Soluble CPG15 expressed during early development rescues cortical progenitors from apoptosis

Putz, Harwell, Nedivi, Nature Neuroscience, March 2005

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What we knew

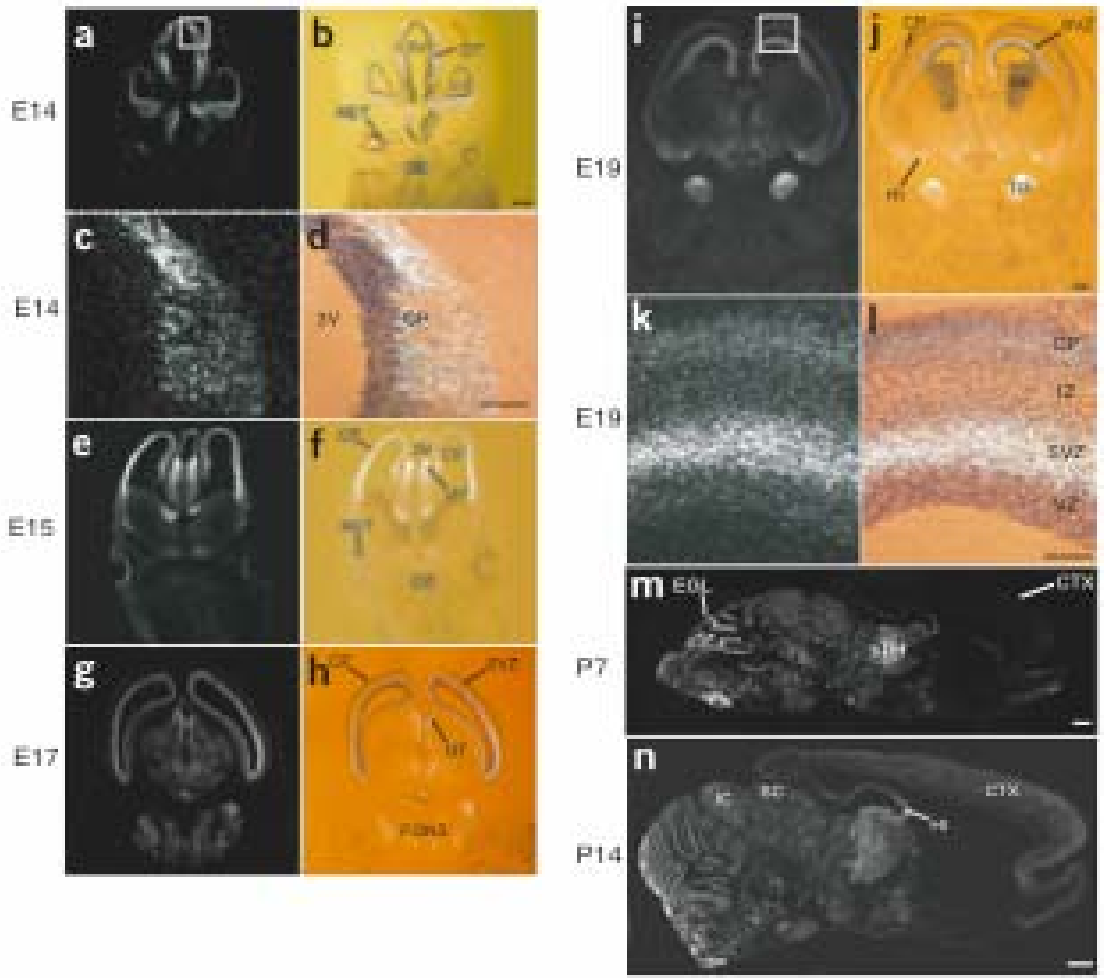
- An activity-dependent protein, *cpg15,* had been identified in late development to regulate growth of dendrites and axons, and promoting maturation, making it a player in synaptic plasticity and the “critical periods” of development.
- However, the protein was also known to be present pre-natally, though its role then was unknown. What is the protein doing at this stage?
- We also knew that apoptosis was occuring early in development, but the survival factors controlling this cell death were unknown.
- **Question**: Is *cpg15* a survival factor--that is, does it interrupt the apoptotic pathway-- in early development?

