Cut homeodomain transcription factor is a novel regulator of cortical glia morphogenesis and niche maintenance around neural stem cells

Running title

Cut regulates cortical glia morphogenesis and neural niche

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Abstract

Cortical glia in *Drosophila* central nervous system forms a niche around neural stem cells (NSCs) and their progeny for necessary signals to establish cross-talk with their surroundings. These cells grow and expand their thin cytoplasmic extensions around neural cell bodies in the nervous system. Although essential for the development and function of the nervous system, how these cells make extensive and intricate membrane networks remain largely unknown. Here we show that Cut, a homeodomain transcription factor, directly regulates the fate of the cortical glial cells impacting NSC homeostasis. Focusing on the thoracic ventral nerve cord (tVNC), we found that Cut is required for normal growth and development of cortical glial cells. We also highlight that levels of Cut expression play an essential role in the cytoplasmic membrane network growth around the neural cells. Loss of Cut in cortical glia results in a substantial reduction in their cytoplasmic extensions and network around cell bodies of NSCs and their progeny, whereas its overexpression induces the overall growth of cortical glia main branches at the expense of side ones. We also note a striking gain in the nuclear size and volume of cortical glial cells upon Cut overexpression. Furthermore, constitutively high Cut levels increase DNA content in these cells more than threefold, indicating an interference with the splitting of nuclei during endomitosis. Since cortical glia makes syncytial membrane networks around neural cells, the finding identifies Cut as a regulator of glial growth and endomitosis to support a functional nervous system. It is the first report that highlights a novel function of Cut in regulating the growth and branching of cortical glial cells and control over endomitosis.

Keywords: Cortical glial cells, endomitosis, Cut, *Drosophila*, polyploidy

Highlights

In this study, we have investigated the role of homeodomain transcription factor Cut in the growth and development of cortical glial cells. We found that Cut regulates cortical glial growth and the formation of complex membrane network around the neural cells. We also note that constitutive high Cut levels in these cells interfere with their normal endomitosis process, decrease nuclei number, and affect glial growth. Thus, Cut is a novel player that regulates the dynamic growth of cortical glial niche around neural cells. Since the human homolog of Cut, CUX1 is considered one of the cancer driver genes which is highly expressed in glioma, our finding highlights the need to explore if activated CUX1 in these tumors enhances chromosomal instability in glial cells and, in conjunction with other mutations, enhances their tumorigenic potential.

Introduction

Glia is an essential and abundant class of cells in the nervous system involved in the maintenance of neural growth and protection, establishing communication, and providing nourishment and support for overall homeostasis of NSC and its progeny (Barres, 2008; Booth et al., 2000; Stork et al., 2012; Trapp and Nave, 2008; Xiong and Montell, 1995). In addition, the glial cells also form the blood-brain barrier and compartmentalize CNS into specialized domains (Abbott, 2005; Awasaki et al., 2008; Oland and Tolbert, 2003). How glial cells grow and develop a dynamic niche around neural stem cells (NSCs) and their progeny is a question of potential interest from normal development as well as disease points of view. Mammalian and *Drosophila* glial cells are comparable and classified into several functional groups based on their morphology and molecular functions (Stork et al., 2012). Based on morphology, the three major classes of glia types are named in *Drosophila* as the surface, cortex, and neuropile-associated glia (Ito et al., 1995; Stork et al., 2012).

