Cell Reports, Volume *22*

Supplemental Information

Activity Regulates Cell Death within Cortical Interneurons through a Calcineurin-Dependent Mechanism

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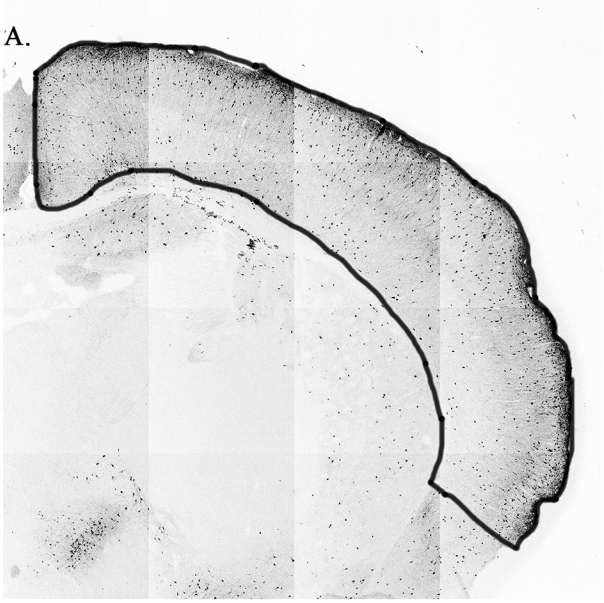
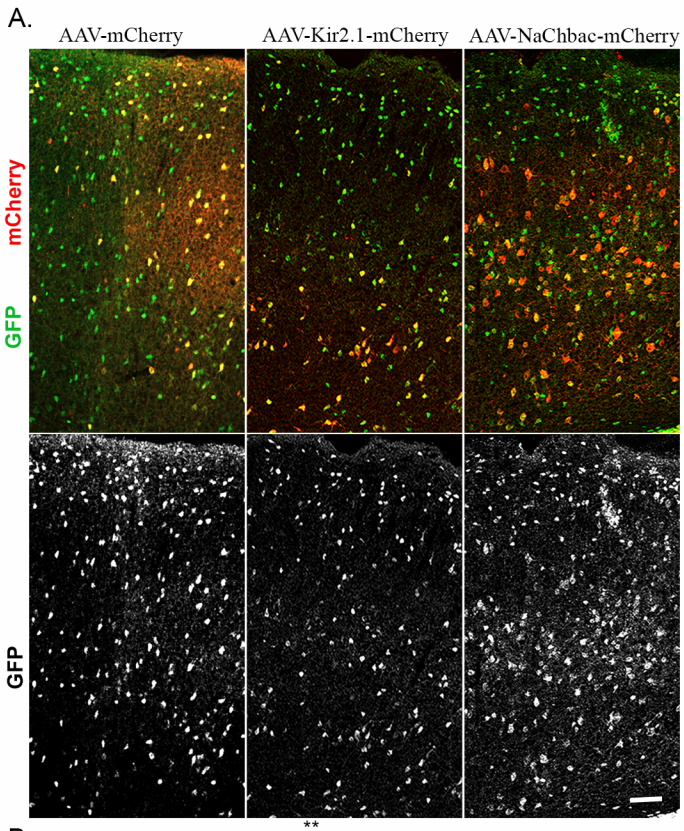
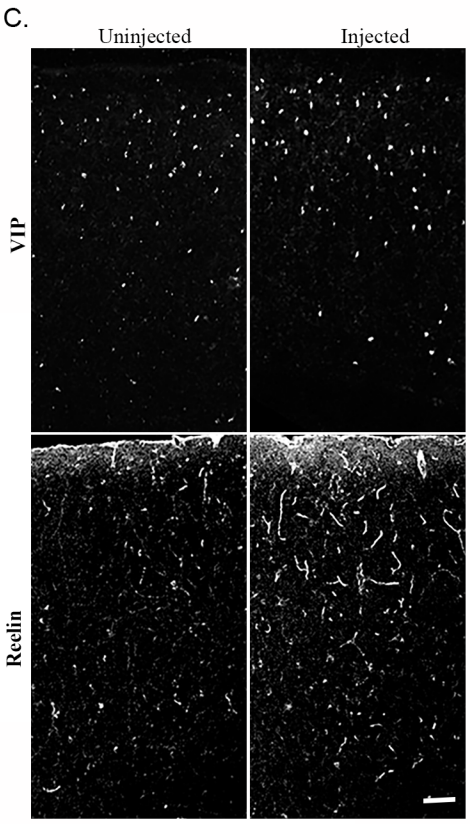
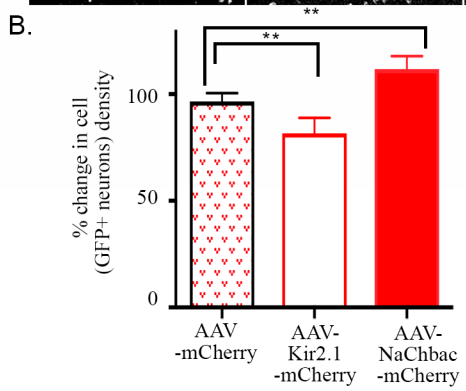


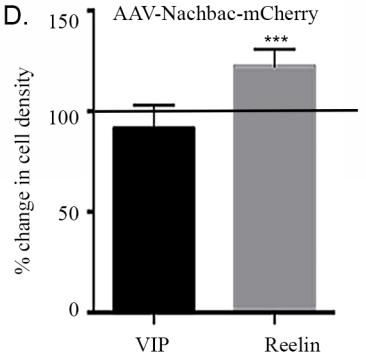
Figure S1.Realated to Experimental Procedure. Representative image of brain slice obtained from 5HT3aR^{eGFP} transgenic animal.