Experimental Strategy

- ***Is cpg15 in the right time at the right place?*** Perform in-situ screens at different stages of development.
- ***Could the cpg15 protein reach many different cells?*** Test *cpg15* protein for its ability to diffuse outside the cell of origin.
- ***Is cpg15 necessary and sufficient to rescue cells from apoptosis?*** Harvest soluble *cpg15* and place it in a culture of starving neurons, and see if the neurons are rescued from apoptosis.
- ***Will cpg15 silencing cause increased progenitor apoptosis in vitro AND in vivo?*** Knock out *cpg15* mRNA with RNAi method in culture and in vivo, assay for progenitor number and apoptosis.
- ***Will cpg15 overexpression cause an increase in the progenitor pool? Will it do this by reducing apoptosis?*** Inject virus containing *cpg15* into developing cortex. Assay for progenitor number, cell mitosis, and apoptosis.

cpg15: right place, right time

- At the earliest stages, E14 and E15, *cpg15* is present in the cortical plate, the ventricular zone of the dorsal thalamus, and the RGCs, correlating with the proliferation of progenitors and high apoptosis of unnecessary neuroblasts.
- At E17-E19, *cpg15* is expressed in SZV of the telencephelon and the diencephelon and the hippocampal primordia (Fig 1g-l), also correlating with the expansion of the progenitor pool in those regions.



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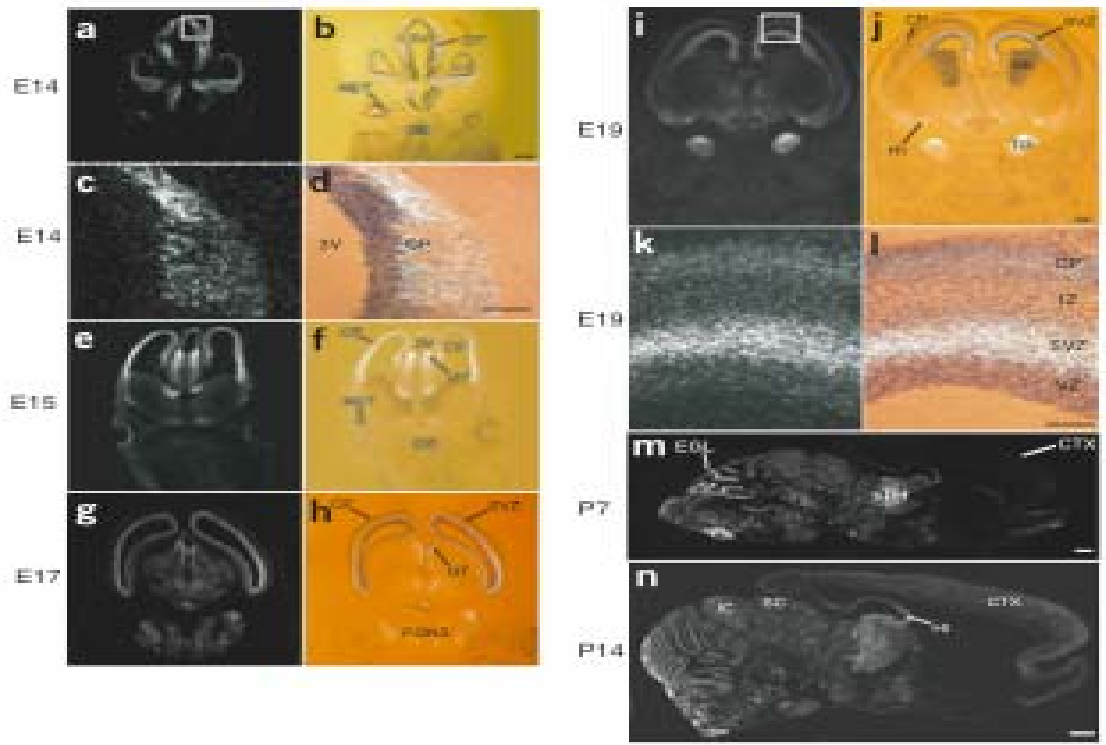
cpg15: right place, right time

- At postnatal day 7, *cpg15* is expressed in the external granular layers of the cerebellum (Fig. m-n, sagittal sections.). Also note that *cpg15* expression returns to the cortex postnatally, this time in the diff. layers. This correlates with the emergence of activity-dependent plasticity in this area.
- **Conclusion**: *cpg15* is present in some regions of proliferation namely, regions of high progenitor proliferation, and in turn, high apoptosis. The fact that is not present in ALL areas suggests cell-type specificity.