Cortical glial cells in *Drosophila* nervous system are born during the mid-embryonic stage. Their cell bodies are irregular and grow massive membrane networks in multiple directions around individual neural cell bodies for trophic and metabolic support (Dumstrei et al., 2003; Ito et al., 1995; Pereanu et al., 2005). In the ventral nerve cord (VNC) of the *Drosophila* larval nervous system, cortical glial cells are mostly found at the ventral and lateral sides and remain closely associated with neural cells and also regulate their fate (Ito et al., 1995). Similar to cortical glia, mammalian astrocytes also extend the membrane extensions towards the outer surface of synaptic neuropils (Awasaki et al., 2008). Growth of the glial cell membrane requires nutrient-dependent activation of Insulin and PI3K signaling (Yuan et al., 2020). In addition, several other signaling pathways involved in overall cell growth, such as Hippo, EGFR, and FGF, are also shown to control the growth and remodeling of the cortical glia cell membrane (Avet-Rochex et al., 2012; Dong et al., 2020; Read et al., 2009; Reddy B. V. V. G. and Irvine Kenneth, 2011; Spé Der and Brand, 2018; Witte et al., 2009). How the fine membrane projections grow out of the cortical glial cell body and how they find their correct path is still an active area of investigation. Here we show that a homeodomain transcription factor, Cut, is required for the developmental growth of cortical glial cells to form a niche around neural cell bodies in the larval CNS.

The Cut family of proteins is highly expressed in the developing *Drosophila* and mammalian CNS and regulates neural identity and proper patterning (Arya et al., 2019; Weiss et al., 2019). From an evolutionary and functional perspective, it is one of the most conserved proteins among all metazoans and is known as CUX1 in humans and CUX1/2 in mice (Neufeld et al., 1992; Quaggin et al., 1996; Tavares et al., 2000; Valarche et al., 1993; Yoon and Chikaraishi,

1994). Cut protein expresses in several tissues and is known for its diverse functions, especially in determining the cell specificity and identity in *Drosophila* and mammals (Andres et al., 1992; Liu and Jack, 1992; Liu et al., 1991; Ludlow et al., 1996; Nepveu, 2001; Pitsouli and Perrimon, 2013; Zhai et al., 2012). It is also known to influence cell proliferation, migration, and response to DNA damage in various tissues (Coqueret et al., 1998; Kedinger et al., 2009; Michl et al., 2005). Cut protein carries multiple DNA binding domains, making several transcription factor isoforms (Blochlinger et al., 1988; Bodmer et al., 1987; Liu et al., 1991). Generally, Cut protein isoforms carry a single homeodomain and one to multiple Cut DNA binding repeat domains. Finally, depending on its interacting partners, Cut acts as a transcriptional activator or repressor of several genes during development (van Wijnen et al., 1996; Yoon and Chikaraishi, 1994). Consistent with its role as a gene regulator, Cut is also identified as a selector gene in several mammalian disorders, including cancer (Zhai et al., 2012,). Loss and gain of Cut/CUX1 mutations have been identified in several cancer types and have been shown to cause multi-organ hyperplasia in mouse models (Ledford et al., 2002; vanden Heuvel et al., 2005, Sansregret and Nepveu, 2008). Despite the diverse roles of Cut in several cellular processes and gene regulation, the detailed molecular understanding of the functional diversity of this protein is an area of active research.

This research reports that homeodomain transcription factor Cut is crucial in regulating proper growth and network formation of cortical glia around neural stem cells and their progeny in the *Drosophila* central nervous system. Cut guides the cortical glial development in several ways. First, Cut protein levels affect the cortical glial extension differentially. Loss of Cut results in stunted cortical glial growth and their eventual loss from the nervous system. On the other hand, ectopically induced high Cut level allows the main branches to develop at the cost of finer extensions that enwrap the individual neural cell bodies. Moreover, high Cut levels also interfere with the normal endomitosis in cortical glia, a process through which their nuclei number increases and a complex membrane network is formed. Intriguingly, continued high Cut expression in cortical glia increases their DNA ploidy to multifold. It is the first report highlighting a novel function of Cut in regulating the growth and branching of cortical glial cells and control of endomitosis.

