Epigenetic control of hippocampal stem cells: modulation by hyperactivation, glucocorticoids and aging

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MicroRNA-124 and -137 cooperativity controls caspase-3 activity through BCL2L13 in hippocampal neural stem cells

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Graphical abstract

Schematic depiction of the initial stages of the neurogenic cascade in control animals (left) or after kainic acid (KA)-induced hyperactivation alterations (right). Phenotypical alterations are: decreased quiescence, increased proliferation, ectopic localization, decreased apoptosis and accelerated differentiation. In this chapter we show for the first time that cooperative action of microRNA-124&137 is a molecular mechanism contributing to the apoptosis and differentiation phenotypes.
Abstract

Adult neurogenesis continuously contributes new neurons to hippocampal circuits and the programmed death of immature cells provides a primary mechanism controlling this contribution. Epileptic seizures induce strong structural changes in the hippocampus, including the induction of adult neurogenesis, changes in gene expression and mitochondrial dysfunction, which may all contribute to epileptogenesis. However, a possible interplay between this factors remains largely unexplored. Here, we investigated gene expression changes in the hippocampal dentate gyrus shortly after prolonged seizures induced by kainic acid, focusing on mitochondrial functions. Using comparative proteomics, we identified networks of proteins differentially expressed shortly after kainic acid-induced seizures, including members of the BCL2 family and other mitochondrial proteins. Within these networks, we report for the first time that the atypical BCL2 protein BCL2L13 controls caspase-3 activity and cytochrome C release in neural stem/progenitor cells. Furthermore, we identify BCL2L13 as a novel target of the cooperative action of microRNA-124 and microRNA-137, both upregulated in the dentate gyrus shortly after kainic acid-induced seizures. This microRNA-mediated fine-tuning of BCL2L13 expression controls casp3 activity, favoring non-apoptotic caspase-3 functions in NSPC exposed to KA and thereby may contribute to the early neurogenic response to epileptic seizures in the dentate gyrus.
Changes in miR expression in epilepsy animal models as well as in the hippocampus of epileptic patients have been identified\(^\text{19-21}\). Interestingly, changes in miR expression may principally impact on proteins involved in neuronal structure, gliosis and apoptosis\(^\text{22}\). Gene regulation by miRs involves a complex interplay between regulatory mechanisms that complicates the elucidation of the actual impact of individual miRNAs\(^\text{9}\). Thus, understanding the coordinated regulation of specific targets by multiple miRs, or miR cooperativity, is key in elucidating the complexity of gene regulation by miRs\(^\text{23}\). Importantly, cooperative miR function could render targets more sensitive to small changes in multiple miRs\(^\text{24}\).

Here, we investigated changes induced in the DG shortly after KA-induced SE (KA-SE), focusing on mitochondrial apoptotic functions in NSPC. Using proteomic, transcriptomic and miR-profiling techniques, we show that particular BCL-2 proteins are downregulated whereas, simultaneously, specific miRs are upregulated. Narrowing down these observation using postnatal hippocampal NSPC cultures as a model to study cell intrinsic molecular mechanisms induced by exposure to KA, we identify the BCL-2 family member BCL-2-Like 13 (BCL2L13) as a novel target of miR-124 and miR-137. We demonstrate that BCL2L13 controls CytC release and casp3 activity in NSPC and that BCL2L13 expression is regulated by the cooperative action of miR-124 and miR-137.

### Results

#### Changes in proteome in the DG after KA-SE

We detected a significant increase in DCX+ cells in the DG of KA-treated animals, starting at 3 days and lasting for at least 7 days after KA-SE, as described before\(^\text{13}\). This increase in DCX+ cells was preceded by an increase in the immunoreactivity for glial fibrillary acidic protein (GFAP), which started 1 day after KA-SE and lasted for at least 7 days (Supplementary Fig. 1), likely reflecting reactive astrogliosis reported by others\(^\text{25}\). To understand the relationships between molecular and cellular changes taking place in the DG shortly after SE, we focused on the 3 days after SE time point, when strong changes in gene expression take place\(^\text{26}\). Comparative proteomics between saline (SAL)- and KA-treated animals identified a total of 2327 proteins in the DG, with good...
sample-to-sample reproducibility in both SAL and KA groups (Supplementary Fig. 1, Supplementary Table 1 and 2). Beta-binomial analysis identified 114 differentially regulated proteins, with 56 up- and 58 down-regulated in the KA group (Figure 1 and Supplementary Table 2).

Global molecular protein networks were identified and visualized using Ingenuity Pathway analysis (IPA, Ingenuity® Systems; Supplementary Fig. 2A and D). These complex networks were reduced into smaller ones, using IPA’s focused gene function (Fig. 2 and Supplementary Fig. 2B-F), revealing one significantly overrepresented network (SON) containing the upregulated protein CLU (Fig. 2A). CLU is upregulated in reactive astrocytes and linked to cell survival27, involved in the regulation of postnatal neurogenesis28 and executes anti-apoptotic functions by interacting with BAX, blocking CytC release from mitochondria and caspase activation29. A second SON included the upregulated protein GFAP (Fig. 2B), linked to astrogliosis. Two others SONs containing upregulated proteins where identified around Nuclear Factor Kappa-B (NFkB) and TNF (Supplementary Figure 2B and 2C, respectively).

In line with an inhibition of mitochondrial apoptosis pathways suggested by CLU upregulation, two of the SONs containing downregulated proteins contained the proapoptotic BAX and NADH Dehydrogenase Ubiquinone 1 Beta 6 and 7 (NDUFB6, NDUFB7, Fig. 2C and Supplementary Fig. 3F, respectively). BAX is linked to the regulation of adult hippocampal NSPC apoptosis30 and NDUFB6 and NDUFB7 are two subunits of the NADH:ubiquinone oxidoreductase complex, involved in ATP generation by oxidative phosphorylation31. Interestingly, within the downregulated proteins we identified a third SON containing the BCL-2 protein BCL2L13, (Figure 2D; Supplementary Table 2). BCL2L13 is a novel atypical BCL-2 protein, localized to mitochondria and whose biological function is associated with CytC release and casp3 activation32,33. A fourth SON containing downregulated proteins was identified around NFkB (Supplementary Fig. 2E). KA-SE-induced downregulation of BAX and BCL2L13 at the protein level was confirmed by western blot (Supplementary Fig. 3A-B).

Next, we used GeneCodis Gene Ontology (GO) analysis to classify the significantly dysregulated proteins into biological processes (BP). Within the BPs overrepresented among upregulated proteins (Supplementary Fig. 4A and Supplementary Table 3), we found transport (containing GABRG2, OSBP, SEC13, SLC25A23, LIN7C, RAB4B and TRPV2), translation (EIF4E2, EIF4G2 and KARS) negative regulation of apoptosis (CLU, ITGAV, MTDH and HSPB1) and nervous system development (ENAH, NPTN and RAB23). GO analysis of the downregulated proteins (Supplementary Fig. 4B and Supplementary Table 4) resulted in the significantly overrepresented BPs transport (SNX12, TNPO3, SLC16A1, CHMP6, SLC32A1, TTYH1 and DYNLRB1), translation (RPL23, RPS15 and MRPL21), nervous system development (JUP, BAX and NDE1), and regulation of apoptotic process (BCL2L13 and BAX). The overrepresentation of BPs linked to apoptosis identified by GO analysis was consistent with BPs identified by IPA, including the downregulated proteins AKT3, BCL2L13 and BAX into the BP mitochondrial apoptosis (Supplementary Fig. 4C). These results suggest that some proteins up- and down-regulated in the DG shortly after KA-SE may converge on the regulation of mitochondrial apoptotic pathways, hallmarked by CytC release from mitochondria and caspase activation.

Correlation between proteome and transcriptome after KA-SE
We hypothesized that changes in protein levels could be explained by changes in corresponding mRNAs. Gene expression profiling identified a total of 52 genes significantly regulated at the mRNA level with 24 up- and 28 downregulated genes (Supplementary Table 5). Next, Pearson’s correlation analysis was used to analyze protein and mRNA levels34. We included in this analysis the 114 differentially expressed proteins in the DG and their corresponding mRNAs (Fig. 1 and Supplementary Table 2 and 5). Overall, protein levels did not correlate significantly with corresponding mRNA (Pearson r -0.165, p = 0.285; Pearson r -0.215, p = 0.172 for up- and downregulated proteins respectively, Supplementary Fig. 3C and 3D), suggesting the involvement of posttranscriptional regulatory mechanisms. BAX and BCL2L13 mRNA levels were validated by real time quantitative PCR (RT-QPCR; Supplementary Fig. 3E and 3F). Unlike BAX protein levels, which corresponded well with its mRNA levels, BCL2L13 protein levels did not (Supplementary Fig. 3C-F).
Figure 1 – Characteristic proteomic and mRNA expression profile observed in the DG after KA-SE.
(A) Normalized relative expression of 55 significantly upregulated proteins and corresponding mRNAs in the DG of mice exposed to KA-SE. (B) Normalized relative expression of 58 significantly downregulated proteins and corresponding mRNAs in the DG of mice exposed to KA-SE. Up- and Down-regulated proteins were sorted on fold change. Colors represent normalized relative protein/mRNA expression arbitrary units (A.U.), green (<1), black (1) and red (>1).
Figure 2 – Significantly overrepresented networks (SONs) containing dysregulated proteins in the DG after KA-SE.

(A) SON depicting nodes around NFκB, including CLU and other upregulated proteins. (B) SON depicting nodes around FOS, including GFAP and other significantly upregulated proteins. (C) SON depicting nodes around NFκB, including BAX and other significantly downregulated proteins. (D) SON depicting nodes around TNF including BCL2L13 and other significantly downregulated. Gene products (nodes) are represented as standard IPA polygons and relationships with lines (edges) between nodes. Full lines indicate a direct interaction and dashed lines an indirect interaction. Intensity of the node color indicates the degree of regulation (SAL vs. KA) and relationship strength is inversely related to line length. Genes represented by uncolored nodes were not differentially expressed in our experiments and were integrated by the IPA knowledge database. Arrows represent activation while non-arrowed lines binding only.
**Changes in miR expression in the DG after KA-SE**

Next, we explored the possible scenario that some of the discrepancies in protein and mRNA expression could be explained by posttranscriptional regulation by miRs. We detected 277 individual miRs in SAL and KA groups. 189 were differentially expressed, with 173 upregulated and 16 downregulated miRs (Fig. 3A and 3C; Supplementary Table 6), showing a distinct miR expression profile in the DG after KA-SE. From a group of previously identified brain enriched or specific miRs, 16 miRs were detected, with 11 up- and 1 down-regulated (Figure 3B and 3C and Supplementary Table 6). Although BCL2L13 is not expressed specifically in brain tissue, we reasoned that its expression in the brain would likely be regulated by brain-specific miRs. However, this approach may have excluded non-brain specific miRs that may have been more strongly upregulated and thus, could be more potent silencers of BCL2L13. We found multiple predicted binding regions for eight of the 11 upregulated brain specific or enriched miRs, including 2 for miR-124, in the mouse BCL2L13’s 3’UTR (Fig. 3F, Supplementary Fig. 5, Supplementary Tables 10-16).

