Final report on Grant No. 116915

ABHD4 serine hydrolase, an enigmatic member of the endocannabinoid system: molecular, anatomical and functional characterization in the developing neocortex

Summary

The original main objective of the grant was to determine the biological function of ABHD4, a hitherto uncharacterized serine hydrolase in the mouse brain. Eventually however, this research program led to the discovery of a novel biological phenomenon which we termed developmental anoikis. Developmental anoikis has a key role in the robustness of cortical development, because it eliminates abnormally misplaced progenitor cells that are delaminated from the ventricular zone due to the loss of their N-cadherin-dependent adherens junctions. We postulated that developmental anoikis have pivotal role in preventing brain malformations, such as heterotopia and dysplasias. By using gain-of-function, loss-of-function and rescue approaches based on in utero electroporation, we discovered that ABHD4 is a sufficient and necessary enzyme for developmental anoikis and as a consequence, it has clinical significance in fetal alcohol syndrome. In addition, we further expanded our studies into cell death mechanisms caused by the loss-of postmitotic N-cadherin during interneuron development. In terms a collaboration with the group of Ádám Dénes, we applied our in utero electroporation approach to support in vivo visualization of functional coupling between cortical neurons and microglia. The study describing the concept of developmental anoikis and the first in vivo function of ABHD4 has received very supportive reviewer opinions at Nature Communications, the revised version is planned to be submitted by April 2020, and is available in full version on bioRxiv. The findings about N-cadherin-related cell death of cortical interneurons in in press and available online in Cerebral Cortex. The in utero electroporation-based microglia contact site results were published in Science in January 2020.

1. Expression of Abhd4

As a first step, we have cloned the *Abhd4* gene open reading frame and studied its spatiotemporal expression patern using in situ hybridization. Surprisingly, we could not detect its expression anywhere in the adult brain. In contrast, we uncovered that *Abhd4* is expressed specifically in the ventricular zones of the telencephalic and third ventricles during development. These zones mainly contain proliferating progenitor cells (Figure 1). This expression pattern is conserved throughout embryonic development, but decays postnatally to undetectable levels in parallel with the diminishing number of proliferating precursors.

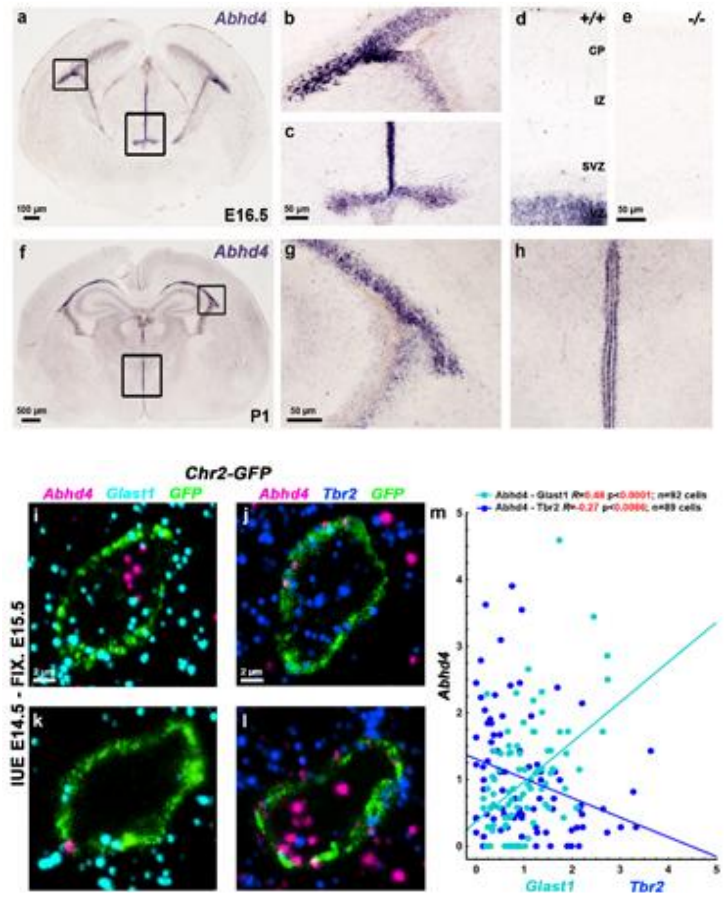

Figure.1 *Abhd4* expression in the E16 and P1 mouse brain and its correlation with RGC (*Glast1*) and IPC (*Tbr2*) markers.