Results

1. Cut expresses in glial subtypes and maintains niche around neural stem cells

Transcription factor Cut is known to express in *Drosophila* central nervous system (CNS) and to regulate the developmental death of NSCs in the abdominal region of VNC (Arya et al., 2019). In addition to NSCs, Cut is expressed in several other cells in the larval CNS. Therefore, the larval CNS was stained with a Cut antibody to profile its expression in the glial cells. In this study, we focussed on the thoracic region of the ventral nerve cord (tVNC) to study the role of Cut in glial biogenesis (Fig. 1A). Since Cut and pan-glia marker Repo antibodies are both rose in mice, and there is no commercial antibody for cortical glia cells, we used the UAS-Gal4 system to mark the glia with GFP using Repo-Gal4 (pan glia) and cyp4g15-Gal4 (cortical glia) drivers (Fig. 1B,C) (Gonzalez-Gutierrez et al., 2019; Rujano et al., 2022). The GFP+, Cut+ cells in the tVNC were counted to estimate the glial cells that express Cut. In Repo>GFP tVNC, where GFP marks all the glial cells, around 55% of total GFP-positive cells were Cut positive (Fig.1B, graph J). On the other hand, when the number of GFP+ cells marking the cortical glia cells (cyp4g15>GFP) that also express Cut were counted, we found that all the tVNC cortical glia express Cut (Fig.1C, J).

The cortical glial networks around neural cells establish the communication between the neural cells to their environment and the survival of newborn neurons (Dumstrei et al., 2003; Freeman and Doe, 2001; Rujano et al., 2022; Spé Der and Brand, 2018). Knockdown of Cut expression selectively in cortical glial cells with cyp4g15-Gal4 driver resulted in substantial membrane network disruption (Fig.1D-F). We validated this finding with two separate Cut-RNAi lines (supplementary fig.1A,B). Since cortical glial cells make a supporting system for the neural cells, the loss of glia and their network around neural bodies also affects NSCs. We used deadpan (dpn) as an NSC marker and noted that NSCs were unusually clumped, irregularly shaped, and were eliminated during development upon Cut ablation in cortical glial cells (Fig.1E, F). Thus, Cut defective cortical glial cells non-autonomously affect the NSC fate. We used four other cortical glia-specific drivers for knockdown of Cut - R54H02, R46H12, Np577 and found similar, significant non-autonomous loss of NSCs in tVNC (supplementary fig.1C). We selected cyp4g15-Gal4 for further studies. We conclude that all cortical glial cells express Cut and it is essential for maintaining the glial niche around the NSCs and their progeny.