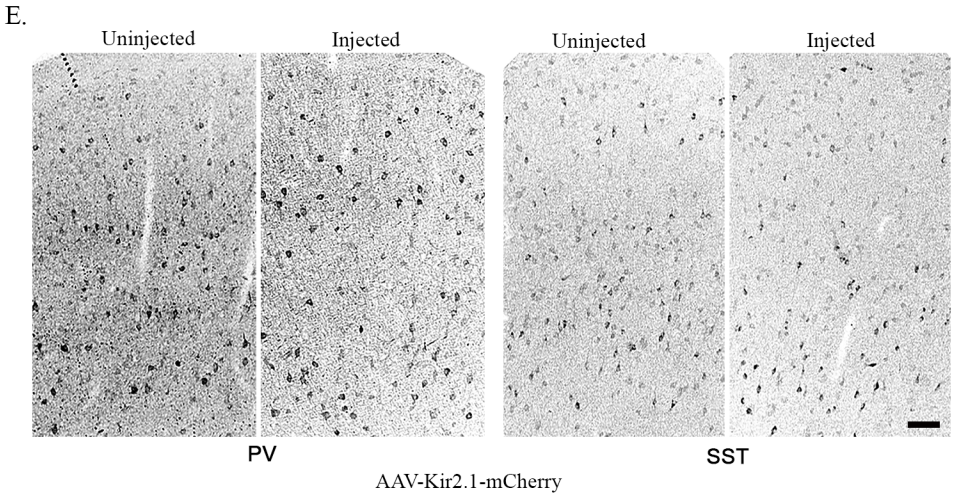
A) GFP+ cells are shown in grayscale. The region of interest is demarcated as shown by the outline.


A.
AAV-mCherry
AAV-Kir2.1-mCherry
AAV-NaChbac-mCherry
mCherry
GFP
GFP


C.
Uninjected
Injected
VIP
Reelin


B.
**
**
% change in cell (GFP+ neurons) density
100
50
0
AAV -mCherry
AAV-Kir2.1 -mCherry
AAV-NaChbac -mCherry


D.
AAV-Nachbac-mCherry
***
% change in cell density
150
100
50
0
VIP
Reelin


E.
Uninjected
Injected
Uninjected
Injected
PV
SST
AAV-Kir2.1-mCherry

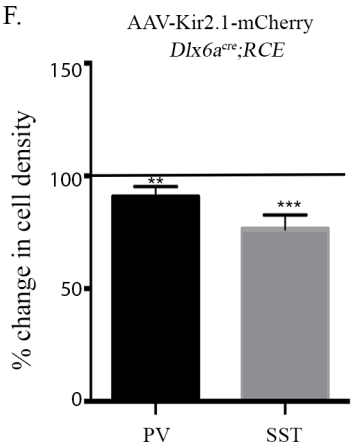

F.
AAV-Kir2.1-mCherry
Dlx6a^cre;RCE
% change in cell density
150
100
50
0
**
***
PV
SST

Figure S2. Related to Figure 2. Cell survival of all interneuron subtypes, except VIP expressing interneurons, are affected by changes in neuronal activity.

A) Representative images of corresponding sections from the Dlx6acre;RCE brains injected with AAV-flex-mCherry (left panel), AAV-flex-Kir2.1-P2A-mCherry (middle panel) and AAV-flex NaChBAC-P2A-mCherry(right panel). **B)** Quantification of the number of GFP+ interneurons upon mCherry, Kir2.1-mCherry and Nachbac- mCherry injections AAV Kir2.1-mCherry: n=5, p=0.0382, AAV-NaChbac-mCherry: n=5, p =0.0045 (Unpaired t- test) **C)** Representative image of the cortical section with unilateral AAV-flex NaChBAC- mCherry injection showing immunostaining with VIP (top panel) and Reelin (bottom panel). **D)** The percent change number of VIP expressing interneurons remain unaffected upon NaChBac overexpression, p=0.2704 and an increase in reelin expressing interneurons is observed on the injected hemisphere, p=0.0019. The number of neurons is normalized to the corresponding values on the uninjected hemisphere. **E)** Representative images of the coronal sections with unilateral injections of AAV-flex Kir2.1-P2A-mCherry, immunostained for PV (left panel) and SST (right panel). **F)** The percent change in the number of PV (p=0.0070) and SST (p=0.0003) expressing interneurons show a decrease upon injections with AAV-flex Kir2.1-P2A-mCherry on the injected side. Scale bar= 50 μm