Moreover, by combining RNAScope in situ hybridization with sparse cell-specific plasma membrane-labeling, we could quantify *Abhd4* mRNA levels at the cellular level and found that its expression correlates with proliferating progenitor marker *Glast1*, but inversely correlates with the mainly SVZ-located intermediate progenitor cell marker *Tbr2* (Figure 1).

2. Loss-and gain of function studies

To unravel its function, we obtained *Abhd4-/-* mice from Benjamin Cravatt (The Scripps Institute, La Jolla, California). Much to our surprise, Abhd4 was required neither for proliferation, nor for radial glia scaffold formation, the two known textbook functions of radial glia cells (Figure 2). Proliferation was examined via BrdU incorporation, while radial glia scaffold was described using nestin immunhistochemistry and its properties quantified via STORM super-resolution microscopy. In addition the intactness of the ventricular zone and its adherens junction belt was demonstrated vith Pax6 and phalloidin immunstaining, respectively.

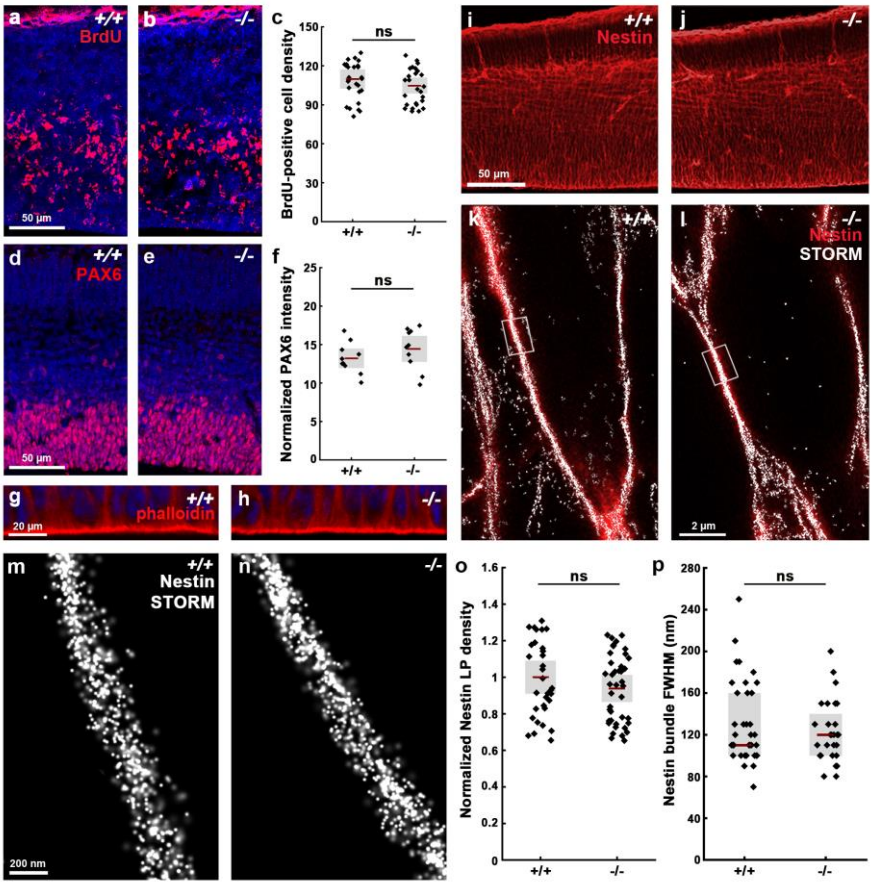

Figure 2. Radial glia structure and function is not affected by loss-of *Abhd4*.

Next, we established in utero electroporation in our laboratory as a tool to utilize in gain-of-function experiments. Ectopic expression of *Abhd4*, but not its hydrolase-dead (Ser159Gly) mutant version outside the ventricular zone resulted in a severe migration defect (Figure 3) accompanied by morphological changes indicative of imminent cell death. Accordingly, cell death levels increased significantly in the *Abhd4*-electroporated area.