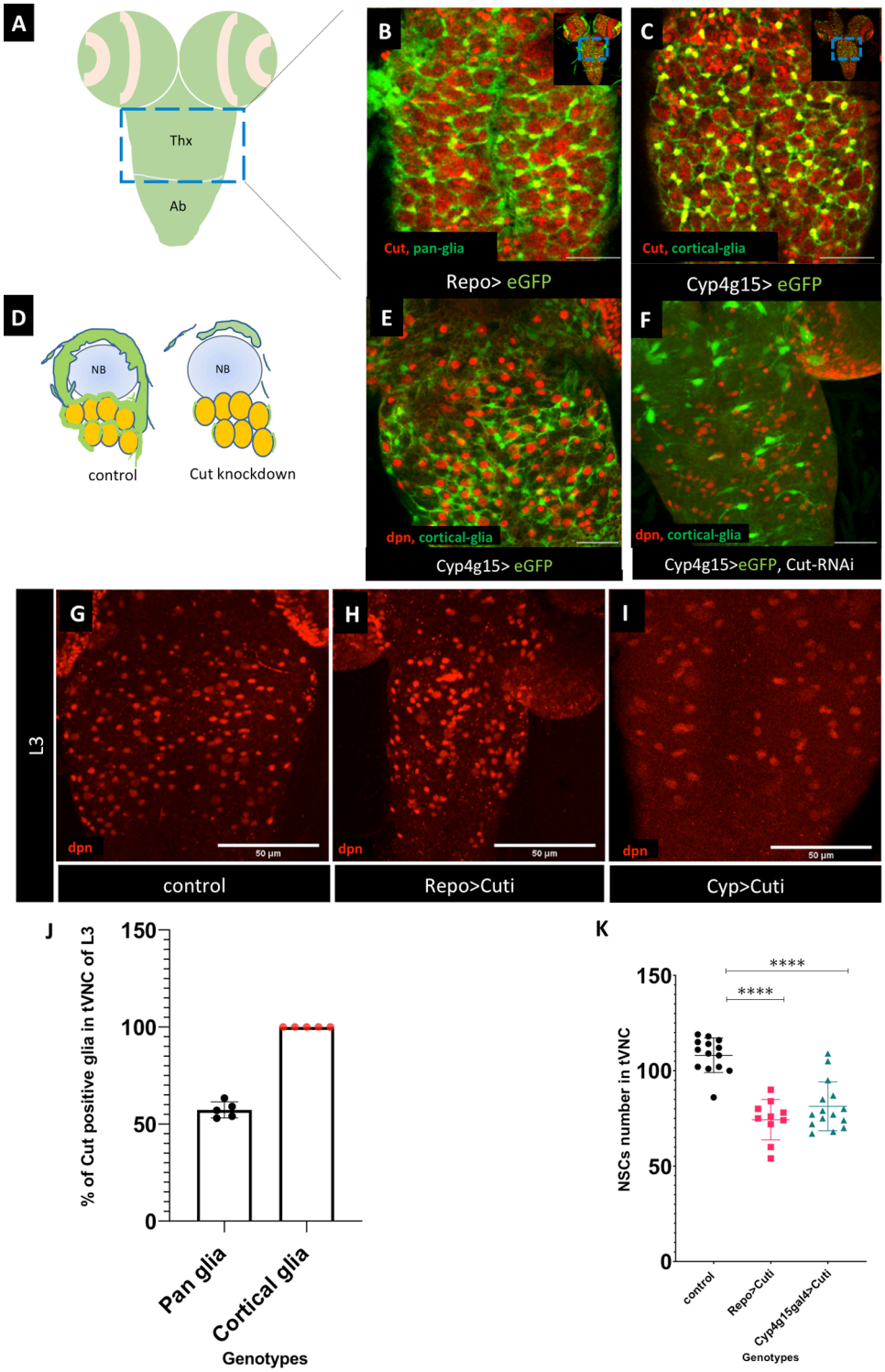
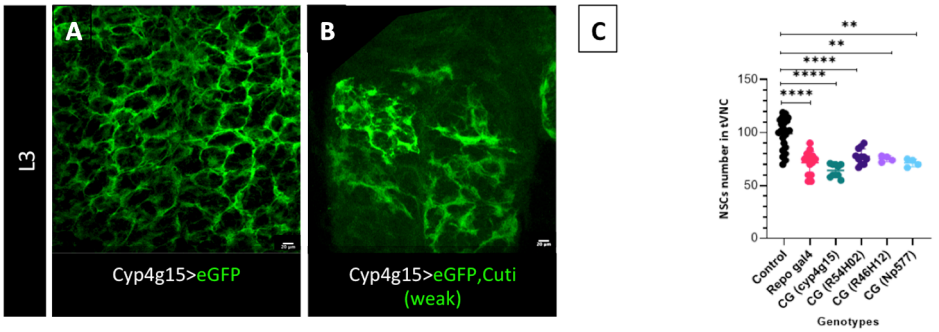


Figure 1: Cut expresses in glial subtypes and maintains niche around neural stem cells
(A) Model of larval CNS indicating the thoracic region (Thx) and abdominal region of (ab)VNC. The blue box marks the late thoracic region of VNC (tVNC), shown in **(B-I)**. **(B-C)** Single confocal sections showing Cut expression (red) in pan glia (Repo>eGFP, **B**) and cortex glia (Cyp4g15>eGFP, **C**), inset in B and C is the whole larval CNS. **(D)** model showing cortical glia enwrapping around a NSC and its lineages in control and disruption of the cortical glial network in Cut-knockdown. **(E),** well-developed glial extensions around NSCs (marked with dpn in red) in control (Cyp4g15>eGFP,+), inset shows glial network around NSC. **(F)** Loss of Cut (Cyp4g15>eGFP, Cut-RNAi) severely disrupts cortex glia and affects the distribution of NSCs; inset is disrupted glial interaction with NSC. **(G-I)**