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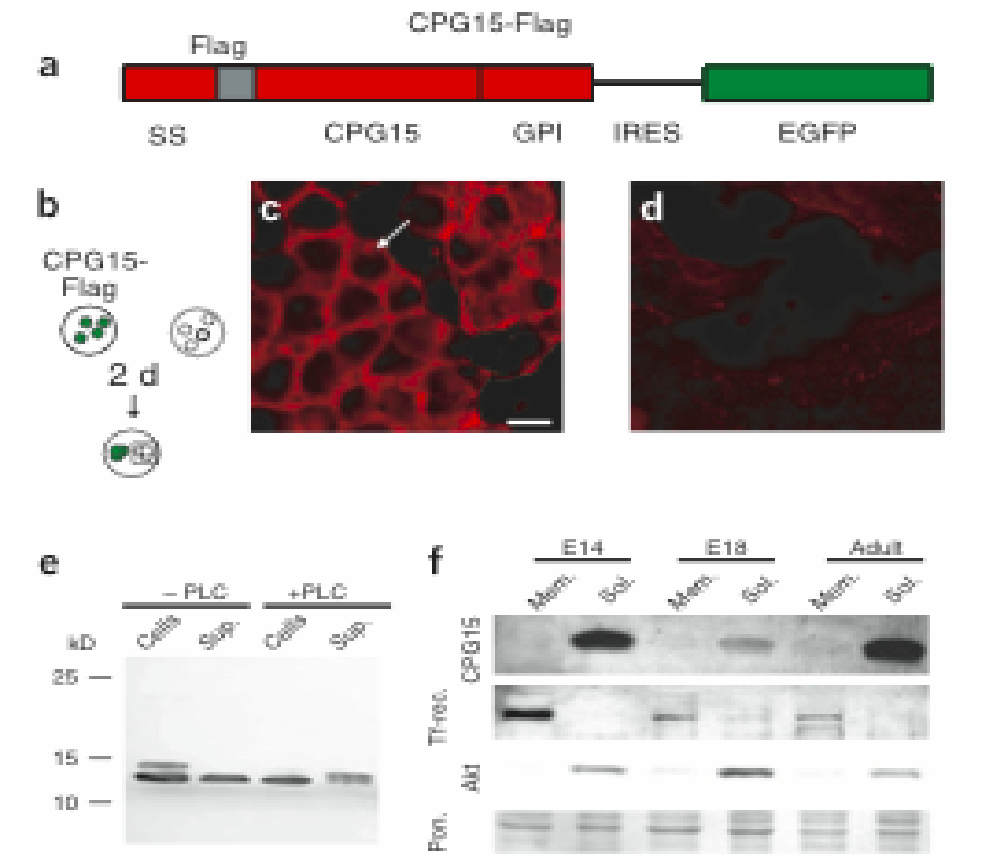
Soluble *cpg15* is secreted

- Construct a *cpg15* gene, tagged with EGFP (to indicate sucessful transfection), and a FLAG epitope (for immunostaining to locate exogenous *cpg15*). Transfect this construct into HEK293 cells.
- Staining (not shown) revealed that *cpg15* was present on the surface of transfected cells, **and** on some untransfected cells, suggesting *cpg15* had travelled to other cells.



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Soluble cpg15 is secreted

- To test if cell-to-cell contact is necessary for *cpg15* transfer, transfected and untranfsected HEK cells were cultured on a coverslip away from each other. Staining revealed *cpg15* membrane staining in the in untransfected population (Fig C) (Figure D is a control FLAG construct=no membrane staining). Suggests *cpg15* is diffusible.
- To isolate the soluble protein, they did a Western on the both the cell cultures and their medium. They found two bands of different MWs in the cell lane. Treatment of each band with phospholipase C, which cleaves cell surface proteins, caused changes in the higher MW protein.

