal neurogenesis

(A and B) The number of $BrdU^+$ (red) proliferating cells was decreased in the dentate gyrus (DG) of 13 weeks old *miR-17-92* knockout (*KO*) mice, compared to wild type (*WT*) mice.

(C and D) The number of $BrdU^+$ cells was significantly increased in *miR-17-92* overexpressing (*OE*) mice.

(E and F) The number of $Ki67^+$ (green) progenitors in the DG was significantly reduced in 13 weeks old *miR-17-92 KO* mice.

(G and H) The number of $Ki67^+$ cells was significantly increased in *OE* mice compared to *WT*.

(I and J) Deletion of *miR-17-92* led to significant reduction in the number of Dcx^+ neurons (red) in the DG.

(K and L) The number of Dcx^+ cells was increased in *OE* mice.

Scale bars, 100μm in A, C, E and G; 20μm in I and K. DAPI (blue) was used to label nucleus. All data were presented as means ± s.e.m. n=6 mice per group. *: $p < 0.05$ and ***: $p < 0.001$. A two-tailed, unpaired Student's t-test was used for comparisons. See also Figures S1 and S3.

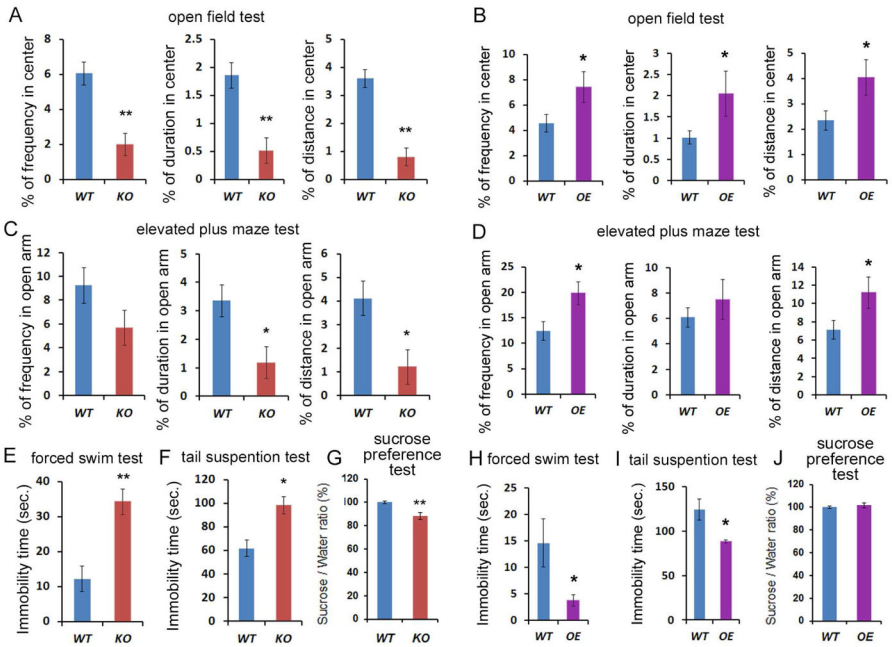

Figure 3. Deletion or overexpression of the miR-17-92 cluster in adult neural progenitors results in altered anxiety- and depression-like behaviors

(A) *miR-17-92* knockout (*KO*) mice displayed less frequency, duration, and distance moved in the center in the open field, compared to wild type (*WT*) mice.

(B) *miR-17-92* overexpressing (*OE*) mice showed significantly anxiolytic behavior in the open field test.

(C) *miR-17-92 KO* mice showed less duration and distance traveled in open arms in the elevated plus maze test.

(D) *miR-17-92 OE* mice showed significantly increased frequency and total distance moved in open arms in the elevated plus maze test.

(E) *miR-17-92 KO* mice showed significantly elevated immobility time in the forced swim test, compared to *WT* mice. Results are shown as the mean of immobility duration (seconds).

(F) *miR-17-92 KO* mice showed increased immobility in the tail suspension test. Results are shown as the mean of immobility duration (seconds).

(G) *miR-17-92 KO* mice showed significantly reduced sucrose consumption in the sucrose preference test, compared to *WT* mice. Results are shown as the percentage of sucrose consumption ratio (% of *WT*).

