SUPPLEMENTAL INFORMATION

SUPPLEMENTAL METHODS

Histology, immunohistochemistry

Embryos and brains were dissected and fixed following the above-mentioned protocol. Paraffin sections were dewaxed, rehydrated and stained with 0.1% cresyl violet.

For deoxyuridine derivatives and p27Kip1, brains were fixed with PFA4%, cryoprotected with 10-30% of sucrose and cryo-sectioned at 10µm. For BrdU-IdU experiments, sections were treated with 2N HCl for 30 min at 37°C, wash in PBS three times. For all immunohistochemical staining, sections were blocked with 10% serum for one hour and incubated more than 2 hours at room temperature with specific monoclonal antibody including mouse anti-BrdU (and IdU) (1/50), p27Kip1(1/1000) primary antibodies from Becton Dickinson Ltd, rabbit anti cleaved-caspase3 (1/200) from Cell Signaling Ltd, goat anti-Cux1 (1/100, Santa Cruz Ltd), mouse anti-Satb2 (1/100), rabbit anti-Tbr1 (1/500), or rat monoclonal anti-BrdU (1/200) from Abcam Ltd. Secondary antibodies used were either Cy3-conjugated goat anti-rat IgM (1/500) or goat anti-mouse conjugates labelled with Alexa-488 (1/1000) or -546 (1/500) purchased from Invitrogen Ltd.

For paraffin-embedded embryos or brains, coronal sections were dipped in ascending grades of ethanol. Slides were microwaved in 10mM sodium citrate, blocked for an hour in 10% serum before an overnight incubation with the following primary antibodies: mouse anti-NeuN (NeuN) (1/500, Chemicon Ltd), rabbit anti-Calretinin (1/2000, Swant Ltd), rabbit anti-Glial Fibrillary Acidic Protein (GFAP) (1/1000, Dako Ltd), rabbit anti-Caspase 3 (1/200, Cell Signaling Ltd), rabbit anti-Doublecortin (DCX) (1/200, Abcam Ltd). All antibodies were polyclonal except for NeuN.

After a first incubation with biotinylated anti rabbit or anti mouse IgG 1% (1/100) followed by a second incubation with avidin-biotin-peroxydase complex (ABC kit, Vectastain, Vector laboratories, Ltd) the final detection of the peroxydase reaction was obtained with diaminobenzidine as chromogen (Sigma, Ltd) according to manufacturer's recommendations.

All images were collected with confocal microscopy or optic microscopy (Zeiss, Ltd) and processed using Axiovision (Zeiss) or Photoshop (Adobe) softwares.

Quantifications

Cortical thickness at P5 was measured on coronal sections at rostral (1) and caudal (2) levels of the primary somatosensory cortex (The mouse brain in stereotaxic coordinates, G. Paxinos and KBJ Franklin, 2004, compact second edition, Elsevier, level: Interaural/Bregma 4.90/1.10 and 1.98/-1.82 respectively). The thickness of primary somatosensory cortical layers II-IV and layers V-VI were measured from coronal sections at the same level as mentioned above (level 2). Cortical surface was measured at the same age, from the cingulate cortex to the rhinal sulcus (at mouse Paxinos Atlas level Interaural/Bregma 3.94/-0.94, n=5 per condition), using Image J (NIH).

Quantification of NeuN positive neurons was performed in the S1 somatosensory cortex area of P5 animals at the same levels than those mentioned above (1, 2). Three or four non serial adjacent sections were assessed at x200 magnification within a 1mm² grid, on at least 4 animals in each group.

Quantification of BrdU-IdU, p27kip1, cleaved-capase3 and DAPI was performed within the Ventricular Zone of the dorsal telencephalon on E11.5. The total number of cells stained was counted at x40 magnification into bins (perpendicular to the apical surface of the ventricular zone: 150µm in height, 90µm in lateral for BrdU and 160µm - 45µm for cleaved-caspase3 and p27kip1 experiments), in three serial non adjacent sections, on at least 6 embryos in each group.