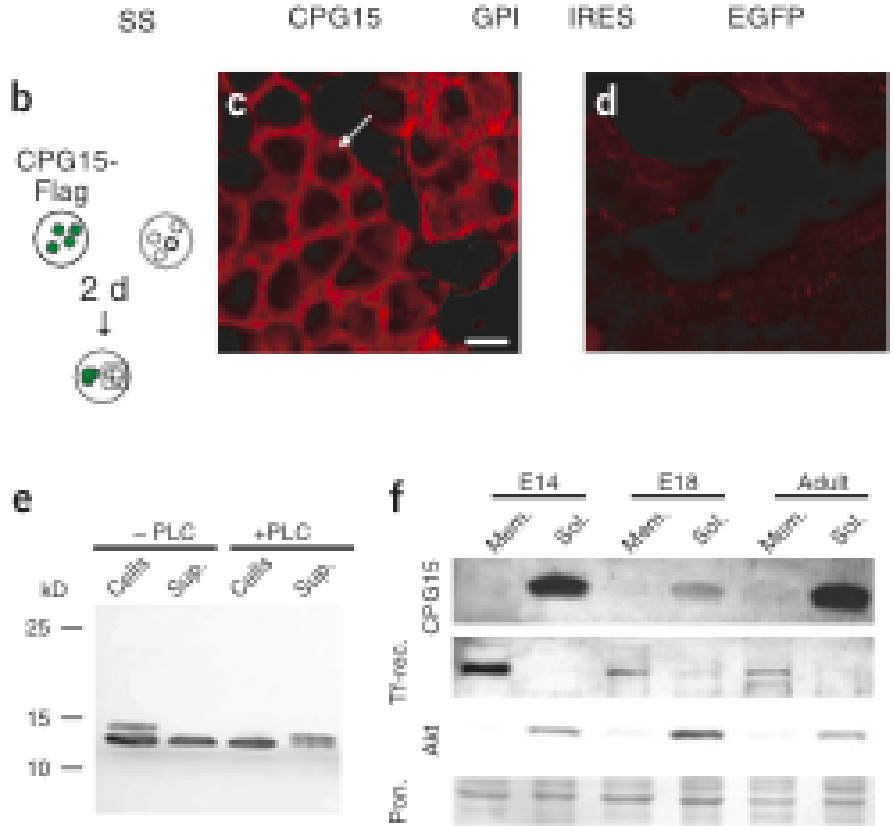
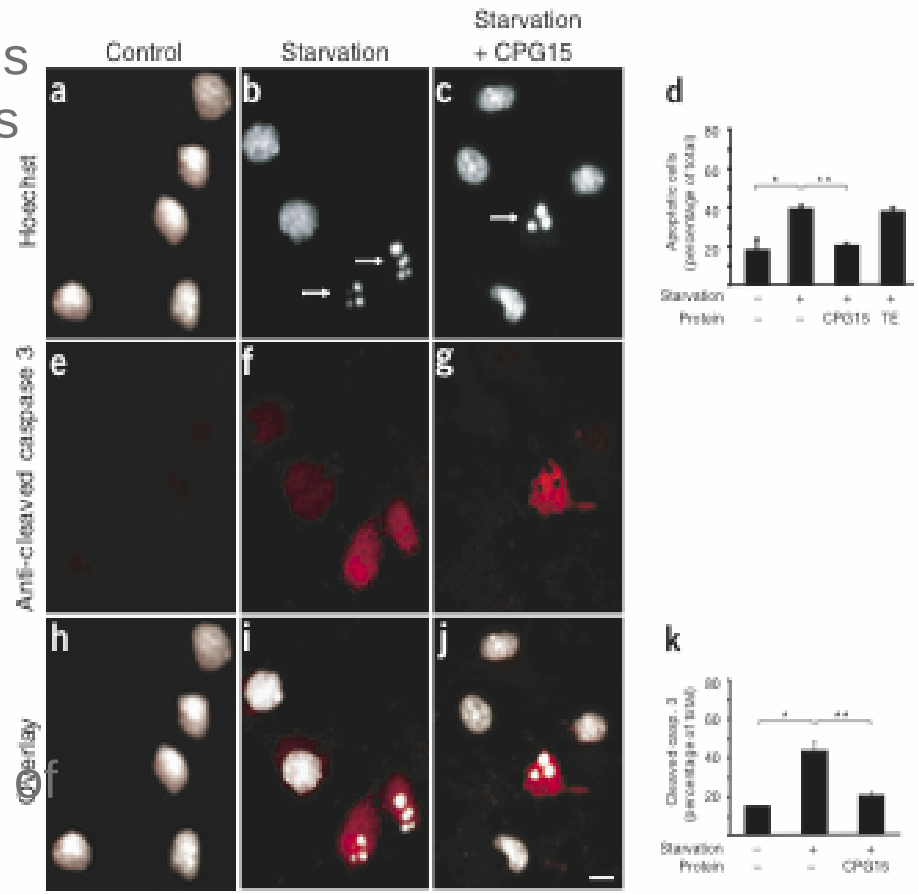


Figure F shows that *cpg15* is present in its soluble form throughout **normal** brain development and into adulthood, suggesting it is the primary form of the protein during development.

Soluble *cpg15* rescues cortical cells from apoptosis

- Harvest soluble *cpg15* from HEK293 cells and see if adding this to cultured neurons prevents induced apoptosis by growth factor deprivation.
- Hoescht staining identifies cells with fragmented nuclei (dying.) Starvation causes 2x more apoptosis in culture, though addition of *cpg15* abolished this effect. (quantified in Figure 3d).
- Stain for caspase 3 showed upregulated levels in the starvation culture, though these levels were reduced upon addition of *cpg15* (quantified in Figure 3k).
- **Conclusion**: Apoptosis is occurring through the default caspase pathway, and *cpg15* appears to interfere with this pathway.