(H) *miR-17-92 OE* mice had significantly reduced immobility time compared to *WT* mice in the forced swim test.

(I) *miR-17-92 OE* mice showed reduced immobility in the tail suspension test.

(J) *miR-17-92 OE* mice showed no difference in the sucrose preference test.

Values plotted are means ± s.e.m, n=13-18 mice per group. *: $p < 0.05$ and **: $p < 0.01$. A two-tailed, unpaired Student's t-test was used for comparisons. See also Figure S3.

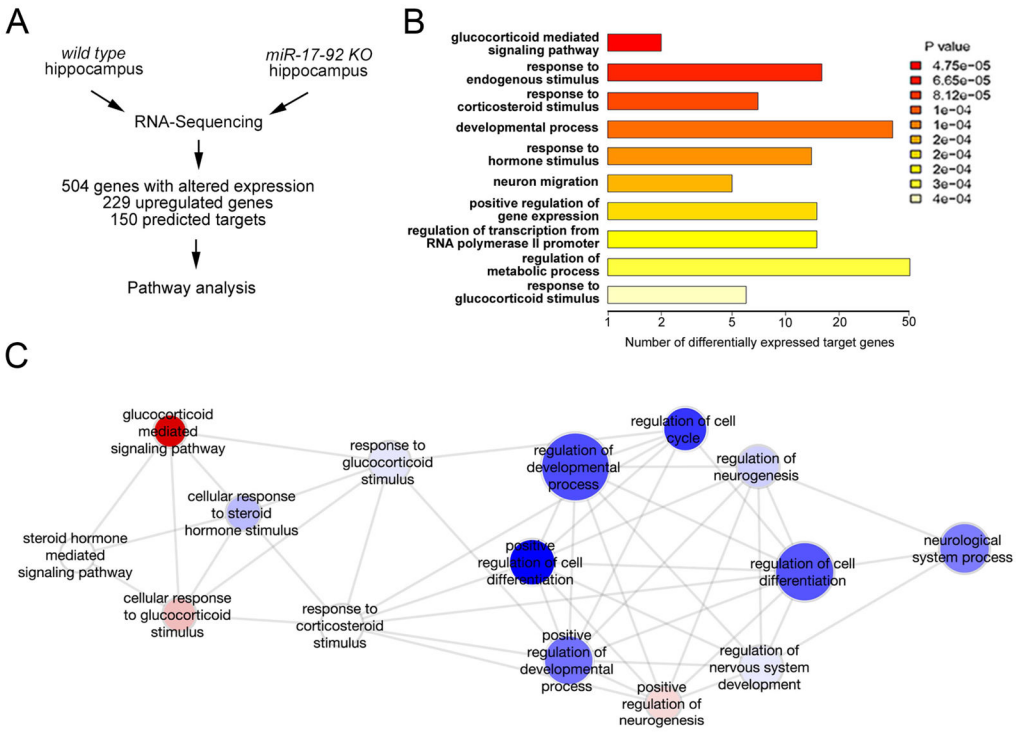

Figure 4. Deletion of miR-17-92 in adult hippocampal neural progenitors alters expression levels of genes in the glucocorticoid pathway

(A) Schematic flow of gene expression analysis. The adult hippocampal samples from 12 weeks old wild type (*WT*) and *miR-17-92* knockout (*KO*) mice were analyzed by RNA-Sequencing to detect genes with altered expression.

(B) Gene ontology (GO) analysis of 150 predicted target genes indicated 10 most significantly overrepresented functions. The glucocorticoid-mediated signaling pathway was the top among altered functions.

(C) Gene ontology analysis of signaling pathways that are associated with the Sgk1 function. See details in Experimental Procedures. See also Figure S4.