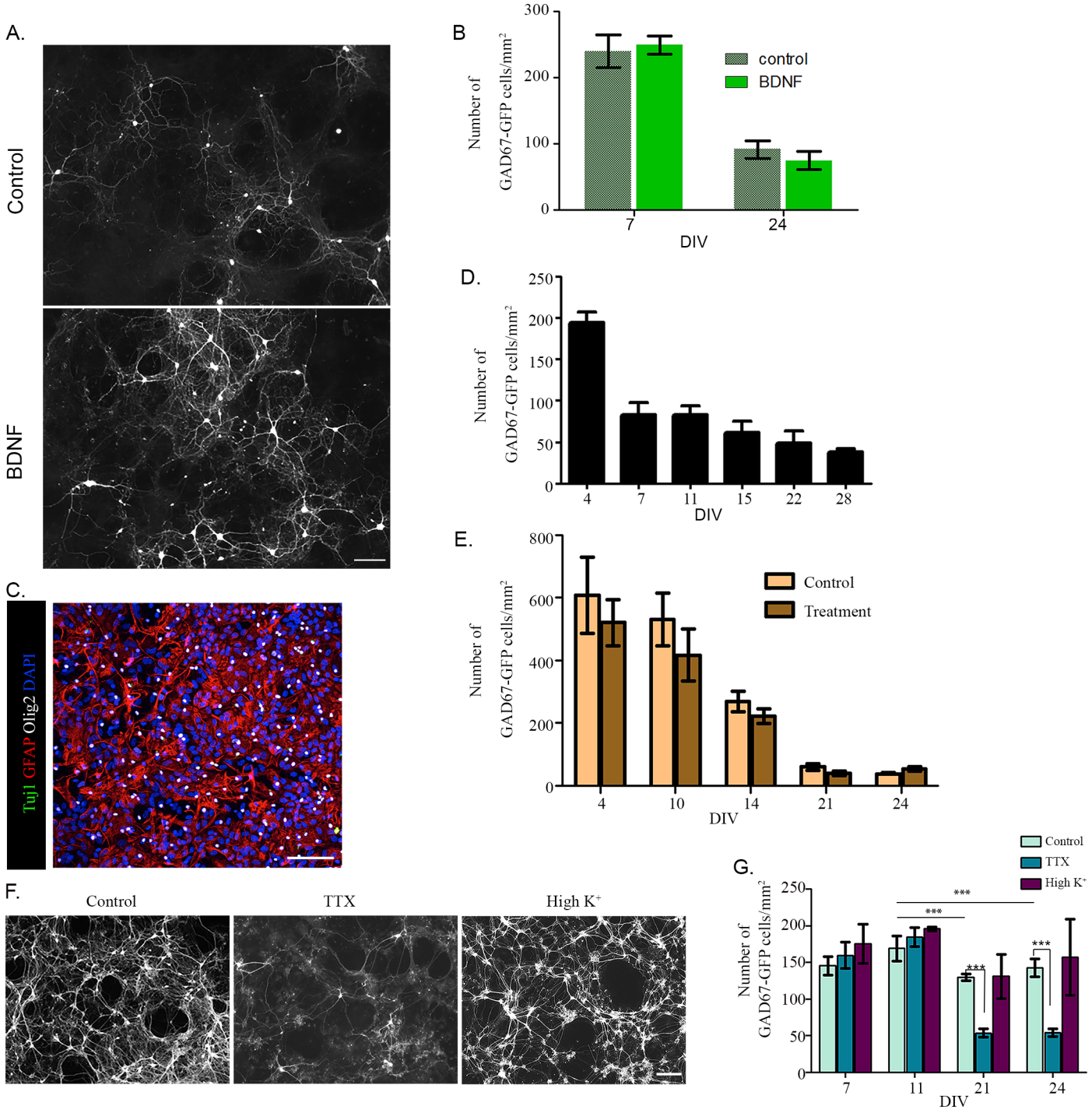


Figure S3.Related to Figure 2. Cell death is not altered by culturing interneurons in BDNF-, Glial- or Neuronal-conditioned medium.

A) GAD67GFP neuronal cultures on Primary feeder layers prepared from P0 to P2 neocortex subjected to control and BDNF conditioned medium. **B)** Quantification of number of GAD67GFP cells at 7 and 24 DIV shows no significant difference in the survival of interneurons in control and BDNF treated conditions (ANOVA, no statistical difference $p>0.5$). **C)** Glial feeder layers prepared from P0 to P2 neocortex.The feeder layer is stained for neurons (Tuj-1, green), astrocytes (glial fibrillary acidic protein (GFAP), red) and oligodendrocytes (Olig-2,white). All cells are labeled by 4′,6-diamidino-2-phenylindole (DAPI, blue). **D)** Temporal profile of the GAD67GFP interneuron cultures on glial feeder layer. The GAD67GFP population exhibits steep decreases in number between 4 and 7 DIV, and continues to decrease by 22 DIV. **E)** Temporal profile of GAD67GFP interneuron cultures on glial feeder layer. The treatment involves exchanging of media with cortical feeder media (ANOVA, no statistical difference and $p>0.5$; $n = 3$ per time point). All error bars represent s.e.m. **F)** Representative image of GAD67GFP population *in vitro*, in control (left panel), TTX (middle panel), and high K+(right panel) treated conditions. Scale bar =50 µm. **G)** Temporal profile of the GAD67GFP population size in vitro. The GAD67GFP population has small but non-significant increase in number between 7 and 11 DIV and then declines by DIV 21. The number of GAD67GFP population decreases upon TTXtreatment by 21 DIV (ANOVA, $p<0.05$). The number of GAD67GFP population trends towards increased survival upon exposure to high K+, at 21DIV and 24DIV (ANOVA, $p<0.05$),$n=3$. All error bars represent s.e.m. Scale bar =50 µm