The prediction of BCL2L13 as common target between miR-124 and 7 other miRs suggested a coordinated action. Therefore, we looked for common targets between these 8 miRs (Fig. 3D and 3E, Supplementary Fig. 5; Supplementary Tables 10-16). The brain-specific miR-124 can trigger apoptosis-inhibitory pathways by targeting pro-apoptotic BCL-2 proteins. Thus, we sought for BPs overrepresented among common targets and focused on miR pairs converging on the regulation of the BP apoptosis (Fig. 3E, Supplementary Fig. 5, Supplementary Tables 10-16). Previous studies of context features present in target 3’UTRs, which influence the targeting efficacy of miR beyond base pairing within “seed” regions, have established that the proximity of sites for coexpressed miRs is an important determinant of cooperative action. We applied this and other possible inclusion criteria for miR cooperativity, as follows: 1) miRs with at least a 6 mer base pairing region (allowing only one G:U wobble) within the first or last quartile of the BCL2L13 3’UTR and 2) 80nt proximity between seed regions (Fig. 3F, Supplementary Fig. 5 and Supplementary Table 9). We adopted these criteria considering distance constraints between miR binding sites known to influence efficacy, and in particular cooperativity, and to include most previously characterized seed-matched binding sites and maximal amount of G:U wobbles allowed in them for translational repression. Following this approach, miR-135a and miR-137 were identified as the two strongest candidates to cooperate with miR-124 in the regulation of BCL2L13 expression.

While only 30 out of 633 (3.1%) common targets between miR-124 and miR-135a were linked to apoptosis, miR-124 and miR-137 shared 336 GO annotated target genes, with 61 (18.2%) involved in the BP apoptosis, included BCL2L13 (Fig. 3D and Supplementary Table 7-9). The upregulation of miR-124 and miR-137 was validated by RT-QPCR (Supplementary Fig. 3G and 3H). Therefore, we decided to investigate further a possible cooperativity between these two miRs on the regulation of BCL2L13.

**BCL2L13 expression in intermediate neuronal progenitors of the DG and in primary post-natal hippocampal NSPC cultures**

The BCL2L13 expression pattern in the DG has not been characterized before. To this aim we used Nestin-GFP transgenic mice, in which GFP expression marks NSPC in the DG. We found that BCL2L13 was expressed in Nestin-GFP+ cells in the DG, and preferentially in Nestin-GFP+/polysialic acid form of neural cell adhesion molecule (PSA-NCAM)+ subpopulation (Fig. 4A and 4B). Only few BCL2L13+ cells were Nestin-GFP-/PSA-NCAM- and these were outside the SGZ (Fig. 4C). Nestin-GFP+/PSA-NCAM+ cells are classified as intermediate neuronal progenitors and a similar cell type is affected by KA-SE. MiR-124 and miR-137 are expressed in NSPC and in the adult DG and control their maturation and fate. In agreement with this, 7 days after miR-124 infusion to the DG, we observed a significant reduction of sex-determining region Y-box 2 positive (SOX2)+ and an increase in DCX+ cells in the SGZ (Fig. 4E-H), with a marked dispersion of DCX+ cells into the granule cell layer (Fig. 4F). This latter cellular phenotype strongly reflected the alterations observed shortly after KA-SE in the DG (Supplementary Fig. 1).
Chapter 3

A  Upregulated miR
- mmu-miR-7b
- mmu-miR-335-3p
- mmu-miR-324-3p
- mmu-miR-708
- mmu-miR-342-5p
- mmu-miR-384-5p
- mmu-miR-543
- mmu-miR-130a
- mmu-miR-15a
- mmu-miR-652
- mmu-miR-15b
- mmu-miR-350
- mmu-miR-409-5p
- mmu-miR-379
- mmu-miR-214
- mmu-miR-16
- mmu-miR-192
- mmu-miR-495
- mmu-miR-30a
- mmu-miR-451
- mmu-miR-132
- mmu-miR-504
- mmu-miR-129-5p
- mmu-miR-434-3p
- mmu-miR-200a
- mmu-miR-27b
- mmu-miR-210
- mmu-miR-339-5p
- mmu-miR-182
- mmu-miR-598
- mmu-miR-221
- mmu-miR-497
- mmu-miR-216b
- mmu-miR-135b
- mmu-let-7i
- mmu-miR-20a
- mmu-miR-411
- mmu-miR-547
- mmu-miR-449a
- mmu-miR-301a
- mmu-miR-674
- mmu-miR-301b
- mmu-miR-370
- mmu-miR-467a
- mmu-miR-124
- mmu-miR-421
- mmu-miR-148a
- mmu-miR-155
- mmu-miR-367
- mmu-miR-340-5p
- mmu-miR-362
- mmu-miR-34c
- mmu-miR-369-3p
- mmu-miR-467c
- mmu-miR-542-3p
- mmu-miR-488
- mmu-miR-344
- mmu-miR-21
- mmu-miR-29b
- mmu-miR-153
- mmu-miR-142-3p
- mmu-miR-27a
- mmu-miR-677
- mmu-miR-540-5p
- mmu-miR-448

B  Downregulated miR

C  Relative expression (dCt)
  - SAL KA

D  GO annotated
  - miR-124 targets
  - 2119 Genes
  - miR-137 targets
  - 1397 Genes

E  Predicted miR-124A137 target genes per BP (N)

F  miR-124 seed regions in BCL2L13 3’UTR
  - 118
  - 137
  - 5’-CUCAUGGUG-GGA-3’
  - BCL2L13 3’UTR
  - Folding energy: -26.900000 Kcal/mol

miR-137 seed regions in BCL2L13 3’UTR
  - 110
  - 102
  - 5’-CUCAUGGUG-GGA-3’
  - BCL2L13 3’UTR
  - Folding energy: -26.000000 Kcal/mol

10-40 10-30 10-20 10-10 100
MicroRNAs control BCL2L13 expression in neural stem cells

Chapter 3

Supporting our observations in vivo, we found detectable levels of endogenous miR-124, miR-137 and BCL2L13 in hippocampal NSPC cultures (Fig. 4I and 4K). The levels of miR-124 and miR-137 changed significantly in cells primed into differentiation (Fig. 4I), suggesting dynamic target regulation in NSPC. We did not observe significant changes in the endogenous levels of miR-124 and miR-137 in cells treated with 30μM KA for 7h (Fig. 4J), the experimental condition used across all our experiments in vitro with NSPC and KA, and previously used by others to model the effects of KA with NSPC and KA, and previously in vitro condition used across all our experiments.

Regulation of BCL2L13 expression and casp3 activity by miR-124 and miR-137 in hippocampal NSPC in vivo

The expression of BCL2L13 in NSPC/intermediate progenitors of the DG suggested a function in the regulation of casp3 activity in this cell type in the context of KA-induced hyperactivation and a possible modulation of this by miR-124 and miR-137. To test this hypothesis we first infused miR-124, miR-137 and an equimolar combination of both to the DG of Nestin-GFP transgenic mice. 2 days after the animals were exposed to a mild SE induced by intrahippocampal injection of KA, which promotes neurogenesis as is the case in our systemic KA injections (Supplementary Figure 1) and analyzed its consequences on BCL2L13 and casp3 activation in NSPC/intermediate progenitors in vivo 3 days after SE. In our hands, infusion of 50nL of 2.22 mM KA to the DG resulted in a mild SE, characterized by single and brief repetitive trains of spike activity as shown in cortical EEG traces obtained from EEG recordings sampled at 500Hz from freely moving mice 2h after KA administration (Fig. 5A and 5B), in agreement with recent observations using similar techniques. Saline-injected controls (SAL) did not display seizures or abnormal trains of spike activity in their EEG recordings at any time during the monitoring (Fig. 5A and 5B). Three days after this mild SE, we observed a significant reduction in the expression of BCL2L13 in Nestin-GFP+/PSA-NCAM+ cells of the DG in mice infused with miR-124, miR-137 or an equimolar combination of both (Fig. 5D and 5F). Interestingly, this miR-induced reduction in BCL2L13 correlated with a significant reduction in activated casp3 expression in the same Nestin-GFP+/PSA-NCAM+ cell type in animals infused with the equimolar combination of both miRs (Fig. 5E and G). These results suggest a regulation of BCL2L13 expression and casp3 activity in NSPC/intermediate progenitors in the context of KA-induced hyperactivation through a possible cooperative action between miR-124 and miR-137, which we decided to characterize further in vitro.

Regulation of BCL2L13 by cooperative action of miR-124 and miR-137

Using a Luciferase-BCL2L13 3’UTR reporter construct (pEZX-MT01-mouse-3kb-BCL2L13-3’UTR), we validated BCL2L13 as miR-124 and miR-137 target. Cotransfection of HeLa cells with the reporter construct and miR-124 or miR-137 resulted in a significant reduction in luciferase expression (Fig. 6A). Increasing miR-124 and miR-137 concentrations beyond 75 and up to 150nM did not further increase luciferase downregulation under these experimental conditions.
MicroRNAs control BCL2L13 expression in neural stem cells

Chapter 3

Figure 4 – BCL2L13 expression in the DG and hippocampal NSPC cultures.