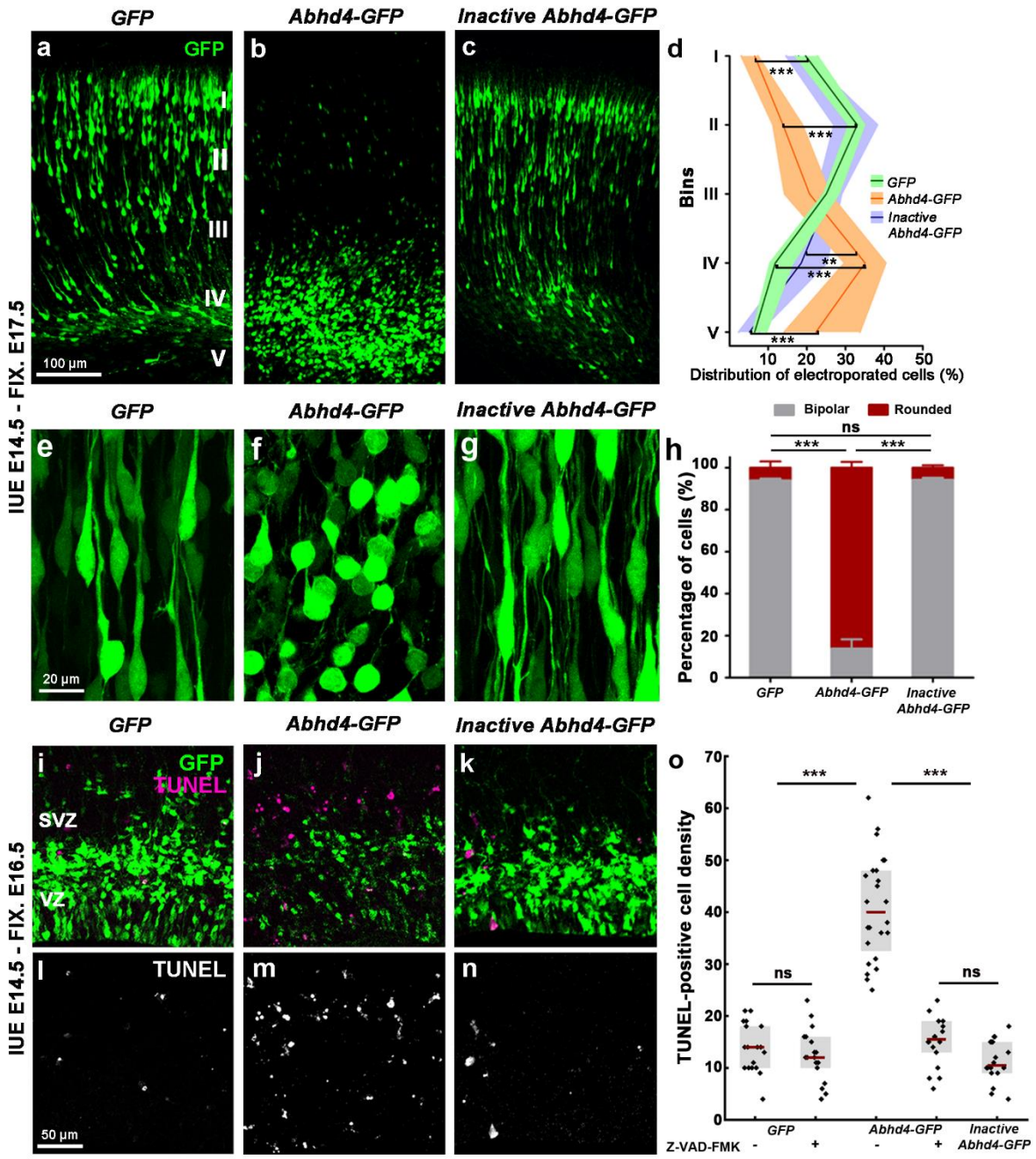

Figure 3. Ectopic expression of *Abhd4* causes migration arrest, strong morphological changes and elevated cell death levels in the Abhd4-electroporated cortex.

3. ABHD4 has a safeguarding function in the mouse embryonic cortex

Due to its pro-apoptotic nature, its restricted localization and its lack of role in normal radial glia progenitor cell functions, we hypothesized that it is a normally passive protein which only becomes active after a pathophysiological event affecting the proliferating progenitors. To model such an event, we disturbed cell-cell connections between ventricular zone cells via disruption of their adherens junctions using in utero electroporation of a dominant-negative version of N-cadherin (*dnCdh2*) the main component of this system.