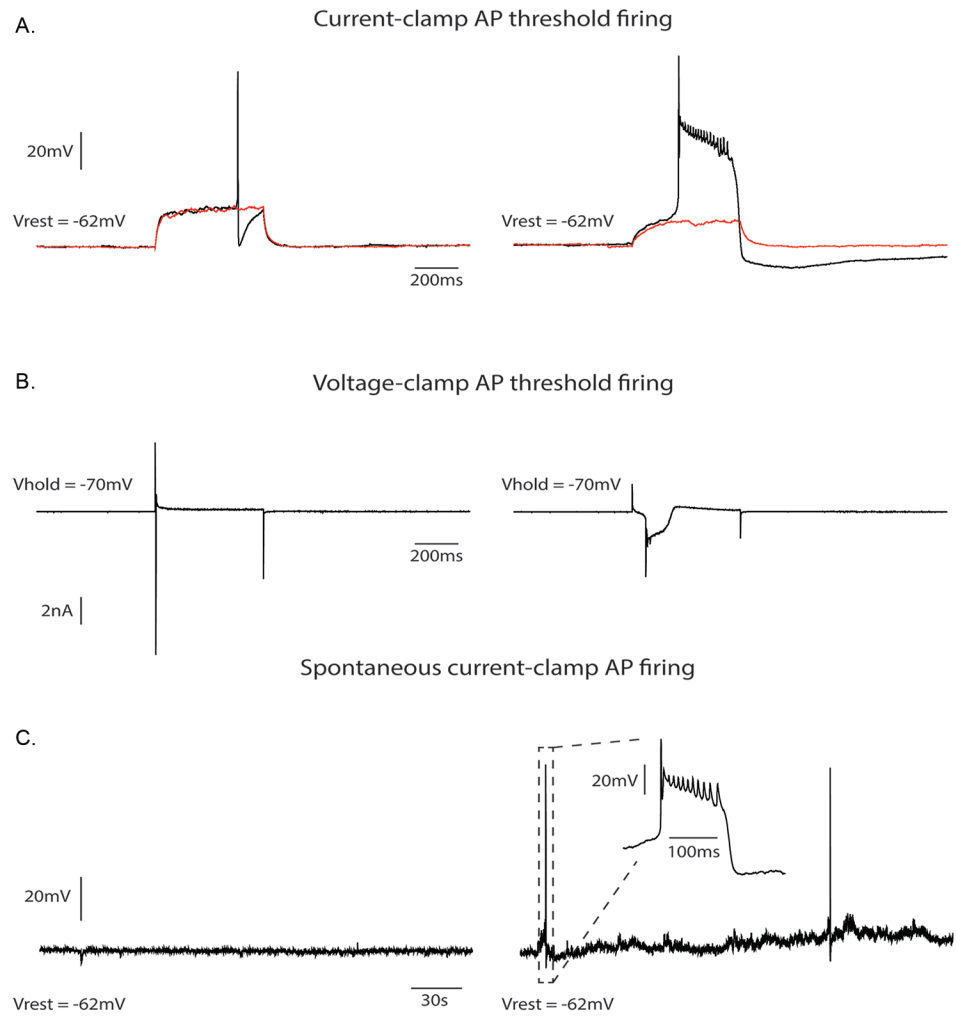
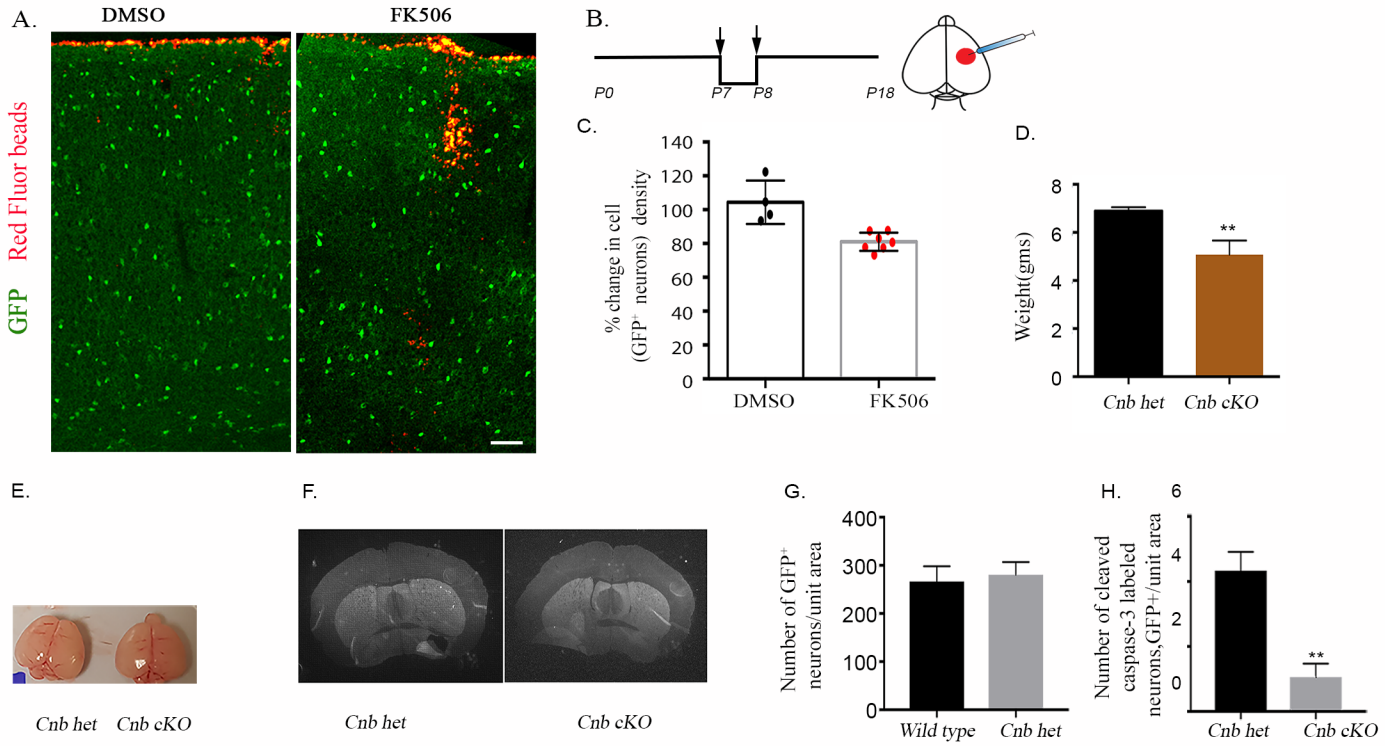
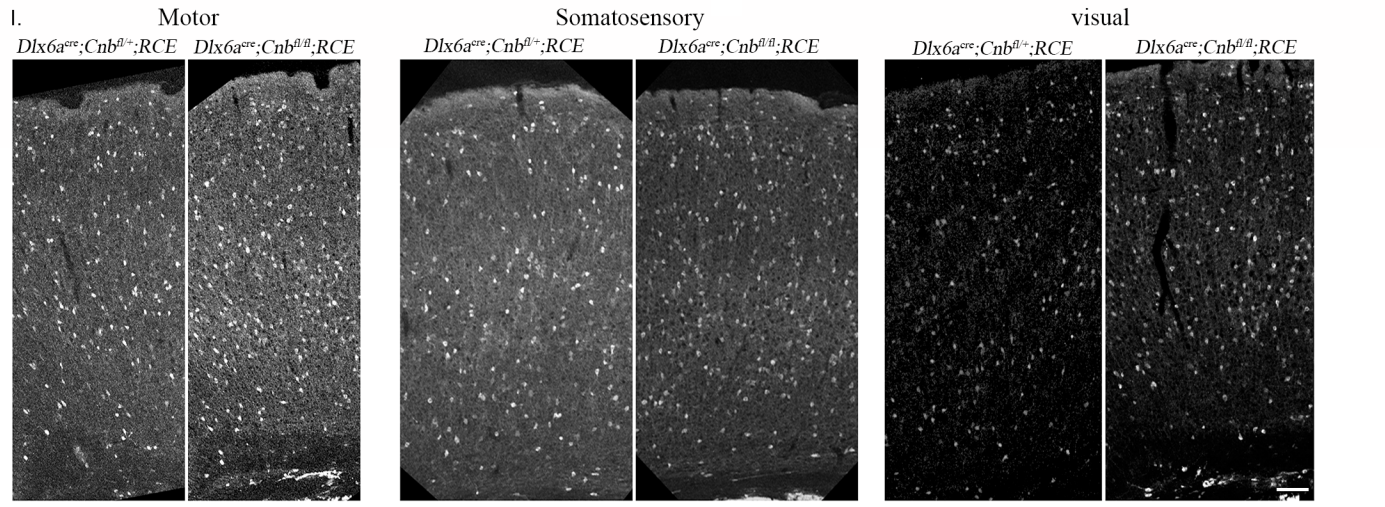
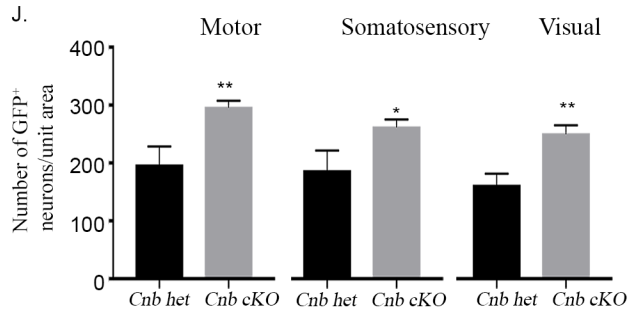