(A) Representative confocal image displaying BCL2L13+/PSA-NCAM+/Nestin-GFP+ cells present in the SGZ (arrows). (B) Representative confocal image displaying BCL2L13+/PSA-NCAM+/Nestin-GFP+ cells present in the SGZ (arrowheads). (C) Representative confocal image displaying BCL2L13+/PSA-NCAM+/Nestin-GFP+ cells outside the SGZ (arrows). (D) Quantification of BCL2L13 protein expression in Nestin-GFP+ cell populations of the DG (**p < 0.001). (E) SOX2+ cells 7 days after infusion with a NT-miR (Ctrl, top) or miR-124 (bottom). Insets: black arrowheads indicate SOX2+ cells within the SGZ, dotted lines indicate the limits of the SGZ. (F) DCX+ cells 7 days after infusion with a NT-miR (Ctrl, top) or miR-124 (bottom). Black arrows: ectopic DCX+ cells in the GCL. (G) Quantification of SOX2+ cells in the SGZ 7dpi with a NT-miR (Ctrl) or miR-124 (**p < 0.01). (H) Quantification of DCX+ cells in the GZ 7dpi with a NT-miR (Ctrl) or miR-124 (**p < 0.001). (I) RNA expression of cell markers and miRs in hippocampal NSPC cultures, (SOX2, * p < 0.05; miR-137, ** p < 0.01; DCX, *** p < 0.001; miR-124, * p < 0.05; Nestin, p > 0.05). (J) miR-124 and miR-137 expression in hippocampal NSPC cultures during and after treatment with 30μM KA, (* p < 0.05 and ** p < 0.01, relative to time=0). (K) BCL2L13 protein expression in hippocampal NSPC cultures. Left lane: molecular weight marker (MW), right: BCL2L13 and GAPDH. Data in bar graphs represent mean normalized numbers of immunoreactive cells (% of Nestin-GFP+/PSA-NCAM+ or Ctrl) ± SEM from three animals. ML: molecular layer, GCL: granular cell layer, SGZ: subgranular zone, GZ: granular zone containing areas GCL+SGZ, dpi: days post injection. Scale bars represent 10μm (A-C and F) or 50μm (E).

Strikingly, an equimolar combination of miR-124 and miR-137 (75nM each) induced the largest decrease in luciferase expression (Fig. 6A). Furthermore, miR-124 concentrations as low as 25nM significantly downregulated luciferase expression only in the presence of miR-137 (Fig. 6B). Next, we followed a 3’UTR truncation approach, comparable to that recently used by others to study luciferase expression only in the presence of miR-137 (Fig. 6B). We then explored the predicted miR-137 binding sites and their proximal 3’UTR context as well, which may be of relevance for miR cooperativity. The excision of a 1.3kb fragment of BCL2L13’s 3’UTR containing the predicted miR-124 and miR-137 binding regions significantly, albeit not completely, rescued luciferase downregulation induced by miR-124 and miR-137 (Fig. 6C). These observations point at BCL2L13 as a target of miR-124 and miR-137. The additive effect in the presence of both miRs and the potentiation of miR-124 downregulation of BCL2L13 3’UTR-driven luciferase by miR-137 supported the hypothesis of miR cooperativity.

Regulation of endogenous BCL2L13 expression by the cooperative action of miR-124 and miR-137 in NSPC exposed to KA

Previous studies have demonstrated that NSPC are responsive to KA in vitro. Following a similar experimental approach, hippocampal NSPC cultures were incubated with vehicle or 30μM KA for 7h. In vehicle treated cells either miR-124 or miR-137 at 150nM or in equimolar combination (75nM each) downregulated endogenous BCL2L13 protein levels as compared to NT-miR (Fig. 6D). In cells exposed to KA, miR-124 or miR-137 alone (150nM) failed to induce significant BCL2L13 downregulation. However, cotransfection with an equimolar (75nM each) combination downregulated endogenous BCL2L13 protein levels (Fig. 6D). Thus, these observations together with those presented in Fig. 4J suggest that the lack of effect of miR-124 and miR-137 individually on BCL2L13 expression may not be simply explained by an induction of endogenous miR levels, and indicate that miR-124 and miR-137 cooperative action on BCL2L13 expression may be a relevant regulatory mechanism in NSPC exposed to KA.

Effect of BCL2L13 overexpression on CytC release and casp3 activation in NSPC exposed to KA

Hippocampal NSPC cultures were treated with increasing concentrations of KA, and morphological alterations were recorded prior to casp3 activity measurements. Low KA concentrations induced low levels of casp3 activation, paralleled by neurite extension, a morphological change associated with NSPC differentiation (Fig. 7A and 7D). High KA concentrations induced higher levels of casp3 activity paralleled by cell shrinkage, a morphological change associated with apoptosis (Fig. 7A and 7E). Likewise, high concentrations of KA reduced CytC localization to mitochondria (Fig. 7F-I), suggesting CytC release associated with the permeabilization of the mitochondrial outer membrane. Vehicle treated cells were used as reference to identify morphological changes induced by KA (Fig. 7C). The effects of KA on casp3 activity were mimicked by Staurosporine (Ssts), a potent activator of casp3, cellular differentiation and apoptosis in NSPC. (Fig. 7B).
Figure 5 – Regulation of BCL2L13 expression and casp3 activity by miR-124 and miR-137 in hippocampal NSPC in vivo after mild SE induced by intrahippocampal KA injection

(A) Representative low (top) and high (bottom) magnifications of cortical EEG recordings 2h after 50nl SAL injection in mice. (B) Representative low (top) and high (bottom) magnifications of cortical EEG recordings 2h after 50nl 2.22mM KA injection, inducing epileptoform activity in mice. (C) Schematic timeline of the experiment. Nestin-GFP mice received miR (1µl, 50µM) injections on day 0, KA (50nl 2.22mM) injections on day 2 and were sacrificed 3 days later, on day 5.
Transfections of exogenous BCL2L13 (validated in Supplementary Figure 6A) aggravated KA-induced CytC release (Fig. 7H and 7J) and casp3 activation (Fig. 7K). Furthermore, treatment of NSPC with specific siRNAs to knockdown BCL2L13 resulted in a significant downregulation of BCL2L13 protein and a concomitant significant decrease in casp3 activation in NSPC cells treated with KA (Supplementary Figure 6B and 6C). Overall these results suggest that the control of BCL2L13 protein levels is relevant for CytC release from mitochondria and casp3 activation in NSPC exposed to KA.

**Functional relevance of the cooperative regulation of BCL2L13 by miR-124 and miR-137**

Finally, we investigated the effects of miR-124 and miR-137 on the expression of active, cleaved casp3 (cl-casp3) in hippocampal NSPC cultures exposed to KA. In vehicle-treated cells, neither miR-124, miR-137, nor the combination of both had detectable effects on uncleaved casp3 (pro-casp3) or cl-casp3 expression levels (Fig. 8A). However, in cells incubated with 30µM KA, co-transfection with an equimolar (75nM) combination of miR-124 and miR-137 reduced cl-casp3 expression. Neither miR-124 nor miR-137 alone (150nM) were able to induce changes in cl-casp3 levels (Fig. 8B). Transfection with a FLAG-BCL2L13 construct devoid of its 3’UTR reverted cl-casp3 downregulation induced by the equimolar combination of miRs (Fig. 8C). These results indicate that miR-124 and miR-137 cooperativity regulates BCL2L13 protein levels and controls casp3 activity in NSPC exposed to KA.

**Discussion**

Here, we investigated relationships between changes in gene expression induced in the DG after KA-SE and alterations linked to mitochondrial function. We show that: 1) a group of 114 proteins is differentially expressed in the DG 3 days after KA-SE; 2) these proteins can be categorized into overrepresented networks and biological functions, including mitochondria-dependent apoptosis; 3) within a group of significantly downregulated proteins linked to mitochondrial function, we identified the BCL-2 protein BCL2L13. BCL2L13 is a hitherto uncharacterized miR-124 and miR-137 target, regulated by the cooperative action of both miRs and 5) the cooperative action of miR-124 and miR-137 regulates BCL2L13 protein levels and controls casp3 activity in NSPC exposed to KA. Our observations suggest that BCL2L13 controls casp3 activation, fine-tuning mitochondria-dependent apoptotic pathways in NSPC.

Previous observations have suggested that protein expression is predominantly controlled at the level of translation in mammalian cells, underscoring the importance of posttranscriptional regulation. In our experiments, changes in protein levels detected by proteomics did not correlate well with changes in corresponding mRNA, thus suggesting posttranscriptional regulation. This conclusion emphasizes the advantage of including proteomics-supported miR targets in our studies.
Figure 6 – Validation of BCL2L13 as a target of miR-124 and miR-137 cooperative action.

(A) Effect of miR-124 and miR-137 alone or in combination on BCL2L13 3’UTR-driven luciferase expression. Red empty bar: 75nM miR-124+75nM NT-miR; * p < 0.05; red dashed bar: 150nM miR-124; ** p < 0.01; (blue empty bar: 75nM miR-137+75nM NT-miR; *** p < 0.001; blue dashed bar: 150nM miR-137. *** p < 0.001; (purple bar: 75nM miR-124+75nM miR-137; *** p < 0.001. All conditions compared to 150nM NT-miR (black bar). The equimolar combination of miR-124 and 137 (purple bar) induced significantly larger downregulation of luciferase, compared to all other conditions (red empty bar: *** p < 0.001; red dashed bar: *** p < 0.001; blue empty bar, * p < 0.05; blue dashed bar, * p < 0.05). Values represent mean normalized expression (RLU) ±SEM of three independent experiments. (B) Effects of increasing concentrations of miR-124 in the presence of 75 nM miR-137. Values represent mean normalized expression to 75nM miR137+ 75nM NT-miR137 (0nM miR-124) ±SEM of three independent experiments (**p < 0.001 compared to 0nM miR-124). (C) Scheme of original and truncated pEZX-MT01-mouse-3kb-BCL2L13-3’UTR and bar graph showing a significant reduction of luciferase expression (OriUTR+NTmiR vs. +miR-124 or + miR-137, * p < 0.05) and a significant rescue (Ori. vs. Trunc. UTR with same miR, * p < 0.05) of miR-mediated luciferase expression in the absence of miR-124 and 137 binding regions. Values represent mean normalized expression (RLU) ±SEM of three independent experiments. In all cases, total miR concentration was kept constant at 150 nM by adding non-targeting miR (NT-miR). (D) Representative immunoblots and bar graph displaying miR induced changes in endogenous BCL2L13 expression in hippocampal NSPC. Black bars: vehicle-treated NSPC cultures, 150 nM miR-124 (miR-124, * p < 0.05), 150 nM miR-137 (miR-137 ** p < 0.01) and 75 nM miR-124+75 nM miR-137 (miR-124&137, ** p < 0.01) significantly reduced BCL2L13 protein expression. White bars: KA-treated NSPC cultures, only 75 nM miR-124 + 75 nM miR-137 significantly reduced BCL2L13 protein expression (miR-124&137, * p < 0.05). All miR treatments compare to 150 nM NT-miR. Values represent means±SEM of three independent experiments performed in triplicates. Bands belong to the same blot, but where re-ordered for clarity of the figure. Cropping lines are indicated by vertical black lines and full-length blots are presented in Supplementary Fig. 7. In all cases total miR concentration was kept constant at 150 nM by adding NT-miR.
Figure 7 – Effect of KA and exogenous BCL2L13 expression on CytC release from mitochondria and casp3 activation in hippocampal NSPC cultures.