This resulted in a remarkably similar migration defect (data not shown) and increased cell death levels to those observed induced by ectopic expression of *Abhd4* (Figure 4.). Carrying out the same experiment in *Abhd4-/-* animals however, completely eliminated the proapoptotic effect of adherens junction disruption. Conversely, co-electroporation of *Abhd4* with □*nCdh2* into *Abhd4-/-* animals rescued the elevated cell death in *Abhd4-/-* animals indicating that ABHD4 enzyme is necessary and sufficient for the adherens junction-loss-induced cell death.

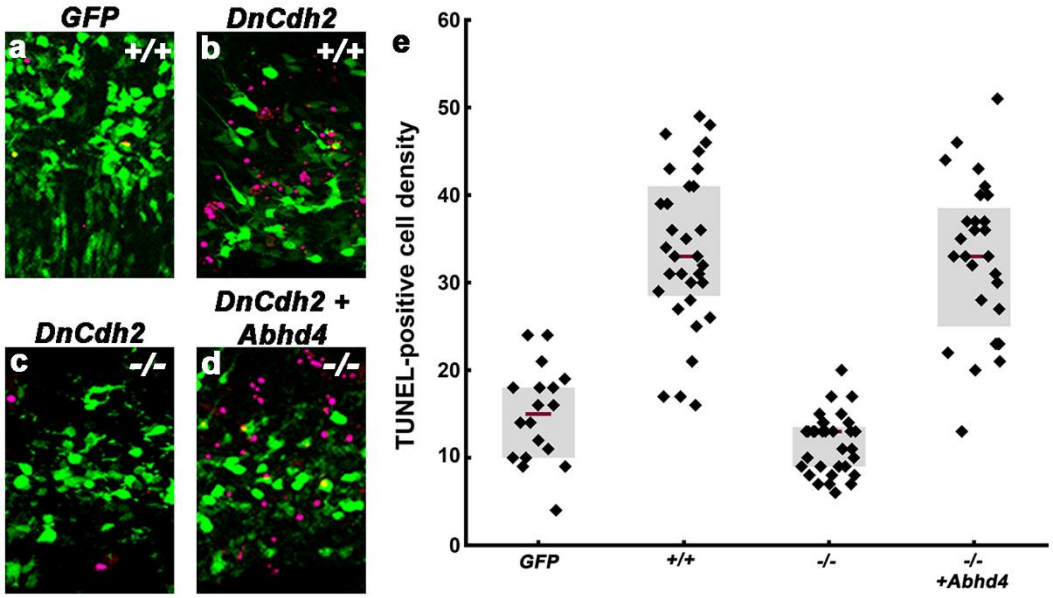

Figure 4. ABHD4 is essential for the adherens junction disruption-induced cell death.

In order to assess the potential clinical significance of ABHD4, we looked at two maternal ethanol exposure models that are known to alter radial migration and elevate cell death levels in the embryonic cortex. By using both an acute (binge-drinking) and a subchronic (3-day treatment) model, we found that ABHD4 is essential for the increased cell death in the embryonic cortex (Figure 6).