Neurosphere-derived progenitors culture

Forebrains were dissected from E10.5 embryos in CO_2 independent medium (Gibco Ltd, US) supplemented with Penicillin (500 Units/ml of medium) Streptomycin (500µg/ml of medium) and L-Glutamine (2mM) was added. Forebrains were incubated in trypsin and DNAse-I solution for 10 min at 37°C. Digestion was stopped by addition of foetal bovin serum (FBS), and the neural tubes were transferred in a petri dish filled with CO_2 independent medium supplemented with FBS 10% and DNAse-I to carefully separate neuroepithelia from surrounding tissues. Neuroepithelia were washed with PBS, collected by centrifugation, then mechanically dissociated by pipetting and resuspended in the culture

medium [Neurobasal medium containing L-Glutamine 2mM, Penicillin (500 Units/ml of medium), Streptomycin (500µg/ml of medium), N2 supplement (Gibco Ltd) and supplemented every two days with Fibroblast Growth Factor-Basic heparin (FGF2) (12 ng/ml Sigma Ltd)]. Cells were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2 in an unprecoated petri dish.

In these culture conditions, neural stem cells proliferate as neurospheres. Neurospheres were kept floating until day in vitro 4 (DIV4) then harvested, dissociated first mechanically then enzymatically (TrypLE express, Invitrogen) for 20 min at 37°C and transferred in 6-, 12- or 96-well plates for quantitative PCR, western blot or BrdU experiments.

Cell proliferation studies

BrdU incorporation assay (cell proliferation kit, Roche Ltd) was performed according to manufacturer's instructions. Following the first passage of neurospheres, cells were seeded in a 96-well plate at a density of 10,000 cells per well (DIV0). On the next day (DIV1), BrdU (10µM) was added and incubation carried out for 4 hours. Then, medium was quickly removed by aspiration and cells were incubated for 30 min with FixDenat for 30 min at room temperature prior to incubation with the anti BrdU-POD working solution for 90 min. Ultimately, after washing, cells were incubated with substrate solution for 20 min. Absorbance was measured at 390 nm on Victor spectrophotometer.

Kinase assay

Chk1 kinase assay was performed using Active Chk1 kinase IP-kinase assay (DuoSet IC, R&D Systems) according to the manufacturer's instructions. Tissue samples (telencephalon or cortices from E12 to P0) or neurosphere cultures were collected and soaked in lysis buffer prior to immunoprecipitation with active Chk1 kinase antibody coated on agarose beads. Active Chk1 contained in IP pellets was finally incubated with the supplied biotinylated kinase substrate in the presence of ATP and Mg^{2+} cations for 45 min at room temperature. The kinase reaction was then stopped and detection of phosphorylated substrate was performed on streptavidin-coated 96-well plates. Colorimetric reaction was finally quantified on a microplate reader set at 450 nm.

Lentiviral-mediated cell transduction

ShRNA expressing lentiviral particles (Mission) were purchased from Sigma Aldrich Ltd. Sequences corresponding to the shRNAs are given in supplemental Table 3. Cell transduction with lentiviral particles was performed according to manufacturer's recommendations. In brief, on day 0 (DIV 0), cells were seeded in a 96-well plate at a density of 10,000 cells/well for 20 hours at 37°C. On DIV 1, medium was changed and lentiviral particles were added to the medium at multiplicity of infection (MOI) 5 for the neurosphere-derived progenitors for 20 hours. To determine the optimal virus-to-cell ratio, the MOI used corresponded to the lowest viral concentrations that still delivered the optimal transduction efficiency as established with the Turbo GFP Control lentiviral particles (Sigma Aldrich, Ltd). On DIV 3, medium was replaced with fresh medium and cells incubate for two more days. Finally, at DIV 5, cells were collected for RNA extraction or BrdU experiments and on DIV12 for protein extraction. Of note, neither puromycine selection nor hexadimethrine bromide enhancer were used in our experimental setup.

SUPPLEMENTAL TABLES

Supplemental Table 1: Calculation of the cell cycle kinetics by the IdU/BrdU double labeling

	P cells (DAPI)		L cells		S cells	
	CTRL	VA	CTRL	VA	CTRL	VA
mean	113,8	96,60	14,25	5,400	51,50	29,00
st. Error	7,793	3,444	0,4787	0,7483	3,663	1,897

CTRL (n = 4) VA (n = 5)

L cells = IdU+/BrdU-

S cells = IdU+/BrdU+

P cells = total number of proliferating cells, estimated by DAPI count in the VZ in the sampling area.