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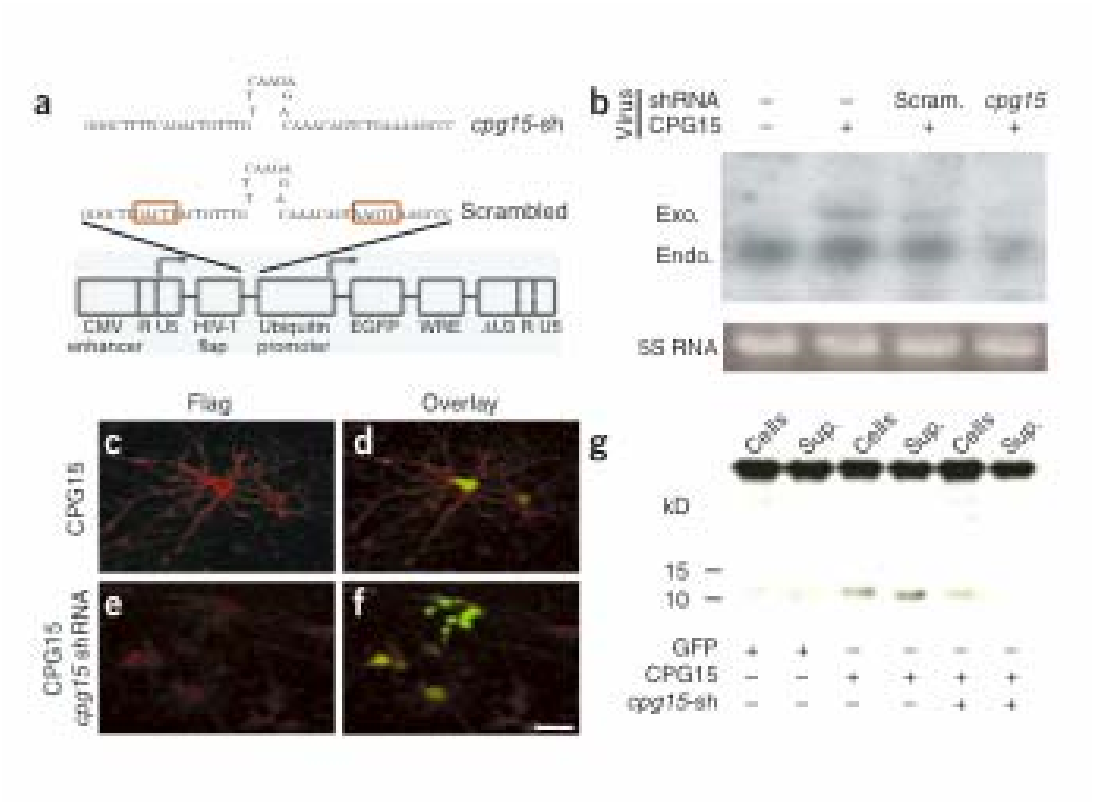
cpg15 RNAi abolishes *cpg15* protein expression



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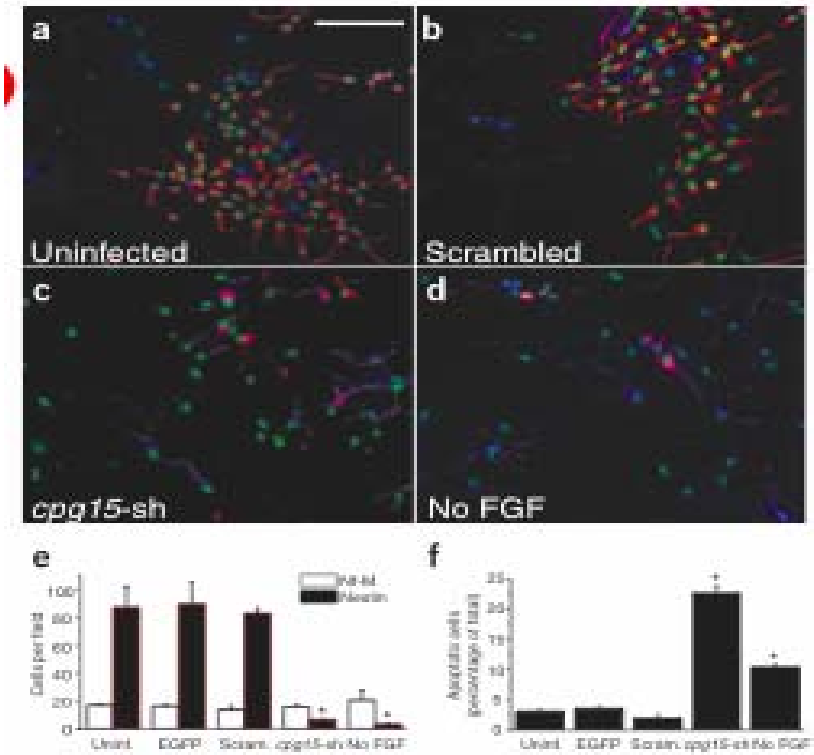
- To test the effects of silencing *cpg15*, we need to confirm that our silencing method actually works.
- Deliver *cpg15* RNAi (small hairpin RNA) via a lentivirus into cells and see if it knocks down expression. To control, also deliver a scrambled RNAi to control population.
- In Figure B, levels of both exogenous and endogenous *cpg15* mRNA are reduced in a Northern blot.
- In Figures c-f exogenous *cpg15* protein is reduced in an immunostaining assay, as well as in a Western blot in Figure G.

cpg15 is necessary for progenitor survival *in vitro*

- Cultures (uninfected, infected with *cpg15*-sh, infected with scrambled RNAi, and FGF deprived) were stained for progenitor markers (**nestin**), neuron markers (**nf-M**), and Hoescht staining (**cell apoptosis**) Figures 5a-d.
- Infection with *cpg15*-sh lentivirus leads to a decrease in neural progenitors and a significant increase in the number of apoptotic cells, quantified in Figures 5e-f.