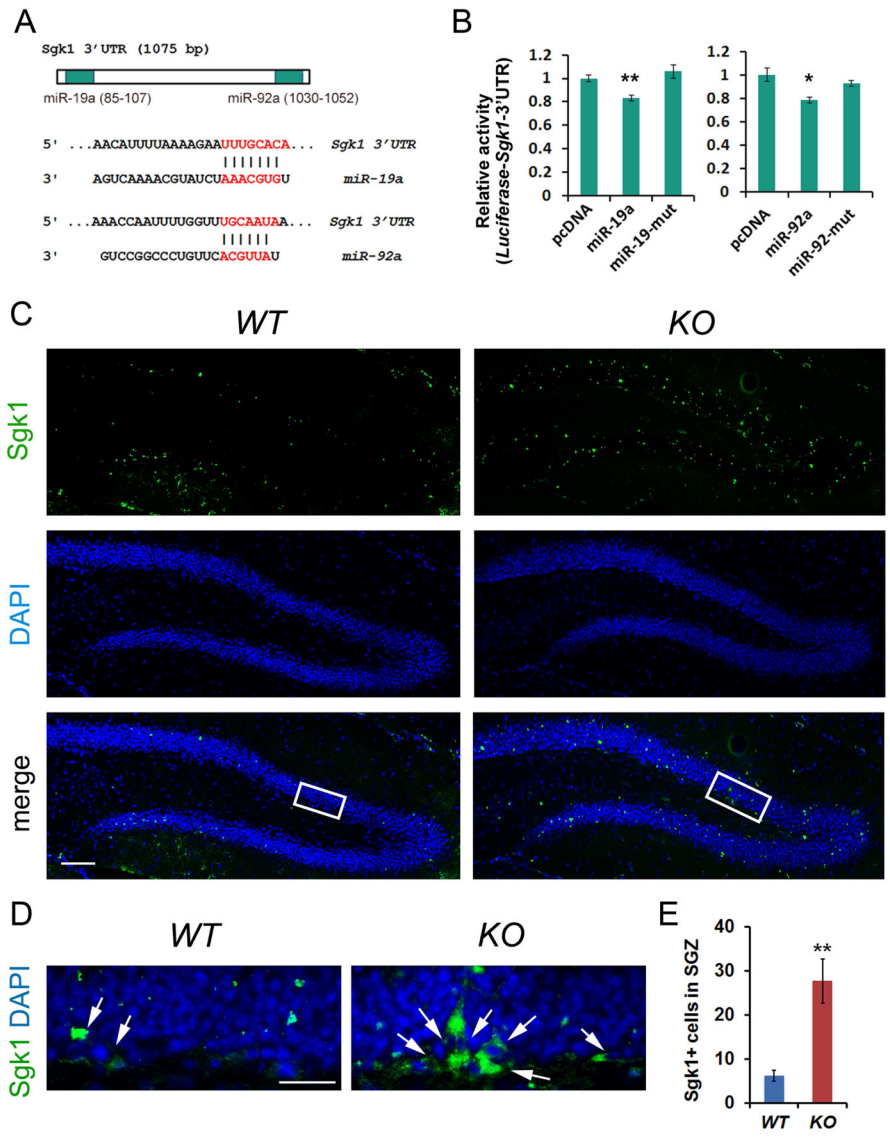

Figure 5. Sgk1 is a target of miR-17-92 in the adult hippocampus

(A) The 3'untranslated region (3'UTR) of *Sgk1* contained targeting sites for miR-19a and miR-92a.

(B) *Luciferase* fused to *Sgk1* 3'UTR was used to examine targeting effects of miR-19a and miR-92a on the *Sgk1* 3'UTR. Both miR-19a and miR-92a, but not their mutants (miR-19-mut and miR-92-mut), recognized the 3'UTR of *Sgk1* and reduced the luciferase activity. n=3.

(C) Sgk1 (green) expression in the dentate gyrus in the hippocampus of the 13 weeks old wild type (*WT*) and *miR-17-92* knockout (*KO*) mice.

(D) High power view of highlighted areas in C. Arrows indicate $Sgk1^{+}$ cells in the subgranular zone (SGZ).

(E) The number of $Sgk1^{+}$ cells was significantly increased in the SGZ in *miR-17-92 KO* mice, compared to *WT* controls. n=6.

Scale bars, 100μm in C and 20μm in D. DAPI (blue) was used to label nucleus. All data were presented as means ± s.e.m. $^{**}p < 0.01$. A two-tailed, unpaired Student's t-test was used for comparisons. See also Figure S5.