showing NSC number and distribution (dpn, green) in tVNC in **(G)** control **(**Repo-Gal4/R+**),** and their affected distribution in Pan glial Cut knockdown in (Repo>Cut-RNAi, **H**) and in cortical glia (Cyp4g15>Cut-RNAi, **I**). **(J)** graph shows the percentage of Cut positive glial cells(n=5). **(K)** Quantification of NSCs upon Cut-knockdown with repo-Gal4 and cortical glia-specific Cyp4g15-Gal4. Statistical evaluation of significance is marked with stars. **** p<0.0001, ** .0041,** .0012. Image acquisition was done using a confocal microscope with 50µm scale bar.



Supplementary. Fig. 1A-B: **A)** compare the membrane network in L3 **(A)** control (Cyp4g15>eGFP, +), **(B)** Cut knockdown weak RNAi line (Cyp4g15>eGFP; Cut-RNAi)**,** the cortical glial networks get disrupted even with weak RNAi line (BL#29625). **(C)** Quantification of NSCs upon Cut-knockdown (strong RNAi line BL # 33967) with Repo-Gal4 and several cortical glia specific Gal4 drivers (Cyp4g15-Gal4, R54H02-Gal4, R46H12-Gal4, NP577-Gal4) and statistical evaluation of significance. **** p<0.0001, ** .0041,** .0012 based on unpaired t-test using GraphPad Prism 9 software.

2. Cut is required for the growth of cortical glia membrane, and it's branching

Following the above result that Cut defective cortical glial cells cannot form a niche around the neural cells, we further explored the role of Cut in cortex glial growth and chamber formation around neural cell bodies. Cortical glia continues to expand their cytoplasmic extensions from the late embryonic stage (Coutinho-Budd et al., 2017; Ito et al., 1995). Therefore, as a first step, we checked the expression profile of the cortical glia Gal4 driver, cyp4g15-Gal4, and found it, also expressed from the late embryonic stage and can be used for the study (data not shown). The cyp4g15-Gal4 driven GFP reporter in the larval instars faithfully marks the glial network from early to late developmental stages (Fig. 2A, 3A-F). As shown in Fig. 2A, A' (yellow arrowhead in 2A'), multiple thinker cytoplasmic extensions emerge from a cortical glia cell body and spread their branches in multiple directions to cover a group of neural cells (Dumstrei et al., 2003; Hartenstein, 2011; Hoyle, 1986; Rujano et al., 2022; Spé Der and Brand, 2018). The cytoplasmic lamelliform extensions of the cortical glia further send out several thinner branches, making a fine meshwork around individual neural cell bodies in the tVNC, known as trophospongium (Fig 2A', red arrow). Interestingly, the membrane extensions coming out from different cells are well connected and self-tilled on one another (Coutinho-Budd et al., 2017; Rujano et al., 2022). Cut knockdown in the cortical glia leads to a massive loss of these lamelliform cortical processes (Fig 2B, B'). Unlike the normal cortical glia, which extends the processes in several directions (Fig 2A, A' yellow arrowheads, and cartoon in inset), the Cut knockdown in these cells leads to only one to two abnormal-looking main

cytoplasmic extensions (compare fig. 2A', B', and cartoon in inset). To further analyze and compare the density of cortical glial extension in the two genotypes, the Image J plot analysis tool was used to evaluate the number of peaks, which indicates the number of cortical glial extensions. In Cut knockdown cortical glia, the number of peaks was far less compared to the control and was of variable intensities, indicating a reduction in cytoplasmic extensions (Fig 2C, D). The plot profile along a line in the tVNC was also performed to further show an evident loss of the glial network upon Cut knockdown (Fig.2E, F).