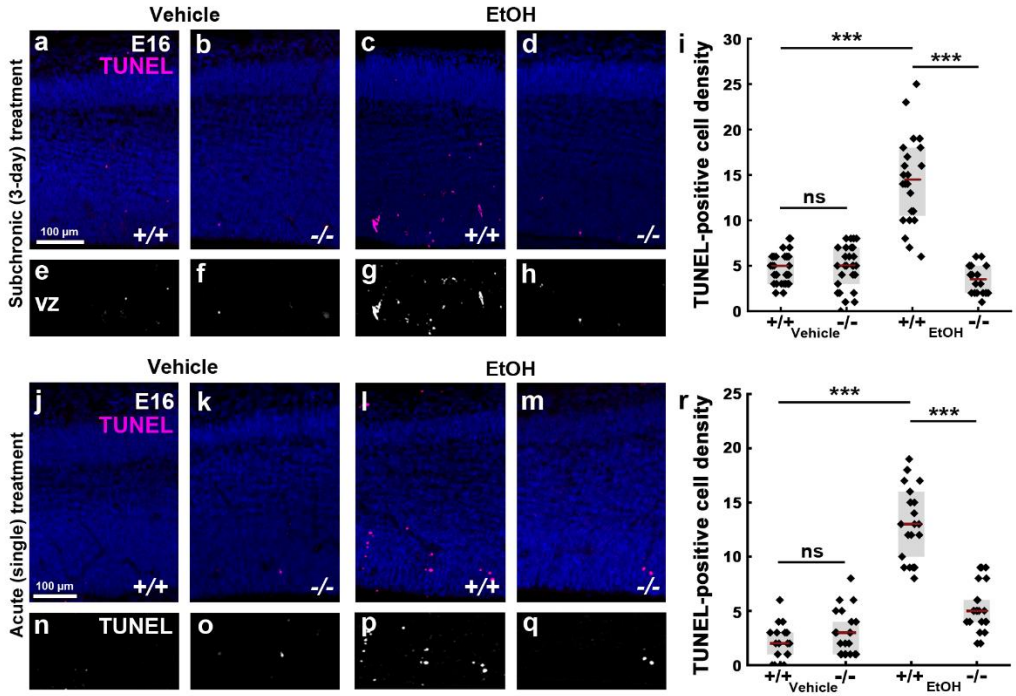

Figure 6. Abhd4 is essential for maternal EtOH-exposure-induced cell death in the embryonic cortex.

The similarity of the results presented here resemble very closely to the endothelial-mesenchymal transition-induced form of cell death that has important significance in cancer research and it is called anoikis. So we termed this adherens junction-loss-induced cell death phenomenon developmental anoikis. Importantly, this process can explain the robustness during cortical development and brain morphogenesis (ie. why such a low percentage of heterotopic mutations occurs despite the astronomical number of cell divisions during corticogenesis). Furthermore, we established ABHD4 as an essential executor protein of this pathway which protects the organism from the consequences of inappropriate delamination of progenitor cells during embryonic development. In addition to these findings that are available on bioRxiv (https://www.biorxiv.org/content/10.1101/2019.12.17.879551v1), and was invited to get resubmitted to Nature Communications. The lab collected several additional experimental findings that suggest that ABHD4 functions together with yet uncharacterized upstream and downstream molecular partners and its specific expression in radial glia progenitor cells is strictly controlled. These findings provide a framework for a longer term research program and will appear in future publications.

4. Disruption of N-cadherin function also results in elevated cell death during interneuron development

We further extended our studies into N-cadherin interference in interneuron precursors as well. We used the Dlx5/6-Cre mice to knock *Cdh2* out selectively in postmitotic interneuron precursors and used Gad65-GFP as a readout for GABAergic interneurons. We found a significant decrease in the number of Gad65-GFP-expressing neurons in the adult knockout

cortex. Furthermore, there was a delay in interneuron precursor entry into the pallial cortex at embryonic day 14 (E14), but embryonic proliferation and cell death levels were not altered. Significantly, we found that although the absence of N-cadherin affects all ganglionic-derived precursors, only the somatostatin- and calretinin-expressing interneuron types exhibited reduced numbers of the adult cortex. Using a temporal expression analysis we managed to trace the event and found that interneuron precursors arriving late to their cortical entry point in the pallial SVZ could start their radial migration. Consequently, these cells got eliminated during the developmental apoptotic wave occuring during early postmitotic stages. We are currently investigating the possibility if this cell death phenomenon is also ABHD4-dependent and conceptually linked to developmental anoikis. The study summarizing these results is in press and it has already appeared on the homepage of Cerebral Cortex (https://doi.org/10.1093/cercor/bhz168).

5. In utero electroporation-based identification of microglial somatic purinergic junctions

In collaboration with the Laboratory of Neuroimmunology led by Dr. Ádám Dénes, the expertise of the Laboratory of Molecular Neurobiology in the optimization of in utero electroporation into the lateral ventricules of the embryonic brain was exploited to visualize microglia together with cortical neurons and to study microglia–neuron interactions in vivo in the mouse neocortex. The cell-type-specific genetic manipulation experiments were instrumental to reveal that microglia processes contact the cell bodies of supragranular cortical neurons and to determine its anatomical and functional properties. The findings were published Science on 31st January, 2020.