(A) Treatment with increasing concentrations of KA. Values are expressed as casp3 activity normalized to vehicle treated cells. (B) Treatment with increasing concentrations of Sts. Values are expressed as casp3 activity normalized to vehicle treated cells. Dashed lines represent the transition from differentiation to apoptosis. (C) Cell morphology under vehicle treatment conditions. (D) Cell morphology after treatment with 10μM KA, inducing low casp3 activity (200% increase) as shown in (B). Arrows: cells displaying thinning and neurite extension. (E) Cell morphology after treatment with 30μM KA, inducing higher amounts of casp3 activity (800% increase) as shown in (B). Arrows: shrunk cells. (F) SIM image showing details of hippocampal NSPC after 10μM KA treatment. Arrowheads: CytC immunoreactivity in Mitotracker+ mitochondria (yellow). (G) SIM image showing details of hippocampal NSPC after 30μM KA treatment. Arrows: CytC immunoreactivity outside Mitotracker+ mitochondria (green). (H) SIM Image showing details of hippocampal NSPC transfected with FLAG-hBCL2L13 after 10μM KA treatment. White arrow points to CytC immunoreactivity outside Mitotracker+ mitochondria (green). (I) Representative SIM micrograph showing hippocampal NSPC transfected with FLAG-hBCL2L13 after 30 μM KA treatment. Arrow: CytC immunoreactivity outside Mitotracker+ mitochondria (green). Cells shown in (F and G) were transfected with EV for comparison to FLAG-hBCL2L13 (H and I). (J) Effect of 30μM KA treatment in combination with EV or FLAG-hBCL2L13 on CytC localization. FLAG-hBCL2L13 transfection decreased the ratio of mitochondrial to cytosolic CytC expression significantly, compared to EV transfection (*** p < 0.001). (K) Effect of 30 μM KA treatment in combination with EV or FLAG-hBCL2L13 transfection on casp3 activation. FLAG-hBCL2L13 transfection decreased the pro-casp3/ cl-casp3 ratio significantly, compared to EV transfection (** p < 0.01). Scale bars: 10μm (C-E); 3μm (F-I).

Values represent normalized mean (% of vehicle or EV) ±SEM of three independent experiments performed in triplicates.
Figure 8 – Effect of miR-124 and miR-137 alone or in combination on endogenous BCL2L13 and casp3 activation in hippocampal NSPC cultures.

(A) Transfection with 150nM miR-124 (miR-124), 150nM miR-137 (miR-137) or 75nM miR-124+75nM miR-137 (miR-124&137) did not result in significant changes in pro-casp3 expression levels in vehicle-treated cultures (p > 0.05, compared to NT-miR). Cl-casp3 expression was below detection levels.

(B) Transfection with 75nM miR-124+75nM miR-137 resulted in a significant reduction in cl-casp3 levels after 30μM KA treatment (* p < 0.05, compared to NT-miR). All other miR transfections led to non-significant differences.

All bands belong to the same blot, but were re-ordered for clarity of the figure. Cropping lines are indicated by vertical black lines and full-length blots are presented in Supplementary Fig. 7.

(C) Effect of 75nM miR-124+75nM miR-137 on cl-casp3 levels was abolished by co-transfection with a BCL2L13 construct devoid of its 3'UTR. There were no significant difference between transfections with 150nM miR-124 (miR-124), 150nM miR-137 (miR-137) or 75nM miR-124+75nM miR-137 (miR-124&137), p > 0.05, compared to 150nM NT-miR.

Values represent mean±SEM of three independent experiments performed in triplicates. In all cases total miR concentrations were kept constant at 150nM adding NT-miR.
Therefore, in a primary effort to understand the contribution of posttranscriptional mechanisms, we focused on miR-mediated control of gene expression. In agreement with recent observations, we found more up- than down-regulated miRs, indicating they may be implicated in the control of some of the changes in gene expression taking place in the DG shortly after KA-SE. Bioinformatics predictions have identified apoptosis as a common pathway among miRs upregulated in various models of SE. Importantly, miR-124 has been implicated in the inhibition of neuronal apoptosis in the DG. Deletion of Rncr3, the dominant source of miR-124a, results in a significant increase in the number of apoptotic cells in the DG without affecting NSPC proliferation, indicating that miR-124 anti-apoptotic function is essential for the maturation and survival of DG neurons. Suggestively, we show that intra-hippocampal delivery of miR-124 induced apoptotic changes in immature cells of the DG that were reminiscent of those induced by KA-SE. Furthermore, intrahippocampal infusion of miR-124, miR-137 or a combination of both prior to KA infusion resulted in a reduction in activated casp3 when both miRs were combined, suggesting a cooperative miR action.

miR cooperativity could explain the coordinated action of multiple miRs, rendering targets more sensitive to relatively small changes in levels of individual miRs, such as those described herein for brain specific or enriched miRS. Therefore, we focused on the hypothesis that miR-124 may cooperate with other upregulated brain specific or enriched miRs to regulate specific targets relevant for NSPC survival. To test this hypothesis we focused on mitochondrial BCL-2 proteins, particularly on BCL2L13, whose protein expression levels did not correlate well with corresponding mRNA levels. Following a bioinformatic pipeline in which we analyzed cooperativity between 11 upregulated miRs, common targets and converging biological pathways, we were able to narrow down the number of potential biologically relevant targets, leading to the identification of BCL2L13 as a novel target of miR-124 and miR-137 cooperativity. We identified multiple predicted binding sites for the brain specific or enriched miR-124 and miR-137 in BCL2L13’s 3’UTR and characterized BCL2L13 as a novel target of these two miRs.

To better understand the functional relevance of miR-124 and miR-137 cooperativity, we focused on the role of BCL2L13 in NSPC. We found that BCL2L13 is preferentially expressed in intermediate progenitor cells of the DG, indicating a functional role in these cells. In NSPC cultures, changes in casp3 induced by low or high KA doses were associated with phenotypic changes indicative of NSPC differentiation or apoptosis respectively, reflecting the dual role of casp3 activation in NSPC. We found that miR-124 and miR-137 cooperatively regulated BCL2L13 in NSPC exposed to KA in vivo and in vitro, indicating that cooperativity between these two miRs is involved in fine-tuning the levels of apoptosis-related proteins. Underlining the functional relevance of miR cooperativity in KA-treated NSPC, miR-124 and miR-137 did not have a significant effect on BCL2L13 protein levels or casp3 activation individually, yet they decreased BCL2L13 levels and inhibited casp3 activation when administered in combination. Moreover, exogenous expression of a miR-insensitive BCL2L13 reverted the reduction of active casp3 mediated by miR-124 and miR-137 and was associated with increased cytosolic CytC localization, leading to the possibility that miR-124 and miR-137 cooperativity fine-tunes BCL2L13 to favor non-apoptotic caspase-3 functions in NSPC. Further demonstration of this concept would require loss-of-function approaches, aimed to knockdown or inhibit miR actions. However, these approaches are difficult to optimize experimentally and all have their advantages and disadvantages, implying that a combination of multiple approaches may be necessary to establish miR function. Furthermore, results obtained with anti-miR oligonucleotides might be difficult to bring into line with those obtained with miR mimics at low endogenous miR expression levels, such as those we observed in NSPC cultures.

Overall, our observations may be relevant to understanding in more detail the regulation of adult hippocampal neurogenesis after SE and its possible consequences for epileptogenesis. Interestingly, recent observations have demonstrated that mitochondria play a key role in adult hippocampal neurogenesis and that mitochondrial dysfunction influences NSPC differentiation and mitochondrial complex I deficiency is observed in...
Chapter 3

hippocampal biopsies of TLE patients, indicating that our experimental model may reflect relevant aspects of the human disorder. Although speculative at this point, BCL2L13 downregulation together with changes in CLU expression and in the mitochondrial NADH:ubiquinone oxidoreductase complex I, suggested by the downregulation of two of its subunits we observed in the DG after KA-SE, may provide a link between mitochondria dysfunction and the regulation of adult neurogenesis in early stages of epileptogenesis. Validation of this hypothesis and the potential impact of reduced BCL2L13 expression on the elimination of excessive cell numbers and network reorganization in the DG require further experiments. However, previous observations have shown that mitochondrial apoptosis pathway are required for normal organization and function newborn neurons in the DG. These, together with our findings, suggest that fine-tuning of key components of mitochondrial apoptotic pathways, mediated by miR-124 and miR-137 cooperativity on BCL2L13 and its associated functions, may contribute to the early response to epileptic seizures in the DG.

Materials and methods

Animals, SE induction and tissue collection

6-8 week-old male C57BL/6j mice were used (Janvier Bioservices, Genest st Isle, France). Mice were housed in groups for one week under a 12-hour dark/light cycle (lights on at 6.30 h) in a temperature-and humidity-controlled room, with free access to food and water and were kept. Animal experiments were approved by the committee of Animal Health and Care, Leiden University (Protocol #08170) and were performed in accordance with the guidelines and regulations of the European Union for the use of animals for scientific purposes. Mice randomly assigned to experimental groups were injected with Kainic acid (KA; Sigma K0250, Kainic acid monohydrate) or Vehicle (Saline; SAL, 0.9% NaCl), following a protocol of multiple, low-dose, intraperitoneal injections of KA. SE was induced by repeated injections of KA (10mg/ml in saline, pH 7.4). The starting dose was 24mg/kg and subsequent injections of 6mg/kg were given every 30min until SE occurred. Behavioural seizures were scored after each KA injection using a modified Racine’s scale. Only animals displaying unequivocally class IV-V seizures that lasted at least 5min were selected for future experiments. Control animals were injected with equal amounts of SAL.

Mice injected with KA or saline (KA and SAL respectively; n=3 per group) were sacrificed 3 days after. Brains were extracted and immediately placed in ice-cold artificial CSF (NaCl: 124; KCl: 2.5; NaH2PO4: 1.25; CaCl2: 1; MgCl2: 1; NaHCO3: 25; D-Glucose: 10; all in mM) constantly bubbled with 95%O2/5% CO2, sectioned with a vibratome and the DG was separated from other hippocampal regions along the hippocampal fissure and avoiding contamination from the third ventricle using a previously described microdissection procedure. For transcriptomics and proteomics analyses, hippocampi of the same individuals were used. The right DG was reserved for genomic and the left for proteomic analyses. For histological preparations brains of three additional mice were used per experimental group.