A.
Current-clamp AP threshold firing
20mV
Vrest = -62mV
200ms
Vrest = -62mV
B.
Voltage-clamp AP threshold firing
Vhold = -70mV
200ms
2nA
Vhold = -70mV
Spontaneous current-clamp AP firing
C.
20mV
Vrest = -62mV
30s
20mV
100ms
Vrest = -62mV
D.
CGE MGE non GFP cells
Cnb
β-actin
E.
CNA1 CNA2 CNA3
β-actin
F.
Wild type
Relative CNA/β-actin expression
CNA1 CNA2 CNA3
G.
VIPcre
Cnb
Tubulin
H.
Dlx6acre
VIPcre
Sham ECS Sham ECS
S774 phos-dyn 1
Tubulin
I.
Dlx6acre
VIPcre
Fold change in ratio of Phos Dyn/Tubulin
Sham ECS Sham ECS
J.
wt
Cnb cKO
S774 phos-dyn 1
Total dyn 1
K.
Fold change in ratio of Phos Dyn/Total Dyn
Wild type CnB cKO

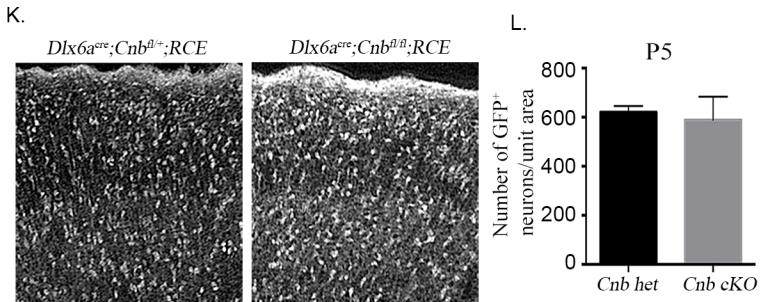
Figure S4. Related to Figure 3. Firing pattern of cortical interneurons expressing NaChBac and expression of CaN in cortical interneurons.