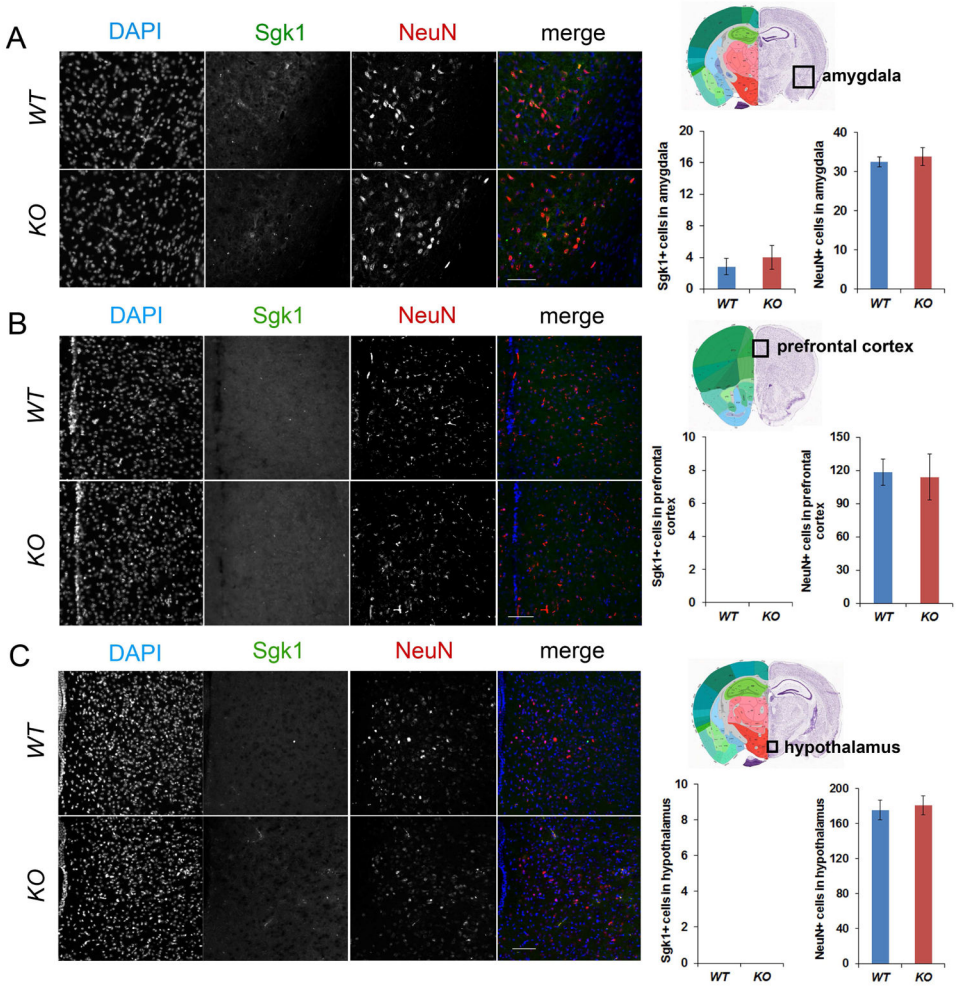

Figure 6. Sgk1 expressions are not changed in other brain regions in *miR-17-92 KO* mice

(A) Sgk1 expressing cells were detected in the amygdala. The numbers of $Sgk1^+$ and $NeuN^+$ cells did not show significant difference between 13 weeks old wild type (*WT*) and *miR-17-92* knockout (*KO*) mice.

(B and C) No Sgk1 expressing cells were detected in the prefrontal cortex and hypothalamus. The number of $NeuN^+$ cells did not show significant difference between *WT* and *miR-17-92 KO* mice.

Scale bars, 100μm. DAPI (blue), Sgk1 (green), and NeuN (red) were labelled. All data were presented as means ± s.e.m. A two-tailed, unpaired Student's t-test was used for comparisons.