The loss of membrane extensions in cortical glial cells upon Cut knockdown is a significant finding since these cells are known to grow extensions and fill the gaps efficiently if a few of their neighbors are ablated (Coutinho-Budd et al., 2017). Since, Cut defective cortical glial cells could not do so, makes it clear that Cut is required for the normal growth and development of lamelliform extensions from the cell body. Upon Cut knockdown, these glial cells cannot grow the processes; therefore, several glial cell bodies might be eliminated during development.

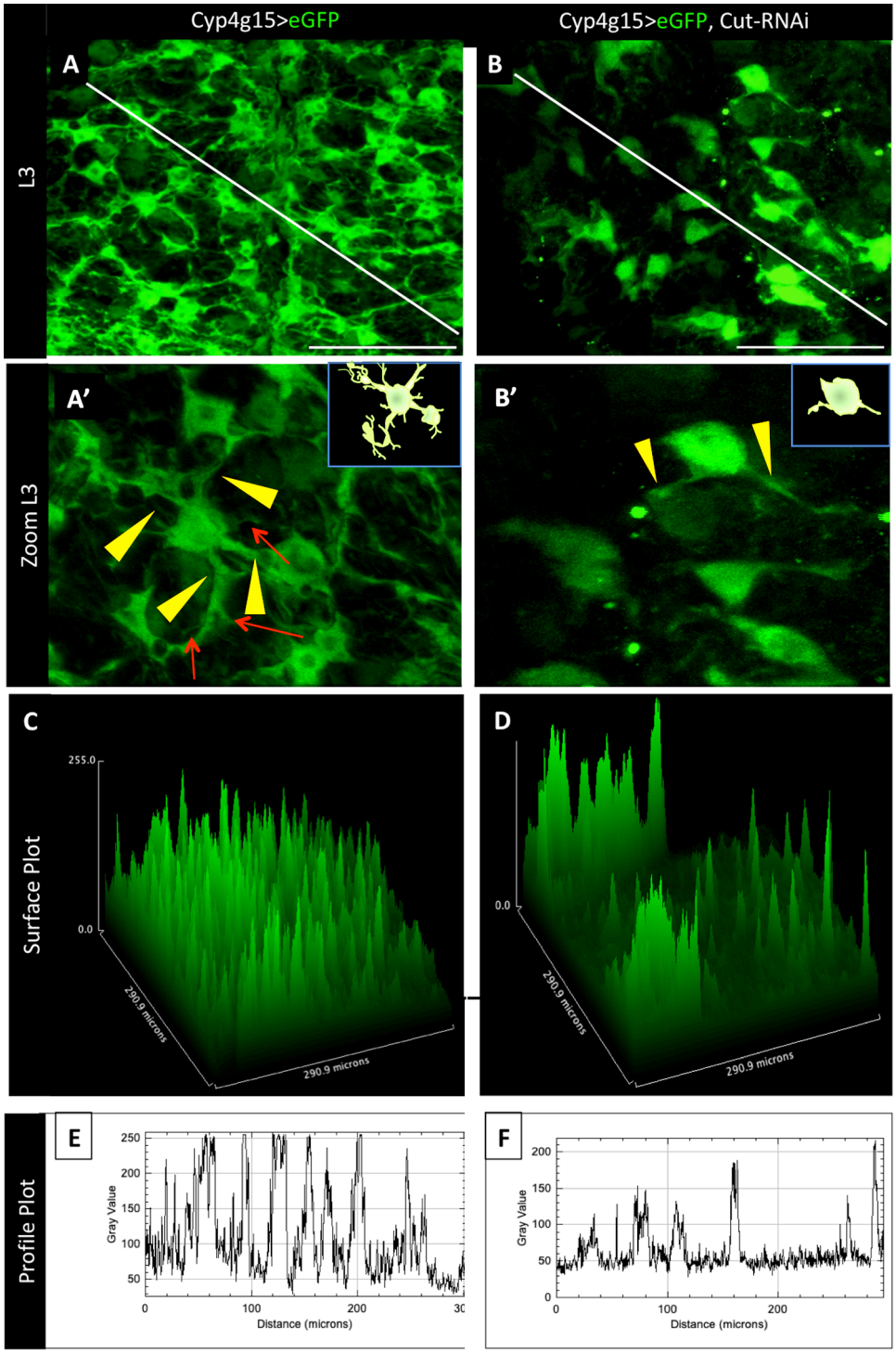


Figure 2: Cut is required for cortical glia growth and branching

(A,) Control (Cyp4g15>eGFP,+) showing fine honeycomb structure of cortical glia. **(A')** Zoom of a small region in **A** shows individual cell body extending thick (yellow arrowheads) and lean processes in multiple directions (red arrows); inset is the model depiction. **(B,B')** Cut defective cortical glia (Cyp4g15>eGFP, Cut-RNAi) with minimum extension growth. **(C-D)** the surface plot in tVNC to compare the intensity and extensions of Cut defective cortical glia with control (along the white line in A, B). **(C)** Control has more peaks representing cell and their thick and thin extensions, **(D)** Cut-Knockdown shows less and variable intensities of the peaks indicating loss of cortical glia. **(E, F)** plot profile along a straight line in tVNC showing that peaks and their intensities are more uniform and close to each other in control compared to Cut knockdown, further emphasizing the loss of glial network.

3. Cut defective cortical glial cells are unable to increase their nuclei number during development

In order to understand when the above-noted reduction in the tVNC cortical glia number is initiated in development and how this occurs, we analyzed early larval CNS. During the second instar larval (L2) stage, a developing network of the cortical glial cells is seen through the VNC (Fig.3A), which appears like intricate honeycomb structures by the late third instar larvae (L3) (Fig. 