Ti = 1,5h

Ti/Ts = L cells / S cells → Ts = Ti / (L cells / S cells)

Ts/Tc = S cells / P cells → Tc = Ts / (S cells / P cells)

Supplemental Table 2: primer sequences used to specifically amplify genes of interest by quantitative RT-PCR

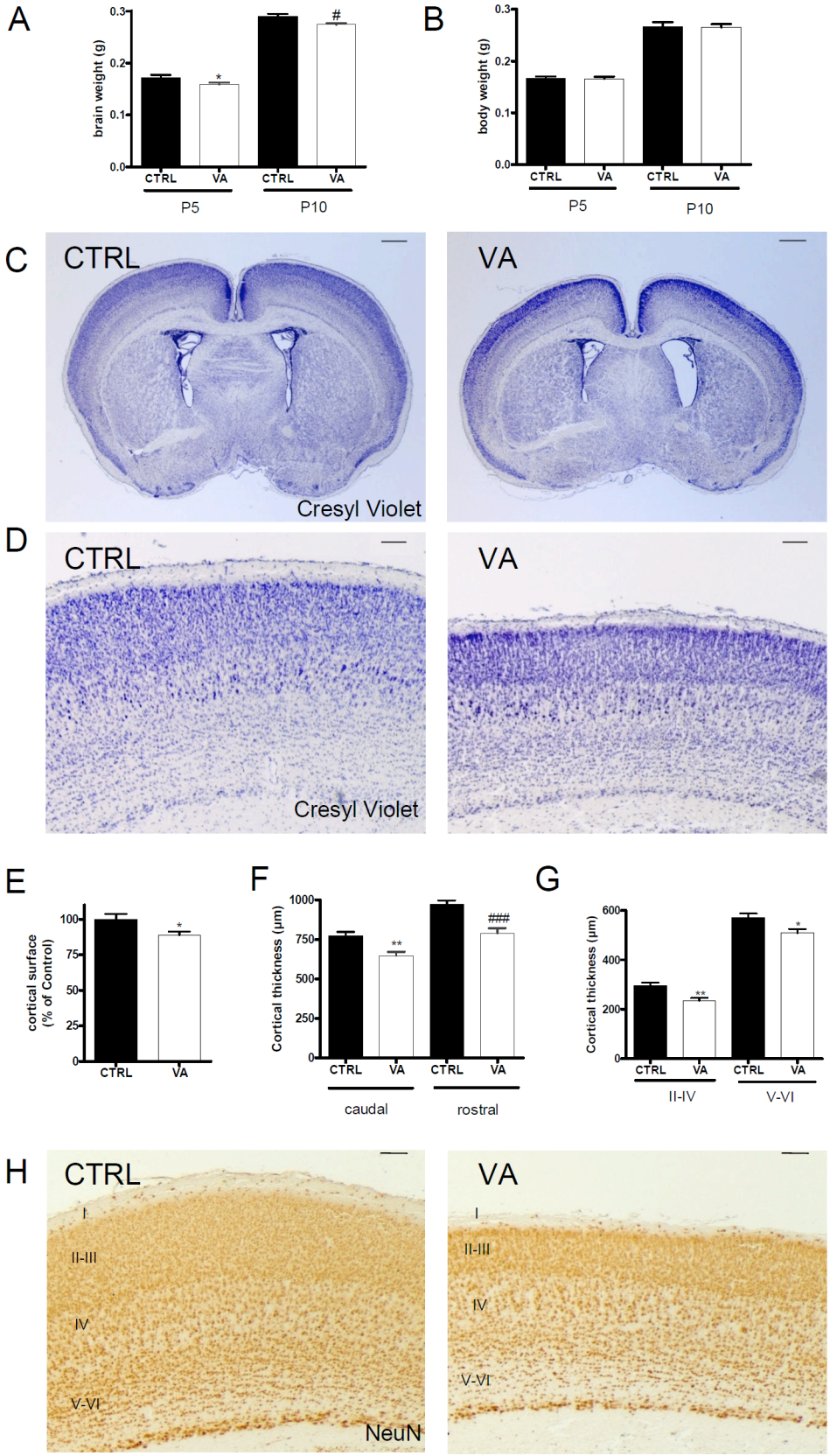
Genes	Primer sequences (sense and reverse)*
Mcph1	5'-GCACAGGCCTTGTCAGACCTC-OH3' 5'-GCTTCTCAGATGGCATGCTTG-OH3'
Chk1	5'-AAGCACATTCATTCCAATTTG-OH3' 5'-TGGCTGGGAACTAGAGAACTT-OH3'
Brca1	5'-CTTGTGCCCTGGGAAGACCTG-OH3' 5'-GCGCTCTTCAAATTTTGGCTT-OH3'
Wdr62	5'-ACAGGAAGCCTCCAACACC –OH3' 5'-GCATGTGAGCTCGTTGTGGAC-OH3'
Cdk5rap2	5'-TACCACCATCTCCTGCCTGA-OH3' 5'-CTGTCTGGCTTCGGGTCTCTA-OH3'
Cep152	5'- TCCGCGGGCAGTACATTA-OH3' 5'- CGCAACACTTCCGCTTTTACC-OH3'