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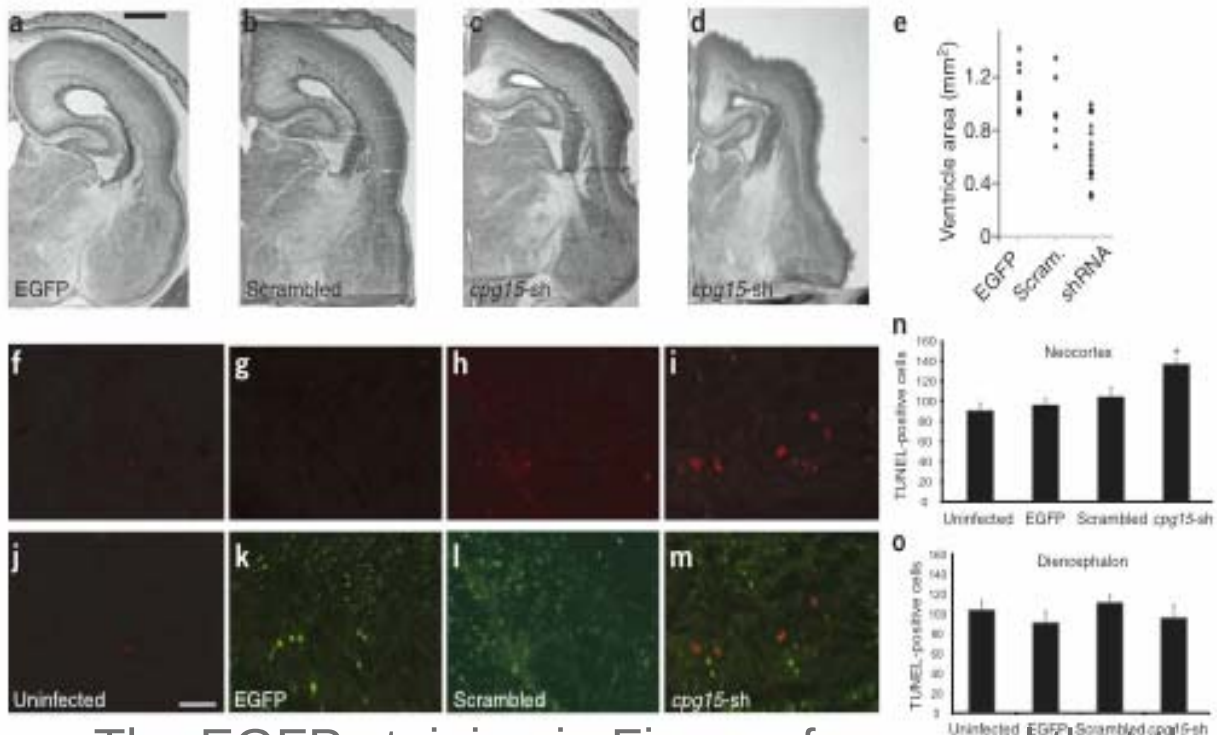
cpg15 block = apoptosis and cortical plate shrinkage *in vivo*

- In Figures 6a-d, we see the cortical plate infected with *cpg15*-sh is severely underdeveloped as compared to controls. Figure 6e quantifies this effect by plotting the area of the ventricles of each tissue sample.

- In Figures 6f-m, **TUNEL** staining reveals that more apoptosis is occuring in the *cpg15*-sh infected ***cortical*** tissue only. This effect is quantified in Figures 6n-o.



The EGFP staining in Figures f-m reveal that the levels of infection of shRNA and scrambled shRNA lentiviruses are comparable.