Nano-LC peptide separation

Tissue homogenization and fractionation was carried out using gel electrophoresis and in-gel digestion. Tissue samples of microdissected DG from the KA or SAL-treated groups were lysed in lysis buffer (per 100mg tissue, one ml buffer containing 7M urea, 2M thiourea, 4% (w/v) CHAPS, and 10μl/ml protease inhibitor mix (Amersham Biosciences, Piscataway, NJ, USA)). Protein (30μg) fractions were loaded on gradient gels (NuPAGE 4–12% Bis-Tris gel, 1 mm × 10 wells, Invitrogen). The gels were then stained with Coomassie Brilliant Blue G-250 (Pierce, Rockford, IL, USA). Before MS analysis, separated proteins were in-gel digested as previously described. Further details on Nano-LC peptide separation, mass spectrometry, protein identification and quantification are described in Supplementary Data.

Microarray gene expression profiling

RNA extraction, sample preparation, hybridization to microarray, and detection were performed as described...
MicroRNAs control BCL2L13 expression in neural stem cells

Chapter 3

miR expression profiling
The same total RNA samples quality-controlled and used for gene expression profiling were used to profile 381 mature mouse miRs using 384-well TaqMan Array Mouse MicroRNA Fluidic v3.0 Cards, in triplicates (Applied Biosystems, Life Technologies), following a method previously used for to the identification of miR expression profiles in rat brain and blood after KA-SE²¹. The U6 small nuclear RNA was used as endogenous control and an unrelated human total RNA sample was included as a negative control. Samples were processed and analyzed by an investigator blinded to treatment using a previously described method⁶⁴. Further details are described in Supplementary Data.

In silico cooperative miR target prediction analysis
The miRecords database, composed of 11 established miR target prediction programs⁶⁵ was used to produce a list of mRNA targets for a group of previously characterized brain enriched or specific miRs, significantly regulated in our experiments. Further details are described in Supplementary Data.

NSPC culturing and transfections
Primary post-natal hippocampal NSPC cultures were prepared, cultured and transfected as previously described⁶⁶. Further details are described in Supplementary Data.

Western blotting
Western blotting was performed as previously described⁶⁶. Further details are described in the Supplementary Data.

3'UTR Luciferase reporter assays
miRs were tested for functional knockdown efficiency with a dual luciferase reporter assay following manufacturers instruction (Dual-Luciferase Reporter Assay System, Promega), luciferase activity was measured in cell lysates using a Spectramax L luminometer, as described before⁶⁷. Further details are described in Supplementary Data.

Quantitative real time polymerase chain reactions
mRNA and miR QPCRs were performed as described before⁶⁷. Further details are described in Supplementary Data.

Stereotactic miR infusion, immunohistochemistry and confocal microscopy
Stereotactic surgery was performed as described before⁶⁵ and was approved by the committee of Animal Health and Care, University of Amsterdam (DEC#236). Further details are described in Supplementary Data.

Electrode implantation, EEG recording, and electrophysiological characterization
Stereotactic surgery was performed as described before⁶⁶ and was approved by the committee of Animal Health and Care, University of Amsterdam (DEC#296). Further details are described in Supplementary Data.

Casp-3 activity
Casp-3 activity was measured using a Caspase 3 Fluorimetric Assay Kit (Cat# CASP3F, Sigma-Aldrich), as previously described⁶³. Further details are described in Supplementary Data.

Immunocytochemistry, mitochondrial staining and structured illumination microscopy (SIM)
50 thousand hippocampal NSPC were seeded per well in 24-well plates containing poly-L-lysine and Laminin coated glass coverslips as described before⁶⁶. The next day cells were transfected with FLAG-tagged human BCL2L13, a kind gift from Dr. Jürg Tschopp, Institute of Biochemistry, University of Lausanne³² using Attractene (Qiagen) or empty vector and were incubated for 48h. In the last 7h of the incubation cells were treated with varying concentrations of KA ranging from 0-300µM KA or vehicle⁴⁶. Cells were treated with 250nM MitoTracker® Red CMXRos (Invitrogen) for 5min to specifically stain mitochondria⁶⁹ and fixed for 15min in 4% PFA. Subsequently, cells were stained with polyclonal sheep anti-CytC (Sigma-Aldrich, 1:100) in combination with donkey anti-sheep Alexa488 (Invitrogen, 1:1000) and coverslips were mounted in vectashield mounting medium (Vector Labs). SIM was performed using a Nikon Eclipse Ti inverted microscope based SIM system as described before⁶⁸.

Statistical analysis
All comparisons were statistically tested using unpaired two-tailed Student’s t-test. When more than two groups were compared, one-way analysis of variance (ANOVA) test with Tukey’s post test was used. For correlative relations Pearson’s correlation analysis was performed. All statistical analyses were performed using GraphPad Prism 5.0.
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References

MicroRNAs control BCL2L13 expression in neural stem cells

Chapter 3


Supplementary information
Supplementary materials and methods

Nano-LC peptide separation
Gels were washed and dehydrated once in 50mM ammonium bicarbonate (ABC) and twice in 50mM ABC/50% acetonitrile (ACN). Cysteine bonds were reduced by incubation with 10mM DTT/50mM ABC at 56 °C for 1h and alkylated with 50mM iodoacetamide/50mM ABC at room temperature (RT) in the dark for 45min. After washing sequentially with ABC and ABC/50% ACN, the whole gel was sliced in 10 bands of equal width for each lane. Gel bands were sliced up into approximately 1mm³ cubes and collected in tubes, washed in ABC/ACN and dried in a vacuum centrifuge. Gel cubes were incubated overnight at 23°C with 6.25ng/mL trypsin and covered with ABC to allow digestion. Peptides were extracted once in 1% formic acid and twice in 5% formic acid/50% ACN. The volume of the peptide extract was reduced to 60μL in a vacuum centrifuge and filtered using a 0.45μm filter to remove gel particles and contaminants prior to LC–MS analysis. Subsequently, peptides were separated by an Ultimate 3000 nanoLC system (Dionex LC-Packings, Amsterdam, The Netherlands) equipped with a 20cm x 75μm ID fused silica column custom packed with 3μm 120Å ReproSil Pur C18 aqua (Dr Maisch GMBH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 6 μl/minute in 1.6% acetonitrile + 0.05% formic acid on a 1cm x 100μm ID precolumn packed with 5 μm ReproSil Pur C18 aqua. Peptides were separated in a 60min gradient (8-32% acetonitrile in 0.05% formic acid) at 300 nl/min. followed by washing (72% acetonitrile in 0.05% formic acid) and equilibration (4% acetonitrile in 0.05% formic acid). The inject-to-inject time was 90min.

Mass spectrometry
Intact peptide MS spectra and MS/MS spectra were acquired on a LTQ-FT hybrid mass spectrometer (Thermo Fisher, Bremen, Germany) as described before1,2. Intact masses were measured at 50.000 resolution in the ICR cell. In parallel, following an FT pre-scan, the top5 peptide signals (charge-states 2+ and higher) were submitted to MS/MS in the linear ion trap (3 amu isolation width, 30ms activation, 35% normalized activation energy, Q value of 0.25 and a threshold of 5000 counts). Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30sec.

Protein identification and quantification
MS/MS spectra were searched against IPI mouse database 3.59 (56692 entries) using Sequest (version 27, rev 12) with a maximum allowed deviation of 10ppm for the precursor mass and 1amu for fragment masses. Methionine oxidation and cysteine carboxamidomethylation were allowed as variable modifications, two missed cleavages were allowed. Scaffold 2.06.01 (Proteome software, Portland, OR) was used to organize the gel-slice data and to validate peptide and protein identifications. Identifications with a Peptide Prophet probability> 95% were retained. Subsequently, protein identifications with a ProteinProphet probability of >99% with 2 peptides or more in at least one of the samples were retained. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped. For quantitative protein analysis across samples, spectral counts (number of identified MS/MS spectra for each protein) were normalized on the sum of the spectral counts per biological sample. Differential analysis of samples was performed using the BetaBinomial test as described previously3. Protein identification and quantification details can be found in2,3.

Ingenuity Pathway Analysis (IPA) were performed as previously described3,4.

Microarray gene expression profiling
Total RNA samples extracted from the hippocampi of each animal of the KA or SAL groups (n=3 per group) using TRIzol reagent (Invitrogen), were checked for their quality and integrity using Nano Lab-on-Chip technology and an Agilent Bioanalyzer. The Illumina TotalPrep RNA Amplification kit (Ambion, Life Technologies) was used to synthesize biotine-labelled cRNA and concentrations of biotinylated cRNA were measured using a Nanodrop spectrometer. A total of 1.5μl of each biotinylated cRNA sample was hybridized onto a MouseWG-6 Expression BeadChip (Illumina) and BeadChips were scanned with the Illumina BeadArray. Gene Expression Analysis was done with Illumina’s Genome Studio software, using default settings suggested by the manufacturer. Transcript signals were subjected to quantile normalization, using the R Bioconductor package Abarray5. Microarray data analyses were performed with the software packages Abarray, BRB Array Tools (Biometric Research Branch of the US National Cancer Institute, Bethesda, MD, USA; http://linus.nci.nih.gov/BRBArrayTools.html) and Spotfire
MicroRNAs control BCL2L13 expression in neural stem cells

Chapter 3

MiRecords’ Predicted Target section with a filter to display putative targets predicted by at least three prediction programs. This filter was established considering previous reports indicating that the total number of programs considered in miR target prediction influences the sensitivity-specificity tradeoff of the prediction. Target predictions for all individual programs are shown in Supplementary Table S7 (miR-124), S8 (miR-137), S11 (miR-9), S12 (miR-125a-3p), S13 (miR-125b-3p), S14 (miR-135a), S15 (miR-135b), S16 (miR-190). Subsequently, miR pairs with predicted overlapping targets were analyzed for common biological processes using the GENECODIS web-based tool, hypergeometrically testing for significantly common processes (cut-off 2 transcripts per biological process, FDR corrected p<0.1).

To further predict the specific miR-mRNA thermodynamic binding properties, RNA22 was used.