Aspm	5'-TCCACTTTACAGCAGCTGCCT-OH3' 5'-CCATGTGCTTCTTAGCGTTCC-OH3'
Cenpj	5'-CCCAATGGAACTCGGAAAGA-OH3' 5'-CATGACCTGCTTCACATCACC-OH3'
Vpac1	5'-TCACTATGTCATGTTTGCCTT-OH3' 5'-GAAAGACCCTACGACGAGTT-OH3'
Vpac2	5'-TCTACAGCAGACCAGGAAACA-OH3' 5'-GTAGCCACACGCATCTATGAA-OH3'
Pac1	5'-ACTGCGTGGTGTCCAACTACT-OH3' 5'-TCTCCTCTCAGGGAAGAAGGT-OH3'
Cdc25	5'-CCCTCGAATGTGCCGTTCTC-OH3' 5'-CCCCCTTTGAGGATATATAGC-OH3'
Cyclin A	5'-GCTGCTAGCTTCGAAGTTTGA-OH3' 5'-AGGTGCTCCATTCTCAGAACC-OH3'
Cyclin B	5'-CAAAATACCTACAGGGTCGTG-OH3' 5'-GTCTCCTGAAGCAGCCTAAAT-OH3'
Cdk2	5'-TCCTTCACCGAGACCTTAAGC-OH3' 5'-AGTTCGGACAGGGACTCCAA-OH3'
Hprt	5'-GGTGAAAAGGACCTCTCGAA-OH3' 5'-CAAGGGCATATCCAACAACA-OH3'