A) Representative traces showing the discharge of an action potential at threshold (red trace) for a control interneuron (left) and a NaChBac-expressing one (right), showing the sustained depolarization and firing in the latter. **B)** Representative traces of the same two cells shown in "a" recorded in voltage-clamp, showing the smaller fast, but more sustained slow inward current in the NaChBac -expressing cell. **C)** Traces from a different set of control and NaChBac-expressing interneurons showing spontaneous action potential firing at very low frequency in the latter and none in the former. The envelope of discharge is qualitative similar to the induced firing seen in "a". The slow firing frequency would be optimal for calcineurin activation in the NaChBac-expressing cells. **D)** Western blot showing the expression of the B regulatory subunit of the CaN in the FAC sorted population of interneurons derived from CGE, MGE and non- inhibitory neurons. **E)** Western blots showing the expression of various isoforms of catalytic subunit of CaN in wild type interneurons. **F)** Quantification showing the relative expression of the three isoforms of the catalytic subunit of CaN. **G)** Western blot of interneuron lysates, FAC-sorted from VIPcre;Ai9 labeled interneurons, showing the presence of CnB. **H)** Western blot of interneuron lysates from VIPcre and Dlx6acre lines, FAC-sorted from electro convulsive shock- or sham-treated animals and probed for phospho-S774 dynamin 1 and tubulin. **I)** Quantification of phospho- dynamin 1 reveals no change in the levels, three hours after an electro convulsive shock treatment was applied to P7 mice as compared to sham-treated littermates for lysates extracted from VIP expressing interneurons: n=3. p=0.6288 (unpaired t- test) **J)** Western blot of interneuron lysates, FAC-sorted from wild type or Cnb cKO animals (n=4 for each condition) and probed for total dynamin, and phospho-S774 dynamin 1. **K)** Quantification shows that the level of phospho dynamin is upregulated in Cnb cKO compared to wild type interneurons. p=0.0252.


A.
DMSO
FK506
Red Fluor beads
GFP
B.
P0
P7
P8
P18
C.
% change in cell (GFP+ neurons) density
140
120
100
80
60
40
20
0
DMSO
FK506
D.
Weight(gms)
8
6
4
2
0
**
Cnb het
Cnb cKO
E.
Cnb het
Cnb cKO
F.
Cnb het
Cnb cKO
G.
Number of GFP+ neurons/unit area
400
300
200
100
0
Wild type
Cnb het
H.
Number of cleaved caspase-3 labeled neurons,GFP+/unit area
6
4
2
0
**
Cnb het
Cnb cKO


I.
Motor
Somatosensory
visual
Dlx6a^cre;Cnb^fl/+;RCE
Dlx6a^cre;Cnb^fl/fl;RCE


J.
Motor
Somatosensory
Visual
Number of GFP+ neurons/unit area
400
300
200
100
0
**
*
**
Cnb het
Cnb cKO


K.
Dlx6a^cre;Cnb^fl/+;RCE
Dlx6a^cre;Cnb^fl/fl;RCE
Sacrificed at P5
L.
P5
Number of GFP+ neurons/unit area
800
600
400
200
0
Cnb het
Cnb cKO

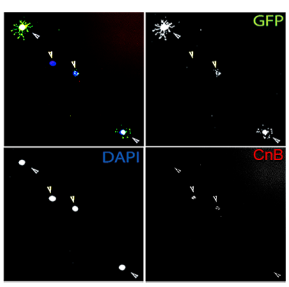

M.
GFP
DAPI
CnB

Figure S5. Related to Figure 4. Differential effect on cell survival upon disrupting calcineurin function at distinct development stages.

A) Representative image of Coronal sections showing the affect of blocking enzymatic activity of calcineurin using a pharmacological blocker, FK506 (mixed with red fluorescent beads) on the interneurons. These neurons are labeled using Dlx6acre allele, driving the expression of eGFP using RCE:loxP reporter allele. **B)** Schematic representation of the experimental strategy for blocking calcineurin activity during the peak of cell death. **C)** Quantification shows a decrease in density of interneurons upon blocking CaN function at P7-8. The student's unpaired t test is performed between DMSO and FK506 injected brains: n=5, p=0.0020 **D)** Analysis of body weight shows a decrease in Cnb cKO animals compared to their control littermates. **E)** Representative image of brain size in het and Cnb cKO animals. **F)** Representative image of coronal section showing similar cortical width of both het and cKO animal. **G)** Quantification of the density of GFP+ interneurons in the somatosensory cortex of Wild type and Cnb het animals. Mean±S.E.M. was calculated and unpaired t test was conducted for the analysis of the data, p=0.5901.**H)** Quantification of the density of cleaved caspase-3 positive interneurons show a decrease in number in Cnb cKO condition as compared to the control. Mean±S.E.M. was calculated and unpaired t test was conducted for the analysis of the data P=0.0014, n=3. **I)** Representative image of the coronal sections showing GFP labeled interneurons using a Dlx6acre;RCE:LoxP reveal that the increase in number of interneurons is consistent along the anterior-posterior axis of the cortex in Cnb cKO mice. Motor cortex (Left panel), somatosensory cortex (middle panel) and visual cortex (right panel) are represented from both cKO and their control littermates. The animals were sacrificed at P21. **J)** Quantification of the density of GFP positive interneurons showing a consistent increase along the rostral caudal axis in the CnB cKOs vs control animals. Mean±S.E.M. was calculated and unpaired t test was conducted for the analysis of the data. Motor cortex, p=0.0064. Somatosensory cortex, p=0.0225. Visual cortex, p=0.0029. **K)** Representative image of coronal sections from control and Cnb cKO brains showing the number of GFP positive interneurons at P5. **L)** Quantification determines no change in the density of neurons between control and cKO at P5.Mean±S.E.M. was calculated and unpaired t test was conducted for the analysis of the data p=0.6122. **M)** Representative image of interneurons in vitro lacking CaN regulatory protein from the conditional knockout of Cnb gene. Scale bar= 50 μm