NSPC culturing and transfections

Cells were cultured in culture flasks in DMEM/F-12 medium supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals), N2 supplement, (Invitrogen), Bovine Pituitary Extract (BPE, Invitrogen), recombinant-human-EGF (20ng/mL, Sigma), and recombinant-human-FGF (10ng/mL, Sigma). For differentiation of NSPC, human-EGF and recombinant-human-FGF deprived medium was used as described before. Hippocampal NSPCs were transfected using Attractene Transfection Reagent, following the manufacturer’s instructions (Qiagen), as described before. 48h after transfection cell lysates were collected for analysis. In miR transfection experiments we used Pre-miR™ miRs (Ambion, Life Technologies) designed to mimic mature endogenous miRs and Nontargeting miR (Cy3 labeled; Ambion, Life Technologies). In siRNA transfection experiments, a GeneSolution cocktail directed against mouse BCL2L13 (Mm_Bcl2l13_5, CACCCTGGAGGTGACAATAA; Mm_Bcl2l13_6, CAGTATAATCATGAAATAA; Mm_Bcl2l13_7, TAGGATTTCACTATGAAACAA; and Mm_Bcl2l13_8, TAGAATTAATTGTTCATTCAA; Qiagen) or negative control siRNA (target decisionsite (Spotfire, Somerville, MA, USA) as previously described. A permutation p-value cutoff < 0.01 was used as inclusion criterion.

MiR expression profiling

Total RNA (800ng/sample) was converted into cDNA using Megaplex RT Primers (Applied Biosystems) and a TaqMan miRNA RT Kit (Applied Biosystems). cDNAs were mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and then loaded on Mouse MicroRNA Fluidic v3.0 Cards (Applied Biosystems). The cards were run using a 7900HT real-time PCR instrument (Applied Biosystems). Statistical analyses and data classification were performed as described. Those miRs for which 95% of individual observations had a raw CT score >35 were excluded from the final data analysis. Fold change filters were applied to select miRs and a t-test with FDR (false discovery rate) of 5% correction for multiple comparisons was used to examine the significance of miRs regulated in the KA group in comparison with the SAL group. miRs with fold change equal or higher than 1.5 and p < 0.05 were selected for further analysis.

In silico cooperative miR target prediction analysis

A list of predicted mRNA targets of the significantly deregulated brain enriched or specific miRs was made using the miRecords database, which integrates the predictions of 11 established miRNA target prediction programs, including DIANAmicroT, Micro-Inspector, miRanda, miR-Target2, mi-Target, NBmir-Tar, Pictar, PITA, RNA22, RNA Hybrid and Targetscan, presenting a balance between rule-based and data-driven prediction approaches, which may improve miR binding region prediction accuracy. For example, conservation across species has been an important parameter used in prediction programs, however, its relevance should be considered together with other possible target recognition parameters, since almost 30% of experimentally supported mammalian miRNA-target gene interactions in a benchmark data set were non conserved, highlighting the potential relevance of nonconserved target sites. Following this approach, lists of predicted targets for the 8 deregulated brain enriched or specific miRs were produced using miRecords’ Predicted Target section with a filter to display putative targets predicted by at least three prediction programs. This filter was established considering previous reports indicating that the total number of programs considered in miR target prediction influences the sensitivity-specificity tradeoff of the prediction. Target predictions for all individual programs are shown in Supplementary Table S7 (miR-124), S8 (miR-137), S11 (miR-9), S12 (miR-125a-3p), S13 (miR-125b-3p), S14 (miR-135a), S15 (miR-135b), S16 (miR-190). Subsequently, miR pairs with predicted overlapping targets were analyzed for common biological processes using the GENECODIS web-based tool, hypergeometrically testing for significantly common processes (cut-off 2 transcripts per biological process, FDR corrected p<0.1). To further predict the specific miR-mRNA thermodynamic binding properties, RNA22 was used.
sequence: AATTCTCGAAGTGTACGT; Qiagen) was used. FLAG-tagged human BCL2L13 or empty vector, kind gifts from Dr. Jürg Tschopp, Institute of Biochemistry, University of Lausanne, were transfected using Attractene (Qiagen) according to manufacturer’s protocol and were incubated for 48h. In the last 7h of the incubation cells were treated with vehicle/KA.

**Intrahippocampal miR and KA infusion in Nestin-GFP mice**

Briefly, 6-week old male Nestin-GFP mice underwent stereotaxic surgery, during which 1.0μL of 50μM mirVana® miRmimic (Ambion, Life technologies) was bilaterally infused into the DG (anterior-posterior: -2.0, medial-lateral: ±2.0, dorsal-ventral: -2.0), miR-124 (miRmimic sequence: U A A G C A C G G G U G A A U G C C ), miR-137 (miRmimic sequence: UUAUUUGCUAAAGAAUACCGUGAAG), or an equimolar mixture of both was infused into the right DG, while NT-miR was infused into the contralateral DG, serving as internal control. 48h after miR infusion, animals underwent a second stereotactic surgery, during which 50nL of 2.22mM KA (Kainic Acid Monohydrate, Sigma Aldrich K0250) was bilaterally injected into the DG, at the same coordinates used for miR infusion. 72h after KA infusion, 8 animals were sacrificed by transcardial perfusion-fixation, brains were extracted and processed for immunohistochemistry as described in the corresponding section.

**Electrode implantation, EEG recording, and electrophysiological characterization**

A separate batch of 6 week old C57BL/6j mice (Harlan) were used to electrophysiologically characterize the intrahippocampal KA infusion. Directly after stereotactic KA/Saline infusion, animals were implanted with epidural gold-plated stainless steel screws. Bilateral recording electrodes were fixed in the burr holes already created for KA injection, while the reference/ground electrode was placed above the right visual cortex. Electrodes were fixed to the skull bone using dental cement (Simplex Rapid, Kemdent), and connected to a common pin connector. At the end of the surgical procedure, a wireless EEG recording device (Neurologger, TSEsystems) was connected to the pin connector, allowing 24/7 EEG recordings sampled at 500Hz from freely moving mice. Data were obtained for 72 hours post KA infusion in freely moving mice and analyzed for the occurrence of epileptiform activity and epileptiform spiking.

**Western blotting**

Cells or tissue samples were lysed in ice-cold 0.5X radioimmunoprecipitation assay (RIPA) buffer (20mM triethanolamine, 0.14 M NaCl, 0.05% deoxycetant, 0.05% SDS, 0.05% Triton X-100) supplemented with protease inhibitors (complete Protease Inhibitor Cocktail tablets; Roche Applied Science, Penzberg, Germany). Subsequently, cell lysates were centrifuged for 30min at 13.000 rpm at 4°C after which the supernatants were collected. Protein content was quantified using the BCATM Protein Assay (Pierce Biotechnology, Rockefort, IL, USA) and 25μg of each sample, were loaded onto an SDS-PAGE gel. After electrophoresis, the samples were blotted overnight onto an Immobilon P membrane (Millipore Corp., MA, USA) and processed as described.

Blots were blocked in 10mM Tris-HCl (pH 8.0), 150mM NaCl, and 0.05% Tween20 containing 5% nonfat dried milk powder. Proteins of interest were subsequently detected using specific primary antibodies: BCL2L13 (polyclonal goat anti-BCL2L13; Santa Cruz, 1:200), pro- and cleaved casp-3 (polyclonal rabbit anti-caspase3; Cell Signalling, 1:1000), CytC (polyclonal sheep anti-CytC; Sigma Aldrich; 1:5000), FLAG-tag (monoclonal mouse anti-FLAG M2/DYKDDDDK; Sigma, 1:1000), BAX (polyclonal rabbit anti-BAX; Abcam, 1:1000) in combination with donkey anti-goat IRdye 800 CW (Li-Cor, 1:10000), goat anti-rabbit IRDye 680 LT (Li-Cor, 1:20000), donkey anti-sheep HRP conjugated (Life Technologies, 1:2000), goat anti-mouse IRDye 680 LT (Li-Cor, 1:10000), and goat anti-rabbit IRDye 680 LT (Li-Cor, 1:20000) respectively as secondary antibodies. Expression levels of α-tubulin (monoclonal mouse anti-α-tubulin antibody; Santa Cruz, 1:200), GAPDH (monoclonal rabbit anti-GAPDH 14C10; Cell Signalling, 1:1000) or β-actin (monoclonal mouse anti-β-actin AC-15; Sigma-Aldrich, 1:2000) in combination with goat anti-mouse IRDye 680 LT (Li-Cor, 1:10000), goat anti-rabbit IRDye 680 LT (Li-Cor, 1:10000) or goat anti-rabbit HRP conjugated (Bio-Rad, 1:3000) and goat anti-mouse IRDye 680 LT (Li-Cor, 1:10000) respectively, were used
MicroRNAs control BCL2L13 expression in neural stem cells

Chapter 3

Restriction enzyme digestion and subsequent Klenow Fragment Large (Fermentas), eliminating the first 1.3kb of the sequence and generating a truncated construct containing no predicted miR-124 and miR-137 binding regions (Figure 6C). Results were expressed as mean normalized expression (RLU) ±SEM of three independent experiments performed in triplicates.

Quantitative real time polymerase chain reactions

RNA was isolated using TRIzol reagent (Life Technologies) according to manufacturers’ protocol. Quantitative real time polymerase chain reactions (QPCR) to detect mature miR-124 (Cat# 4427975, Assay ID 001182, Life Technologies) and miR-137 (Cat# 4427975, Assay ID 006016, Life Technologies) were performed using a TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies) combined with TaqMan® 2x Universal PCR Master Mix (Life Technologies) and were normalized against RNU6B (Cat# 4427975, Assay ID 001093, Life Technologies). QPCR to detect mRNA was performed as described before using the following primer sequences:

3'UTR Luciferase reporter assays

The first 3kb of the mouse BCL2L13 3'UTR mRNA (NCBI Reference Sequence NM_153516.2), containing predicted miR-124 and miR-137 binding regions, was cloned into the multiple cloning site of a reporter vector (pEZX-MT01, Genecopoeia) containing the firefly Luciferase open reading frame as depicted in Figure 3F (pEZX-MT01-mouse-3kb-BCL2L13-3'UTR). HeLa cells were cotransfected with pEZXMT01-mouse-3kb-BCL2L13-3'UTR and mature miRs and/or non-targeting homologues (Pre-miRTM, Ambion, Life Technologies) using Attractene Transfection Reagent (Qiagen). Cells were lysed 48h after transfection, and ratios between Firefly luciferase and Renilla luciferase activity were calculated and expressed as relative light units (RLU). In this cloning strategy 3 out of 4 binding regions for miR-124 and miR-137 have been effectively relocated from the first quartile of the original 5.4kb 3'UTR to the center of the truncated 3kb-long 3'UTR (pEZX-MT01-mouse-3kb11 BCL2L13-3'UTR, Figure 3F). This could have disfavored miR activity, resulting in a possible underestimation of knockdown efficiency. EcoRI and BbvCl (New England Biolabs) restriction enzyme digestion and subsequent Klenow Fragment Large (New England Biolabs) blunting allowed for a religation of the truncated pEZX-MT01-mouse-3kb-BCL2L13-3'UTR, using T4 DNA Ligase treatment (Fermentas), eliminating the first 1.3kb of the sequence and generating a truncated construct containing no predicted miR-124 and miR-137 binding regions (Figure 6C). Results were expressed as mean normalized expression (RLU) ±SEM of three independent experiments performed in triplicates.