*(all primer sets were designed to generate 85-110bp amplicons)

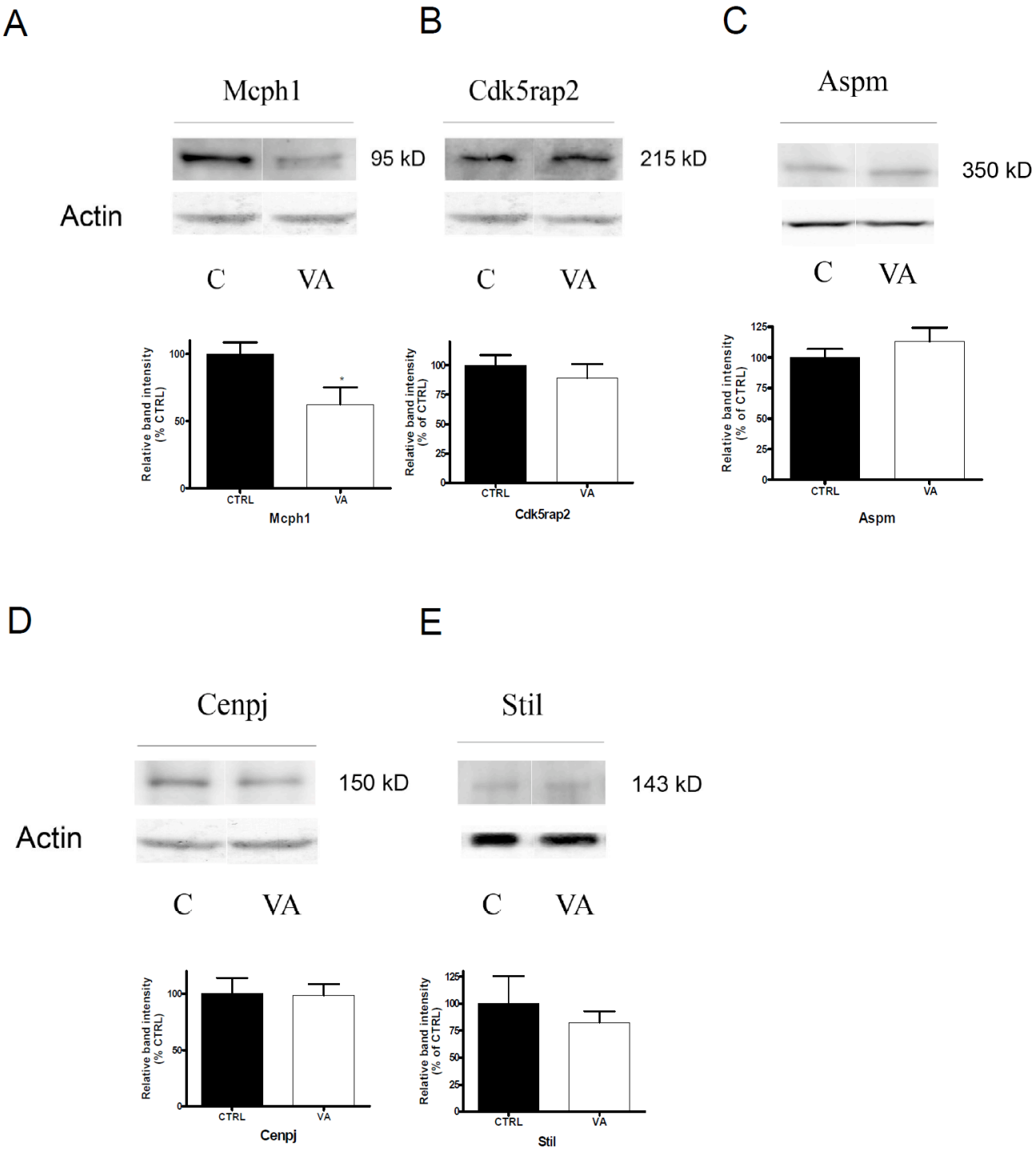
Supplemental Table 3: Lentiviral particles (controls and specific inserts) designed to assess transduction efficacy and silencing efficiency of *Chk1* expression in neurosphere-derived progenitors.

Knockdown gene	**Sh sequence/Vector description**
Empty Vector	No shRNA Insert
non target shRNA control	CCGG**CAACAAGATGAAGAGCACCAA**CTC GAG**TTGGTGCTCTTCATCTTGTTG**TTTTT
Turbo GFP Control	No shRNA insert. Contains TurboGFP gene, under the control of the CMV promoter.
Mcph1-sh1	CCG**GGCACTTGTTGATGAGTCTTTG**CTC GAG**CAAAGACTCATCAACAAGTGC**TTTTTG
Mcph1-sh2	CCG**GTGCCGACTTGAACGCCA**TTTACTC GAG**TAAATGGCGTTCAAGTCGGCA**TTTTTG
Mcph1-sh3	CCG**GCTCCTACGATGTCCATCATAG**CTC GAG**CTATGATGGACATCGTAGGAG**TTTTTG
Mcph1-sh4	CCG**GAGATACTTGTTCCCAATTATA**CTC GAG**TATAATTGGGAACAAGTATC**TTTTTTG
Mcph1-sh5	CCG**GCCACTCATCTTTCGGTGATTC**CTC GAG**GAATCACCGAAAGATGAGTGG**TTTTTG
Chk1-sh1	CCGG**GTGGAAGAAGAGTTGTATGAA**CTC GAG**TTCATACAACTCTTCTTCCAC**TTTTT
Chk1-sh2	CCGG**GCTGTGAATAGAATAACTGAA**CTC GAG**TTCAGTTATTCTATTCACAGC**TTTTT
Chk1-sh3	CCGG**GCAACGGTATTTCGGCATAAT**CTC GAG**ATTATGCCGAAATACCGTTGC**TTTTT
Chk1-sh4	CCGG**GCCACGAGAATGTAGTGAAAT**CTC GAG**ATTTCACTACATTCTCGTGGC**TTTTT
Chk1-sh5	CCGG**CCCATGTAGTAGTATCACTTT**CTC GAG**AAAGTGATACTACTACATGGG**TTTTT