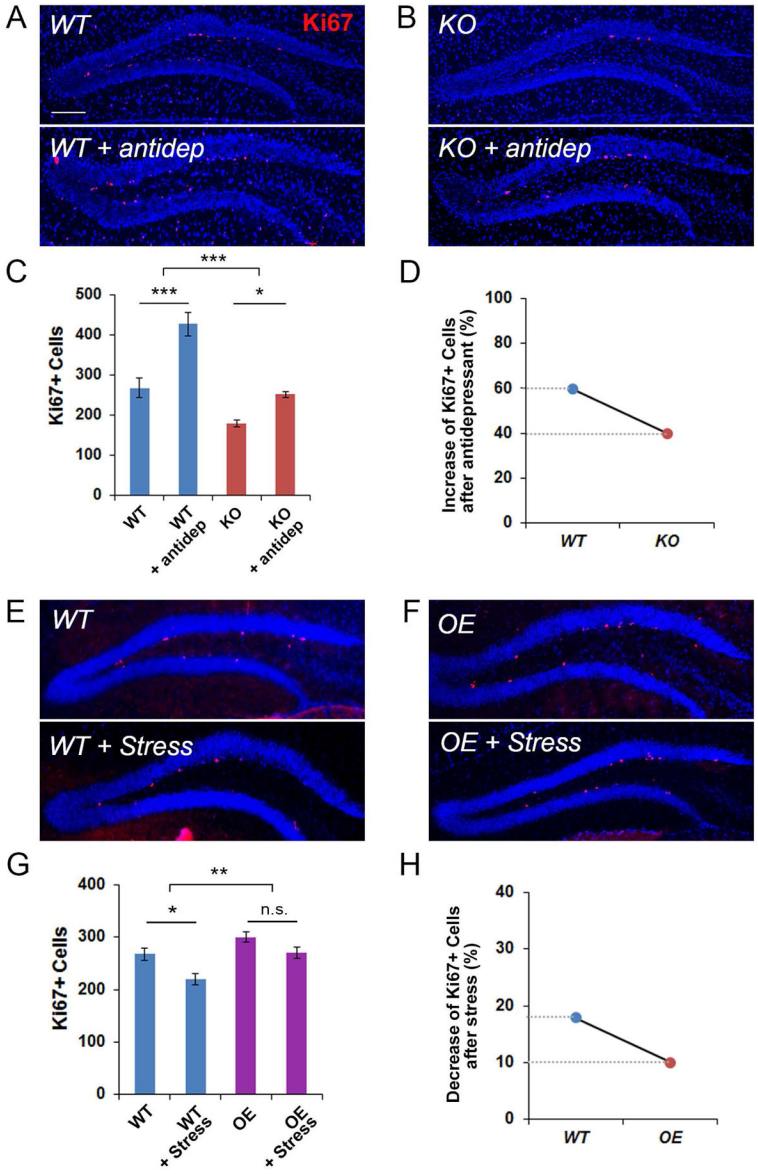

Figure 7. miR-17-92 mediates antidepressant- and stress-regulated adult hippocampal neurogenesis *in vivo*

(A–C) The number of Ki67$^+$ (red) progenitors in the dentate gyrus (DG) was significantly increased in antidepressant-treated 13 weeks old wild type (*WT*) mice, compared to vehicle-treated *WT* mice. Moreover, the number of Ki67$^+$ progenitors in the DG was significantly increased in antidepressant-treated 13 weeks old *miR-17-92* knockout (*KO*) mice, compared to vehicle-treated *miR-17-92 KO* mice.

(D) *miR-17-92 KO* mice showed less degree of increase in the number of Ki67$^+$ progenitors in the DG than *WT* mice after antidepressant treatment.

(E–G) The number of Ki67$^+$ progenitors in the DG was significantly reduced in stress-administered 13 weeks old *WT* mice, compared to *WT* mice without stress treatment. However, stress-administered *miR-17-92* overexpressing (*OE*) mice showed significant rescue of reduced number of Ki67$^+$ progenitors.

(H) *miR-17-92 OE* mice showed less degree of decrease in the number of $Ki67^+$ progenitors in the DG than *WT* mice after stress treatment.

Scale bars, 100μm. DAPI (blue) was used to label nucleus. All data were presented as means ± s.e.m. n=6 per group. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, and n.s.: not significant. Statistic analyses were assessed using two-way ANOVA with Bonferroni's *posthoc* test. See also Figures S6 and S7.