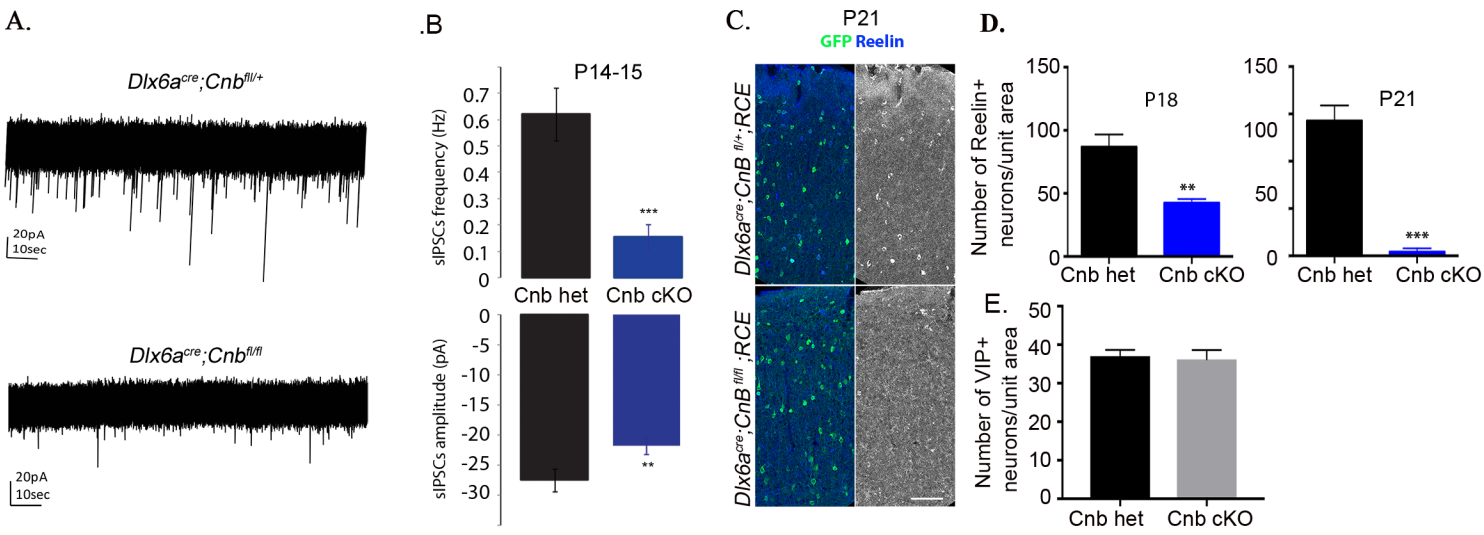


Figure S6. Related to Figure 6. Embryonic removal of calcineurin specifically regulates the maturation of Reelin expressing interneurons.

A) Representative traces of sIPSCs recorded from layer II/III pyramidal neurons in vitro (top, control;bottom, *Cnb cKO*). **B)** Quantification of sIPSCs reveals a decrease in both frequency, $p < 0.0001$ and amplitude, $p=0.034$.(Mann-Whitney non-parametric test) , n=3. Number of cells recorded= 13 cells **C)** Representative image of coronal sectionsstained for Reelin in *Cnb cKO* and control animals.Green represents GFP positive interneurons. Blue (left panel) and gray scale (right panel) represents Reelin immunostaining.**D)** Quantification of the density of Reelin- expressing interneurons in Cnb cKO, shows a reduction as compared to controls. Quantification at P18, $p=0.0019$ (left panel) and quantification at P21,$p<0.0001$(Right panel),(Mean (±sem values were calculated,unpaired t test) **E)** Quantification of the density of VIP+ interneurons in control and *Cnb cKO* animals at P21,$p=0.5874$.(unpaired t test). $n\geq3$ for each condition. Scale bar= 50 μm