SUPPLEMENTAL FIGURES

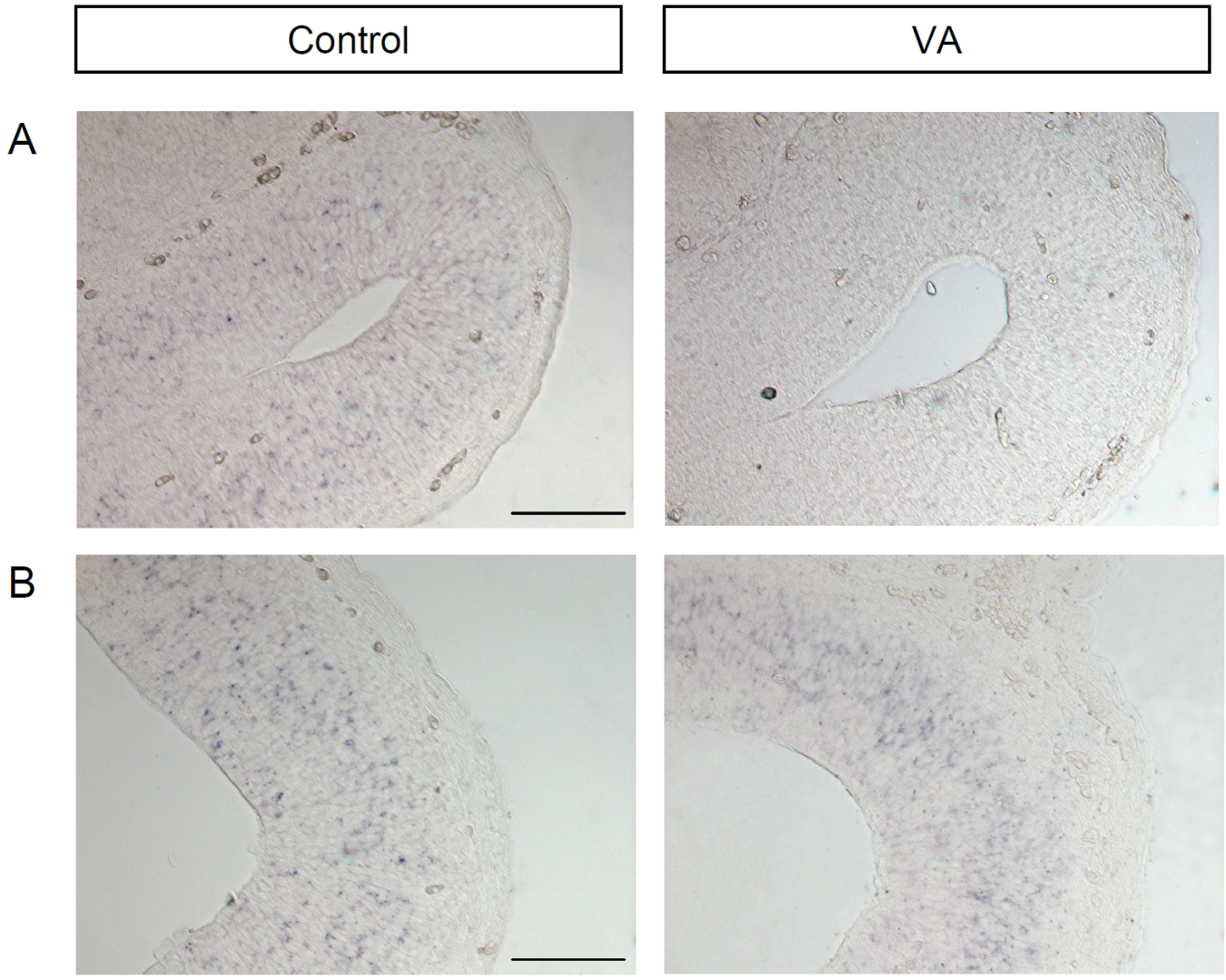


Supplemental Figure 1: VIP blockade during neurogenesis induces microcephaly with thinner cortex.
A-B: Brain and body weight analyzes. VA injection in pregnant mice results in a selective reduction in brain weight of litters at P5 and P10 (1A, n=15 per group, unpaired t-test, * or #$p<0.05$) without significant body weight loss from controls (1B, n=15 per group, unpaired t-test, * or #$p<0.05$).
C-D: Coronal P5 brain sections labeled by Cresyl Violet staining shown at low (x2.5, 1A) and high (x10, 1B) magnifications, reveal a dramatic reduction in cortical surface and thickness in VA-treated animals when compared to age matched controls (Scale bars= 500 and 100µm, respectively).
E-G: At P5: quantification of cortical surface (C) and cortical thickness (D, E) in primary somatosensory cortex (S1) of Control and VA brains.