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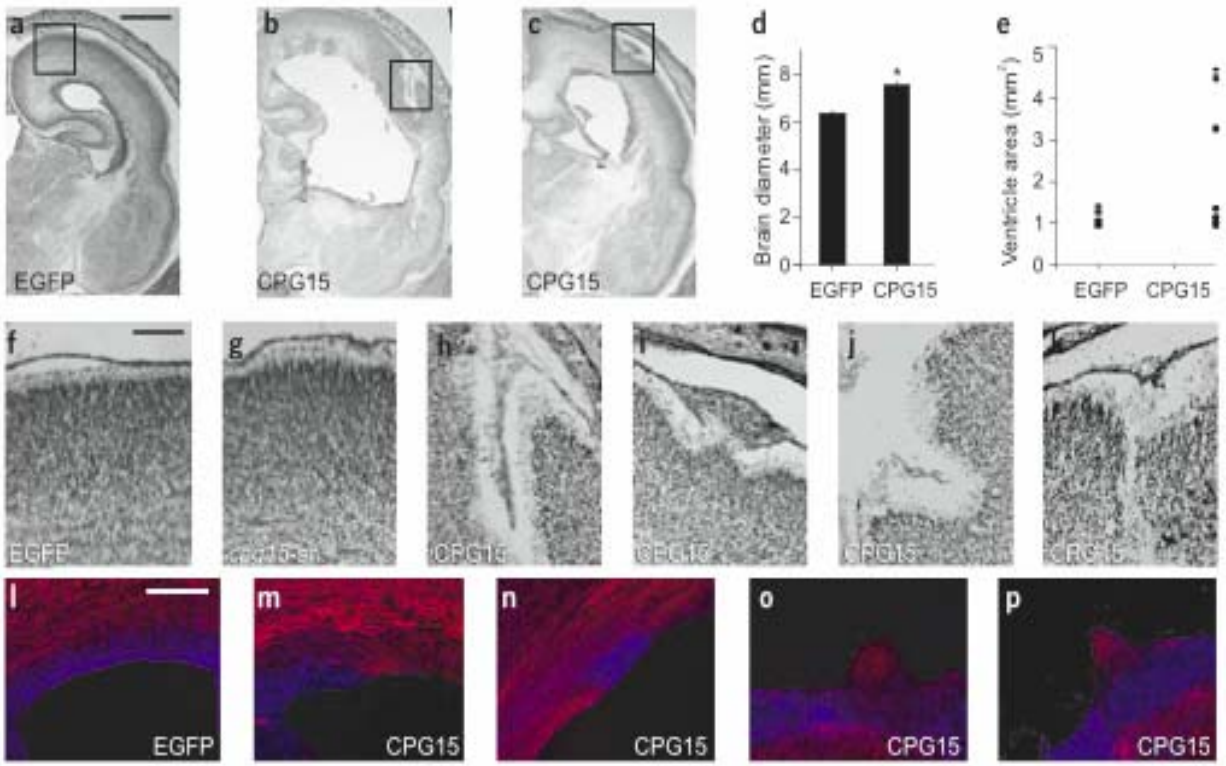
cpg15 overexpression = enlarged cortical plate and svz cell masses *in vivo*

- Next, we perform the complimentary experiment by overexpressing *cpg15 in vivo.* To do this, the authors injected *cpg15* containing lentivirus into the ventricles of the developing brain.

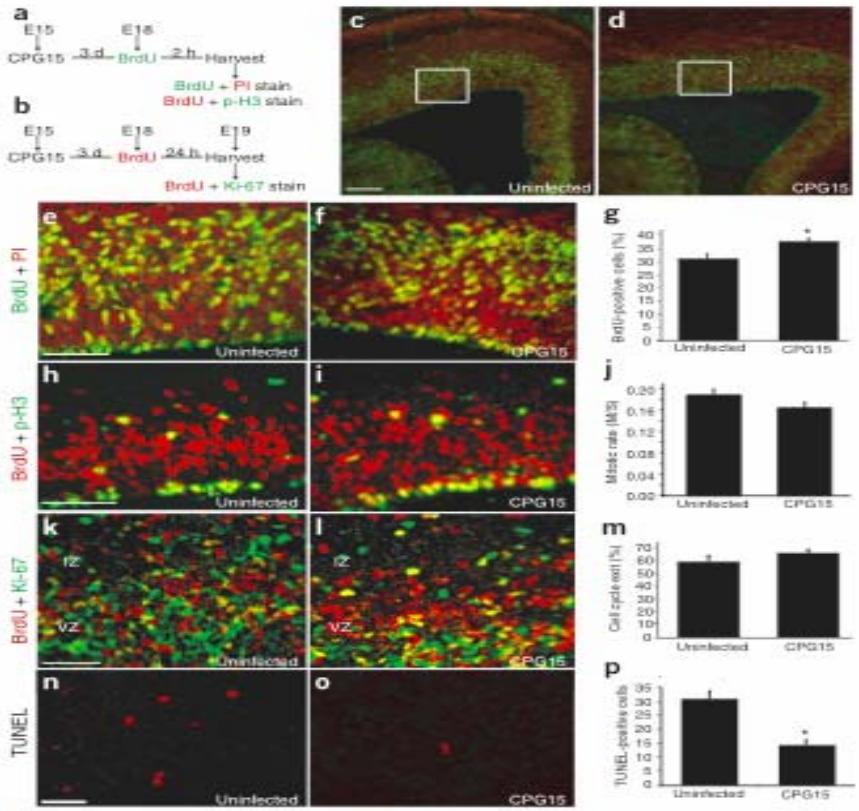
- Overexpression leads to an enlarged cortical plate and cell masses in the vz. Brain diameter and ventricle areas are quantified in Figures 7d and e.