3D). Even in L2 stage knockdown of Cut in these cells severely perturbs the menbrane network of cytoplasmic extension, (compare Fig.3A with 3B) which by the L3 stage is almost lost (compare Fig.3D with 3E). We quantified the total number of cortical glial nuclei in the tVNC of L2 and L3 tVNC. During these early stages, there was a significant reduction in their nuclei number upon Cut knockdown (Fig 3G). Furthermore, the loss of network and glia was more profound in the late L3 (compare Fig.3D with E, Graph G). To evaluate if the canonical cell death pathway is helping the defective cortical glial cells to eliminate, we tried to rescue the cortical glial loss by co-expressing P35 genetically, a well-known promiscuous inhibitor of caspases. The L2 stage shows a partial yet significant rescue in glial nuclei number (Fig.3A-C, Graph G). Interestingly, these rescued cortical glial cells still do not grow regular fine membrane extensions and are unable to connect as seen in Cut-knockdown alone (Fig.3C). Thus, P35 only partially rescues cell death but had no impact upon branching networking defects.

Further, during the L3 stage when the loss of cortical glia and network is more profound, we note that co-expression of P35 is largely ineffective in rescuing the cells and profound loss of cells is visible (Fig.3D-E, G). We also evaluated the volume of cortical glia in these genotypes through thresholding and network density through image J. The co-expression of P35 does not improve the network's coverage and volume of cortical glia (Fig. 3H). It indicates that the cells are developmentally defective in growing network and thus are eliminated partly by apoptosis and other unknown process/s (Fig.3). Recently, it has been shown that cortical glia increases their nuclei number and extend membrane network through endomitosis (Rujano et al., 2022). We see the reduced nuclei and defective membrane growth, which is only partially restored by P35 expression; thus, we conclude that the loss of Cut in cortical glial cells inflicts growth defects likely by interfering with endomitosis.