H: NeuN (F) immunostainings at high (x10) magnification. Note the proper lamination of the primary somatosensory cortex into six layers remains unchanged in VA as in control brains (Scale bars= 500µm).



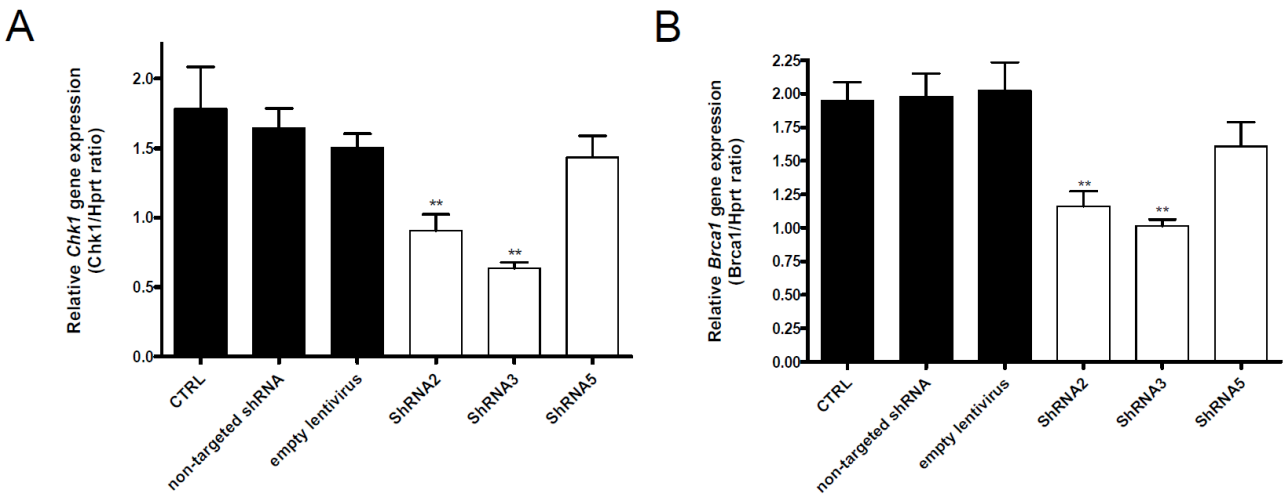
Supplemental Figure 2: VA treatment reduces the expression of Mcph1 protein at E12.5

A-E: Expression levels of Mcph1, Cdk5rap2, Aspm, Cenpj and Stil assessed by western blot on protein extracts from telencephalon of E12.5 embryos (CTRL or VA treated). Parallel to the reduction of *Mcph1* transcripts, VA induces a significant reduction in Mcph1 protein expression (n=8, t-test, $p<0.05$*) compared to CTRL at E12.5 (**A**). In contrast, no clear difference is observed between CTRL and VA extracts for Cdk5rap2, Aspm, Cenpj and Stil (**B-E**). The lanes were run on the same gel but were non-contiguous.