- Note the human-like gyri and sulci shown in Figures 7f-k.



Labeling for **progenitor** and **neuronal** markers is shown in Figures 7l-p. The expanding neuronal population is becoming so crowded, it is pushing itself into the progenitor layer.

cpg15 overexpression = reduced apoptosis



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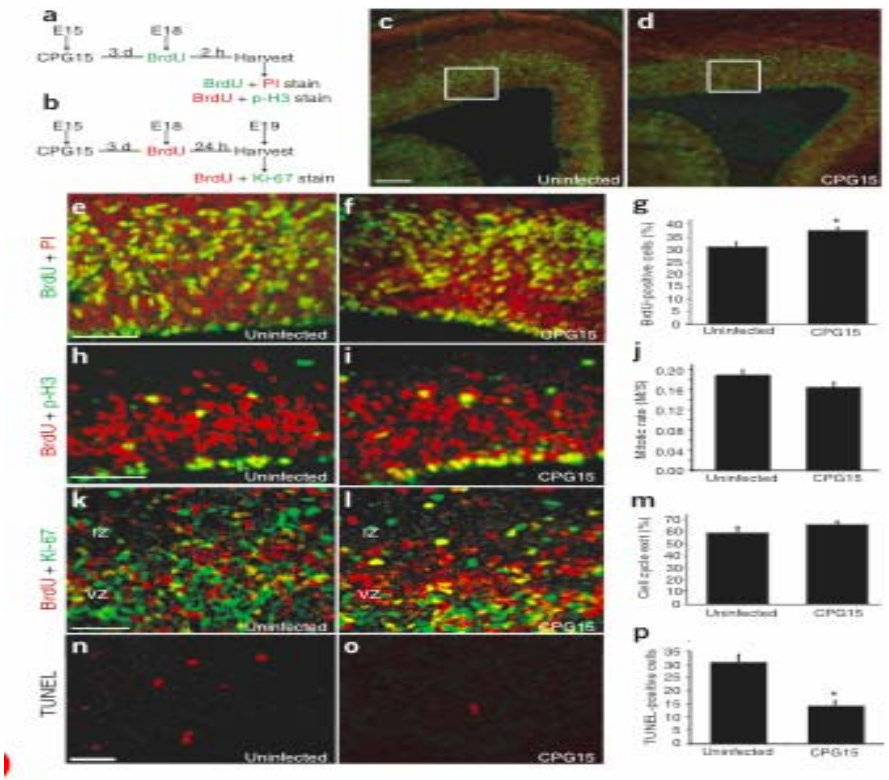
- Now we need to make sure that the increase in the progenitor is not the result of an increase in mitotic rate, a decrease in cell-cycle re-entry, but *strictly* reduced apoptosis.
- *cpg15-Flag* embryos at different stages were injected with **BrdU and PI** labels, which when overlapped mark **dividing cells**--in this case, the progenitors. In Figure 8g, we see infected embryos have more progenitors.
- *cpg15-Flag* embryos were injected with BrdU at E18, then harvested and stained for **BrdU** (M phase) and **p-H3** (S phase). The ratio of the two gives a measure of mitotic rate. No change in mitotic rate of progenitors.

cpg15 overexpression = reduced apoptosis



- Next, embryos were stained with **BrdU** (M phase) and **Ki-67** (marks progenitors at any phase) to assess percent of cells undergoing cell cycle exit. Levels were identical for control and infected embryos.

- Finally, embryos underwent **TUNEL** staining to assess levels of apoptosis. As shown in Figures 8n-o and quantified in Figure 8p, levels of apoptosis are reduced in our embryos overexpressing *cpg15*.

- Since we have ruled out the other possibilities for why we would see increased progenitor number, we can be sure that the reason is because *cpg15* is reducing the levels of caspase apoptosis during development.

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Summary

- *cpg15* is a soluble secreted factor that rescues cortical neurons from starvation-induced apoptosis by preventing caspase-3 activation.
- Decreased *cpg15* levels in vivo results in the shrinking of the cortical plate due to apoptosis of progenitors, while overexpression causes cortical plate expansion and convolution as a result of an enlarged progenitor pool.
- Therefore, *cpg15* is a survival factor for progenitors during development.
- ***Next steps:*** What are the other survival factors? What would happen to a *cpg15* knockout mouse, where *cpg15* can be eliminated even earlier in development?

Thank you!