Quantitative real time polymerase chain reactions

RNA was isolated using TRIzol reagent (Life Technologies) according to manufacturers’ protocol. Quantitative real time polymerase chain reactions (QPCR) to detect mature miR-124 (Cat# 4427975, Assay ID 001182, Life Technologies) and miR-137 (Cat# 4427975, Assay ID 006016, Life Technologies) were performed using a TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies) combined with TaqMan® 2x Universal PCR Master Mix (Life Technologies) and were normalized against RNU6B (Cat# 4427975, Assay ID 001093, Life Technologies). QPCR to detect mRNA was performed as described before using the following primer sequences:

α-tubulin (for normalization)

fwd: CCCCCTGGCTTCAAGCGTGGTC;
rev: TGTTCTTGTCACTTGGCATGGC;
Nestin

fwd: GGGCAGCACTGGCACHACCTC;
rev: TGCAGCTTCAGCTTGGGGTGAGG;
SOX2

fwd: GGAGACGGAGCTGAGCCGC;
rev: CCAGGAGCATACCATGAGGAGCG;
DCX

fwd: CCCCCGTGGAGTGCTGACTACA;
rev: ACCAGTTGGGGTTGACATTCTTGGT;
BCL2L13

fwd: TGGAGTGGAGTGGTGGCTAGATTCTCTTCT;
rev: TTGAACTCCTGGGGGTGG;
BAX

fwd: GCGTGGTTGCCTTCCTTCTAC;
rev: CCAAGCCACCCCTGCTTTG.
Immunohistochemistry and (confocal) microscopy
The following antibodies were used: polyclonal chicken anti-GFP (Abcam, 1:500), monoclonal mouse anti-PSA-NCAM (Chemicon, 1:500) and polyclonal rabbit anti-BCL2L13 (Abcam, 1:200) or rabbit anti-cl-casp3 (Cell Signalling, 1:600) in combination with goat anti-chicken Alexa488 (Invitrogen, 1:500), goat anti-mouse Alexa647 (Invitrogen, 1:500) and goat anti-rabbit Alexa568 (Invitrogen, 1:500) respectively. Sections were counterstained for DNA using Hoechst (Invitrogen, 1:20000) to detect nuclei. The SOX2 staining required an antigen retrieval treatment, performed by heating the sections in 0.1M citrate buffer (pH 6.0) in a standard microwave (Samsung M6235) to a temperature of approximately 95°C for 15min (5min at 800Watt, 5min at 400Watt and 5min at 200Watt). Antibodies used were polyclonal rabbit anti-SOX2 (Abgent, 1:500) and polyclonal goat anti-doublecortin C18 (DCX, Santa Cruz, 1:800) which were amplified with biotinylated goat anti-rabbit (Vector, 1:200) and biotinylated donkey anti-goat (1:500; Jackson ImmunoResearch Laboratories) respectively and avidin-biotin enzyme complex (ABC kit; Elite Vectastain, 1:1000) and developed with diaminobenzidine (DAB; 20 mg/100 ml tris buffer; 0.01% H2O2). Images were acquired using a Leica CTR5500 microscope with the Leica MM AF program (MetaMorph, version 1.6.0), and stack images were acquired using the multidimensional acquisition. Brain sections from KA or SAL 13 groups (n=3 each) were stained to detect the immature neuron marker DCX and the astrocyte marker GFAP and DCX+ cells were automatically identified and counted per section in z-projected confocal images using Cell Profiler24. Immunoreactive cells were counted as described before using every tenth hippocampal section (of 40μm from 8 series) starting at the hippocampus, within each collected set of approximately 11 slices, as described before25. The SGZ was defined as a two-cell body band around the limit of the GCL and the hilus, as described before26. The granular zone (GZ) was defined as the area containing GCL+SGZ. Bar graphs represent normalized mean±SEM (% of BCL2L13+/Nestin-GFP+/PSA-NCAM+ of ctrl miR) or mean intensity±SD (background subtracted average gray value of BCL2L13 or cl-casp3 in Nestin-GFP+/PSA-NCAM+).

Casp-3 activity
Hippocampal NSPC were treated with varying concentrations of KA (Cat# K0250, Sigma-Aldrich). Staurosporine (Cat# A4400, Sigma-Aldrich) was used as a positive control for casp-3 activity. Lysates were collected according to manufacturer’s protocol and measured using a FLUOStar Optima plate reader (BMG Labtech GmbH).

References


Supplementary Figure 1 – Hallmarks of KA-SE in the mouse DG and proteomics quality control.

(A-D) Representative confocal images showing the temporal increase in DCX+ immunoreactive cells observed in the DG 0, 1, 3 and 7 days after KA-SE. Right images show higher magnifications of the images depicted left. (E) Bar graph showing the quantification of DCX+ cells three days after KA-SE. Values represent normalized mean (% of control) ±SEM (n=3) and were tested using unpaired Student’s t-test (* p < 0.05). (F) Representative confocal images showing the temporal increase in astrocytic marker GFAP immunoreactivity observed in the DG shortly after KA-SE. Control: 0 days after KA-SE. Scale bars in (A-D) and (F): 50μm. (G) SDS PAGE gel image showing separation pattern of KA and SAL proteins. (H) Venn diagrams indicate the overlap between replicates (n=3) for the SAL and KA groups and between the two groups. (I) For the proteins identified in 3/3 samples the average coefficient of variance (CV) for protein quantification was calculated to be 21.5% for the SAL group and 24.6% for the KA group, indicating good quantitative reproducibility.
Supplementary Figure 2 – Gene networks identified around the significantly dysregulated proteins in the DG shortly after KA-SE.

(A) Global protein network of upregulated proteins in KA-SE samples. (B) and (C) Two of the most significantly overrepresented gene networks depicting nodes around (non-regulated; white) NFkB and TNF with direct (solid edge) and indirect (dashed edge) interactions of significantly (red) upregulated proteins. (D) Global protein network of downregulated proteins in KA-SE samples. (E) Significantly overrepresented gene network depicting nodes around NFkB and significantly downregulated proteins with direct or indirect interactions. (F) Significantly overrepresented gene network depicting nodes around TP53, including NDUFB6, NDUFB7 and significantly downregulated proteins with direct or indirect interactions. Gene products (nodes) are represented as standard IPA polygons and relationships with lines (edges) between nodes. Intensity of the node color indicates the degree of regulation (SAL vs. KA) and relationship strength is inversely related to line length. Genes in uncolored nodes were not identified as differentially expressed in our experiments and were integrated by the IPA knowledge database. Full lines indicate a direct interaction and dashed lines an indirect interaction. Arrows represent activation while non-arrowed lines binding only.
Supplementary Figure 3 – Validation of relevant changes detected in proteomics, transcriptomics and miR profiling experiments.

(A) Validation of changes in BCL2L13 protein levels by WB. Representative immunoblots and quantifications show that KA-SE induced a significant reduction in BCL-RAMBO protein levels in the DG (*p < 0.05). (B) Validation of changes in BAX protein levels by WB. Representative immunoblots and quantifications show that KA-SE induced a significant reduction in BAX protein levels in the DG (*p < 0.05). (C) Pearson correlation analysis of all the proteins significantly upregulated by KASE and their corresponding mRNAs showing no significant correlation (Pearson r = -0.2147, p > 0.05). (D) Pearson correlation analysis of all the proteins significantly downregulated by KA-SE and their corresponding mRNAs showing no significant correlation (Pearson r = -0.1647, p > 0.05). (E) Validation of changes in BCL2L13 mRNA levels by Q-PCR, showing that KASE didn’t induce significant changes in levels of BCL2L13 mRNA in the DG after 3 days (p > 0.05). (F) Validation of changes in BAX mRNA levels by Q-PCR, showing that KA-SE induced significant changes in levels of BAX mRNA in the DG after 3 days (*p < 0.05). (G) Validation of changes in miR-124 levels by TaqMan Q-PCR, showing that KASE induced significant changes in levels of miR-124 in the DG after 3 days (**p < 0.001). (H) Validation of changes in miR-137 levels by TaqMan Q-PCR, showing that KASE induced significant changes in levels of miR-137 in the DG after 3 days (**p < 0.001). Values in bar graphs represent normalized mean expression or fold changes±SEM (N=3) and were tested using unpaired Student’s t-test. Pearson correlation analysis was performed using GraphPad Prism 5.0.
Supplementary Figure 4 – Biological pathways overrepresented among significantly dysregulated proteins in the DG.

(A) GO analysis of the proteins upregulated in the DG after KA-SE. (B) GO analysis of the proteins downregulated in the DG after KA-SE. BPs were identified with Genocodis modular enrichment GO analysis. Red bars: number of annotated members per BP. Blue bars: hypergeometric FDR corrected p-values. Two or more regulated proteins per process were used as inclusion criterion. (C) IPA showing the downregulated proteins Akt3, BAX and BCL2L13 within a mitochondrial pathway of apoptosis. Gene products, relationships, degree of regulation, relationship strength and interactions are represented as described in Fig. 2.
Chapter 3 - Supplementary information

**A**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125a-3p targets (2423 Genes)

**B**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125b-3p targets (2403 Genes)

**C**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125a-3p targets (2403 Genes)

**D**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125b-3p targets (2403 Genes)

**E**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125a-3p targets (2403 Genes)

**F**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125b-3p targets (2403 Genes)

**G**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125a-3p targets (2403 Genes)

**H**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125b-3p targets (2403 Genes)

**I**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125a-3p targets (2403 Genes)

**J**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125b-3p targets (2403 Genes)

**K**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125a-3p targets (2403 Genes)

**L**

GO annotated miR-124 targets (2113 Genes)  
GO annotated miR-125b-3p targets (2403 Genes)
Supplementary Figure 5 - Genecodis GO analysis of mmu-miR-9, mmu-miR-125a-3p, mmu-miR-125b-3p, mmu-miR-135a, mmu-miR-135b and mmu-miR-190 that share common predicted targets with mmu-miR-124.