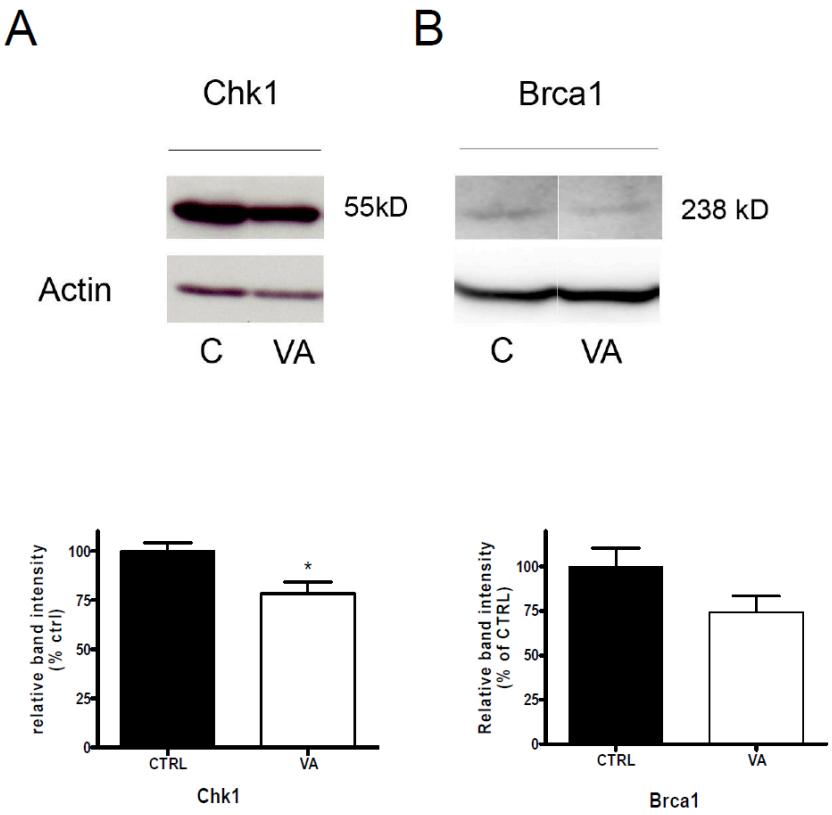


Supplemental Figure 3: VA treatment decreases the level of *Mcph1* expression and changes its pattern of expression

A-B: Expression of *Mcph1* gene by in situ hybridization in CTRL and VA treated embryos at E12. Expression of *Mcph1* gene is lost in the caudal area of the dorsal telencephalon of VA treated embryos when compared to CTRL at E12.5 (**A**). VA treatment changes the pattern of expression of *Mcph1* gene expression in the rostral area of the dorsal telencephalon relative to CTRL: *Mcph1* is not expressed in the neuroepithelium close to the ventricle when compared to CTRL (Scale bars= 40µm).



Supplemental Figure 4: Silencing of *Mcph1* gene expression reduces both *Chk1* and *Brca1* transcript levels
A-B: *Chk1* and *Brca1* expression levels assessed by quantitative RT-PCR on extracts from neurosphere-derived progenitors transduced by *Mcph1* specific lentiviral-mediated shRNA. Knock down of *Mcph1* expression by sequences shRNA 2 and 3 significantly reduces both *Chk1* and *Brca1* gene expression (n=3 per group, run in duplicate, one-way ANOVA, $p<0.05$*, $p<0.01$**, $p<0.001$***), while sequence shRNA5 failed to induce any significant silencing.



Supplemental Figure 5: VA treatment reduces Chk1 protein level at E12.5
A-B: Expression levels of Chk1 and Brca1 assessed by western blot on protein extracts from telencephalon of E12.5 embryos (CTRL or VA treated). Parallel to the reduction of *Chk1* transcripts, VA induces a significant reduction in Chk1 protein expression (n=8, t-test, $p<0.05$*) compared to CTRL at E12.5 (**A**) and, although not reaching statistical significance, a decrease of Brca1 protein expression (**B**). The lanes were run on the same gel but were non-contiguous.