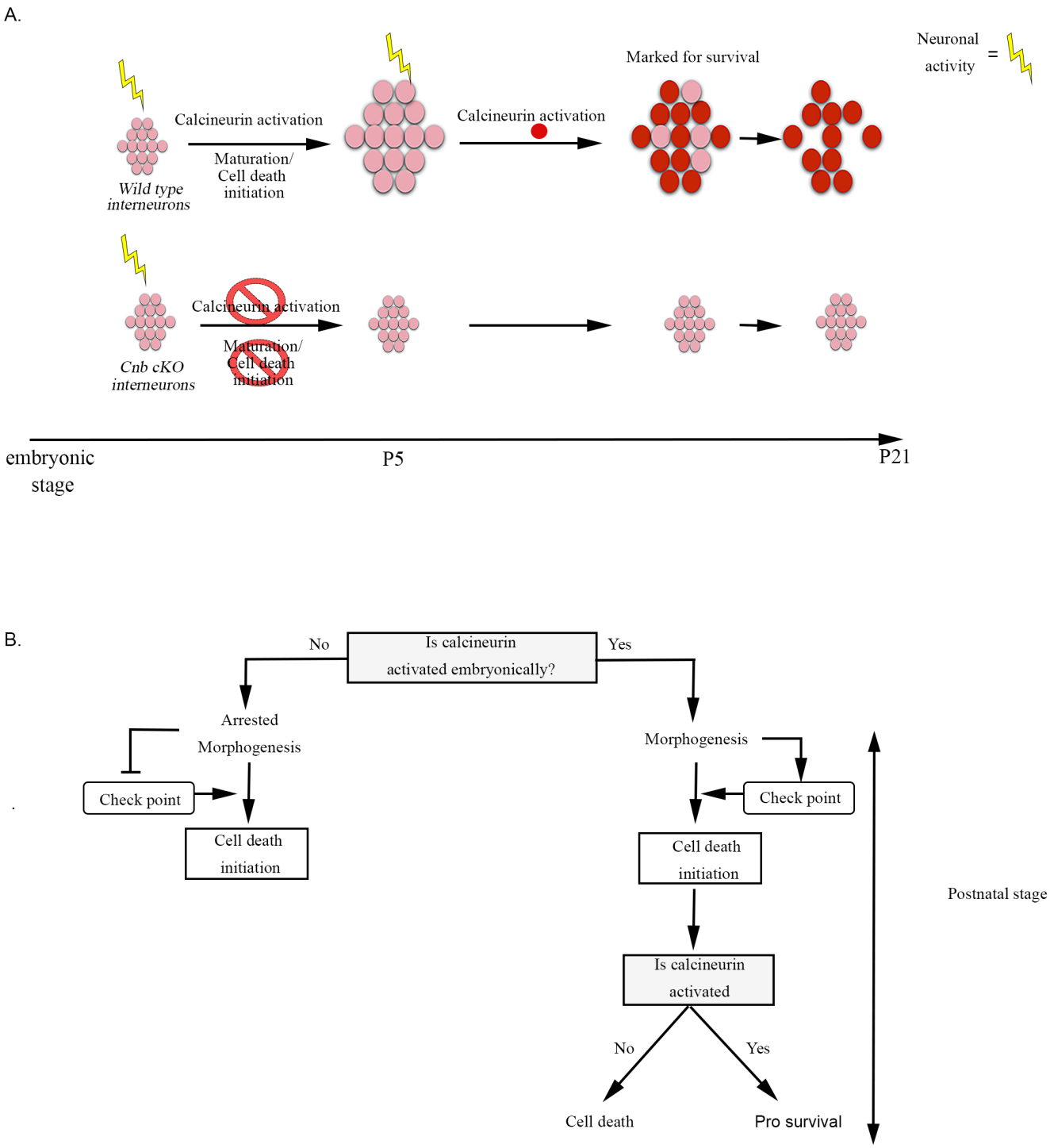


Figure S7.Related to Figure 7. Model

A) Schematic depicting the regulation of cell death via calcineurin mediated signaling.

B) Model showing the requirement of neuronal activity via calcium signaling for distinct developmental stages. CaN activation is essential for both, neuronal maturation, and regulation of cell death. The model presents the coupling between maturation and cell death program. When interneurons are not mature they do not initiate the apoptotic pathway. Subsequent to morphological differentiation, the apoptotic pathway is activated and requires appropriate activity level to prevent interneurons from undergoing developmental cell death.

Supplemental Experimental Procedures (Related to Experimental Procedures)

Cultures

Primary cortical cultures were prepared as previously described (Xu et al., 2004). The neocortex was dissected from P0 to P2 CD1 mice, macerated using fine forceps, then trypsinized in the presence of Leibovitz L-15 medium (UCSF Cell Culture Facility) and DNase (1 U/ml; Pro-mega, Madison, WI). The tissue was triturated using a pipette, and then resuspended in DMEM-F12 media (UCSF Cell Culture Facility) containing 10% fetal bovine serum (Hyclone). Fifty thousand cells were added to each well of 8-well chamber slides (70 mm^2; BD Falcon) coated with polylysine (10 g/ml) and laminin (5 g/ml; UCSF Cell Culture Facility). Cultures were maintained at 37°C in the presence of 5% carbon dioxide and ambient oxygen.

Ganglionic eminences (MGE and CGE) were dissected from E13.5 $GAD67^{GFP}$ embryos and mechanically dissociated in a solution of Leibovitz L-15 medium and DNase. The resultant cell suspension was then concentrated by brief centrifugation and placed in N5 medium (DMEM-F12 with glutamax, 100× N2 supplement (Invitrogen)), containing DNAse, bovine pituitary extract (35 ug/ml; Invitrogen), human epidermal growth factor (20 ng/ml), human fibroblast growth factor-2 (20 ng/ml; Preprotech), and fetal 5% bovine serum (Hyclone). Cells were added to wells containing feeder layers grown for 24 hours (5×10^3 cells per well). The cultures were thereafter maintained in Neurobasal/B27 medium (Invitrogen).

To generate glial feeder cultures, the neocortex was dissected and prepared as cortical feeders. To remove neurons and some oligodendrocytes precursors, cells were allowed to grow to near confluence (typically 24- 48hours) and then the plate or flask was shaken vigorously for 30 seconds with top closed. Media was aspirated and adherent cells, glial, were washed several times with sterile PBS before use.

Experiments were repeated 3 times with minimum of 2-3 treatment replicates per experiment. For BDNF treatments, 20ng/ml BDNF (Preprotech) in media was used. Note that treatment did have an effect on morphology and arborization of treated cells. For high potassium exposure, cells were exposed to 25mM increase from 5mM of KCL in NB media for 2hour intervals, twice daily, for a week, from 5DIV to 12DIV. For TTX treatment, 10uM TTX to was added to the media for 2 weeks from 7DIV to 21DIV. For validating the loss of Cnb protein from the conditional *Cnb* knockout cells, interneurons were plated on Poly-D-Lysine coated plates and immunostained with Cnb antibody after 24 hours.

Subdural injections.

For the subdural injection of FK506, the CaN blocker was dissolved in DMSO.20mM stock that was further diluted in PBS to get a final concentration of 20nM. A volume of 30-40nl red fluorescent beads were mixed with the FK506 solution just before injection. In the control condition a similar volume of DMSO was diluted with PBS and mixed with the red fluorescent beads to visualize the site of injection. The subdural injections were done on two consecutive days, i.e.

at P7 and P8.

Supplemental Reference:

Xu, Q., Cobos, I., La Cruz, De, E., Rubenstein, J.L., Anderson, S.A. (2004). Origins of cortical interneuron subtypes. J. Neurosci. 24, 2612–2622.