(A) Venn diagram showing miRecords database predictions of miR-mRNA interaction mining for miR-124 (2119 Genecodis GO annotated targets) and miR-9 (2823 Genecodis GO annotated targets) with ≥3 algorithms positively predicting an miR-mRNA binding. The miR-124 and miR-9 pair had 690 predicted common Genecodis GO annotated targets. The bar graph depicts Genecodis Comparative Modular Gene Enrichment GO analysis of the 690 predicted common targets between miR-124 and miR-9. Apoptosis is among the significantly (Hypergeometric FDR corrected p = 1.26*10^{-4}) overrepresented GO biological processes with BCL2L13 as a member. (B) Schematic representation of the BCL2L13 3’UTR with miR-124 and miR-9 binding regions highlighted, none of which meet the required inclusion criteria for cooperative action with miR-124. Details of cooperativity predictions are given in Table S9. (C) Venn diagram showing miRecords database predictions of miR-mRNA interaction mining for miR-124 (2119 Genecodis GO annotated targets) and miR-125a-3p (2380 Genecodis GO annotated targets) with ≥3 algorithms positively predicting an miR-mRNA binding. The miR-124 and miR-125a-3p pair had 585 predicted common Genecodis GO annotated targets. The bar graph depicts Genecodis Comparative Modular Gene Enrichment GO analysis of the 585 predicted common targets between miR-124 and miR-125a-3p. Apoptosis is among the significantly (Hypergeometric FDR corrected p = 1.55*10^{-3}) overrepresented GO biological processes with BCL2L13 as a member. (D) Schematic representation of the BCL2L13 3’UTR with miR-124 and miR-125a-3p binding regions highlighted, none of which meet the required inclusion criteria for cooperative action with miR-124. Details of cooperativity predictions are given in Table S9. (E) Venn diagram showing miRecords database predictions of miR-mRNA interaction mining for miR-124 (2119 Genecodis GO annotated targets) and miR-135a (2439 Genecodis GO annotated targets) with ≥3 algorithms positively predicting an miR-mRNA binding. The miR-124 and miR-135a pair had 633 predicted common Genecodis GO annotated targets. The bar graph depicts Genecodis Comparative Modular Gene Enrichment GO analysis of the 633 predicted common targets between miR-124 and miR-135a. Apoptosis is among the significantly (Hypergeometric FDR corrected p = 2.30*10^{-3}) overrepresented GO biological processes with BCL2L13 as a member. (H) Schematic representation of the BCL2L13 3’UTR with miR-124 and miR-135a binding regions highlighted. The predicted miR-124 binding region at position 916 and the predicted miR-135a binding region at position 848 met the required inclusion criteria for cooperative action. Details of cooperativity predictions are given in Table S9. (I) Venn diagram showing miRecords database predictions of miR-mRNA interaction mining for miR-124 (2119 Genecodis GO annotated targets) and miR-135b (2473 Genecodis GO annotated targets) with ≥3 algorithms positively predicting an miR-mRNA binding. The miR-124 and miR-135b pair had 643 predicted common Genecodis GO annotated targets. The bar graph depicts Genecodis Comparative Modular Gene Enrichment GO analysis of the 643 predicted common targets between miR-124 and miR-135a. Apoptosis is among the significantly (Hypergeometric FDR corrected p = 3.88*10^{-3}) overrepresented GO biological processes with BCL2L13 as a member. (J) Schematic representation of the BCL2L13 3’UTR with miR-124 and miR-135b binding regions highlighted, none of which meet the required inclusion criteria for cooperative action with miR-124. Details of cooperativity predictions are given in Table S9. (K) Venn diagram showing miRecords database predictions of miR-mRNA interaction mining for miR-124 (2119 Genecodis GO annotated targets) and miR-190 (1025 Genecodis GO annotated targets) with ≥3 algorithms positively predicting an miR-mRNA binding. The miR-124 and miR-190 pair had 277 predicted common Genecodis GO annotated targets. The bar graph depicts Genecodis Comparative Modular Gene Enrichment GO analysis of the 277 predicted common targets between miR-124 and miR-190. Apoptosis is among the significantly (Hypergeometric FDR corrected p = 1.12*10^{-3}) overrepresented GO biological processes. (L) Schematic representation of the BCL2L13 3’UTR with miR-124. The mouse BCL-RAMBO 3’UTR was not predicted to contain miR-190 binding regions, instead a putative binding region was found in the ORF. Details of cooperativity predictions are given in Table S9.
Supplementary Figure 6 – Validation of exogenous expression of FLAGhBCL2L13 in hippocampal NSPC cultures and casp-3 as a target of BCL2L13.

(A) Representative immunoblots and bar graph showing quantifications of endogenous BCL2L13 (endogenous-BCL2L13) expression or FLAG-tagged hBCL2L13 (FLAG-hBCL2L13) after transfection. Transfection significantly increases FLAG immunoreactivity (** p <0.01) without affecting endogenous- BCL2L13 (ns, p > 0.05).

(B) Representative immunoblots and bar graph showing quantifications of BCL2L13 expression after siSCR (scramble siRNA) and siBCL2L13 transfection and subsequent KA treatment. siBCL2L13 transfection significantly decreases BCL2L13 immunoreactivity (** p <0.01). (C) Representative immunoblots and bar graph showing quantifications of pro- and cl-casp3 expression after siSCR (scramble siRNA) and siBCL2L13 transfection and subsequent KA treatment. When treated with 30μM KA, siBCL2L13 transfected NSPC showed a significant abolishment of the KA induced increase in cl-casp3 levels (* p < 0.05, compared to siSCR+veh; ** p < 0.05, compared to siSCR+KA).
MicroRNAs control BCL2L13 expression in neural stem cells

Supplementary Figure 7 - Full-length blots corresponding to Figure 6 and 8B. Representative full-length immunoblots showing the effect of miR-124 and miR-137 alone or in combination on endogenous BCL2L13 protein levels (A, corresponding to data presented in Figure 6) and on pro- and cleaved-caspase3 levels (B, corresponding to data presented in Figure 8B) in hippocampal NSPC cultures. Lanes marked with a dash (-) contained samples irrelevant for the experiments described in the figures. All samples were run on the same gel and transferred to the same membrane, which was cut after transfer at the position indicated by the horizontal black line to avoid possible antibody cross-reactivity during immunoblotting. Molecular weights (kD) were estimated using Precision Plus Dual Color Standards (Bio-Rad), indicated as MWM.

Supplementary Table 1 - List of peptide identifications measured by proteomics after KA-SE in the DG.
Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s9.xls.

Supplementary Table 2 - Significantly dysregulated proteins measured by proteomics after KA-SE in the DG.
Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s10.xls.

Supplementary Table 3 - Genecodis GO analysis of KA-SE induced significantly upregulated proteins.
Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s11.xls.

Supplementary Table 4 - Genecodis GO analysis of KA-SE induced significantly downregulated proteins.
Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s12.xls.

Supplementary Table 5 - List of transcriptomic changes after KA-SE in the DG.
Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s13.xls.

Supplementary Table 6 - miR profile after KA-SE in the DG.
Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s14.xls.

Supplementary Table 7 - List of miRECORDs predicted mmu-miR-124 targets.
Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s15.xls.

Supplementary Table 8 - List of miRECORDs predicted mmu-miR-137 targets.
Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s16.xls.
Supplementary Table 9 - miRECORDs and RNA22 lists of predicted miR seed regions in BCL2L13’s 3’UTR.
Inclusion criteria for predicted cooperative action together with miR-124 were: miR among the upregulated brain enriched or specific miRs (Figure 4C), miR with at least a 7mer seed base pairing in the UTR, miR seed region within 80nt proximity of miR-124’s first or last bp seed regions (between 1520-1700 or between 2318-2498). Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s17.xls.

Supplementary Table 10 - Genecodis GO analysis of the 336 mmu-miR-124 and mmu-miR-137 miRECORDs predicted common targets.
Apoptosis is among the significantly (p = 1.65*10^{-10}) overrepresented GO biological processes with BCL2L13 as a member. Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s18.xls.

Supplementary Table 11 - List of miRECORDs predicted mmu-miR-9 target mRNAs and Genecodis GO analysis of the 690 mmu-miR-124 and mmu-miR-9 predicted common targets.
Apoptosis is among the significantly (p = 1.26*10^{-4}) overrepresented GO biological processes with BCL2L13 as a member. Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s19.xls.

Supplementary Table 12 - List of miRECORDs predicted mmu-miR-125a-3p target mRNAs and Genecodis GO analysis of the 585 mmu-miR-124 and mmu-miR-125a-3p common targets.
Apoptosis is among the significantly (p = 1.55*10^{-3}) overrepresented GO biological processes with BCL2L13 as a member. Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s20.xls.

Supplementary Table 13 - List of miRECORDs predicted mmu-miR-125b-3p target mRNAs and Genecodis GO analysis of the 139 mmu-miR-124 and mmu-miR-125b-3p common targets.
Apoptosis is not among the significantly overrepresented GO biological processes (p > 0.05). Bioinformatics analysis predicted seed regions for mmu-miR-125b-3p were predicted in BCL2L13 3’UTR. Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s21.xls.

Supplementary Table 14 - List of miRECORDs predicted mmu-miR-135a target mRNAs and Genecodis GO analysis of the 139 mmu-miR-124 and mmu-miR-135a common targets.
Apoptosis is among the significantly (p = 2.30*10^{-3}) overrepresented GO biological processes with BCL2L13 as a member. Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s22.xls.

Supplementary Table 15 - List of miRECORDs predicted mmu-miR-135b target mRNAs.
Genecodis GO analysis of the 643 mmu-miR-124 and mmu-miR-135b common targets.
Apoptosis is among the significantly (p = 3.88*10^{-4}) overrepresented GO biological processes with BCL2L13 as a member. Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s23.xls.

Supplementary Table 16 - List of miRECORDs predicted mmu-miR-190 target mRNAs and Genecodis GO analysis of the 277 mmu-miR-124 and mmu-miR-190 common targets.
Apoptosis is among the significantly (p = 1.12*10^{-2}) overrepresented GO biological processes with BCL2L13 as a member. Data can be found at: http://www.nature.com/article-assets/npg/srep/2015/150724/srep12448/extref/srep12448-s24.xls.