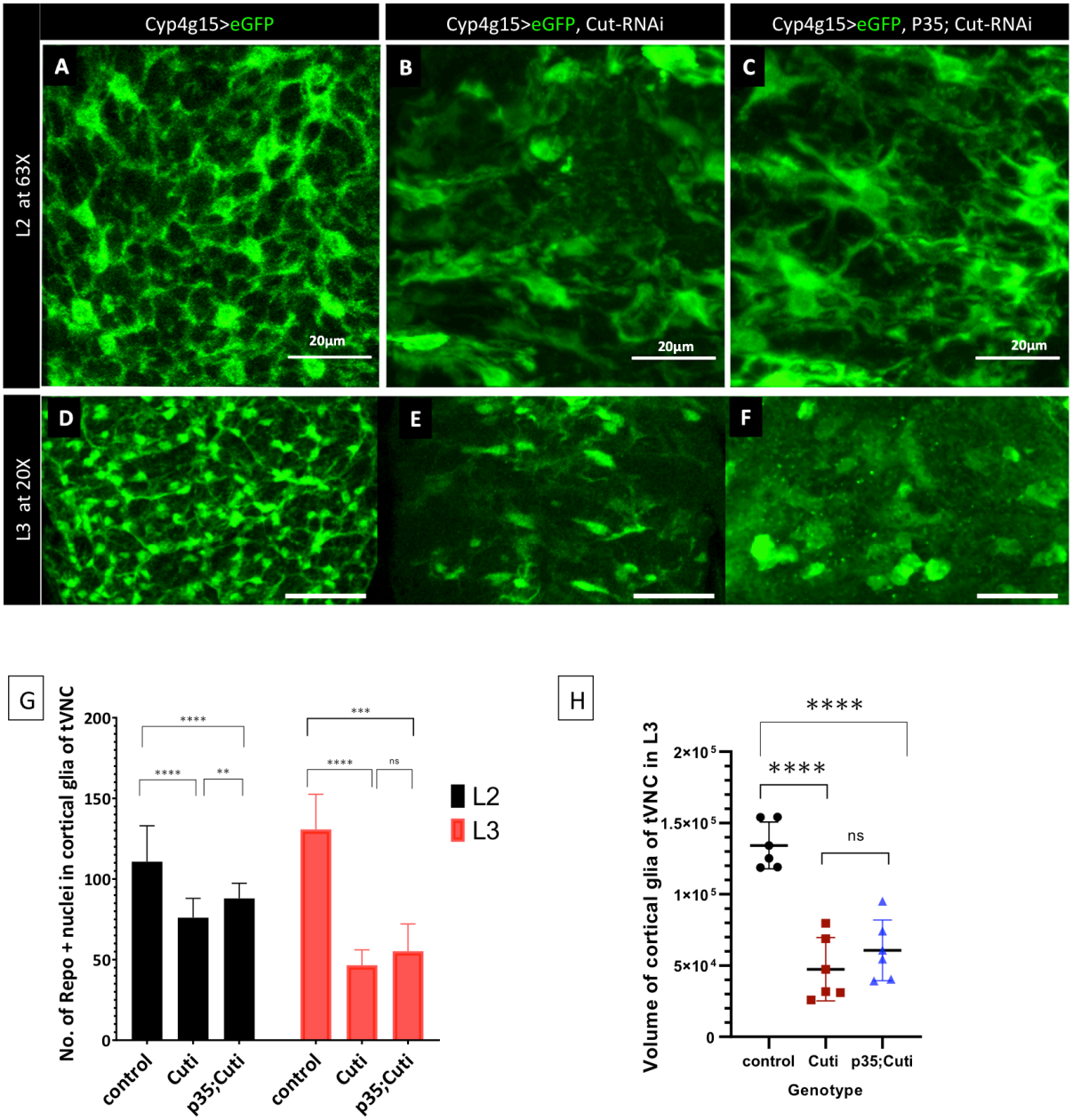


Figure 3: Nuclei number and membrane volume are adversely affected in Cut defective cortical glia. (**A-C)** compare the membrane network in L2 control (Cyp4g15->eGFP, **A**) Cut knockdown (Cyp4g15>eGFP; Cut-RNAi, **B**) and Cut knockdown co-expressing cell death inhibitor P35 (Cyp4g15>eGFP; Cut-RNAi, **C**). Disruption of the network is apparent in (B), which gets only partially restored upon P35 co-expression (**C**). **(D-F)** compare the cortical glia network in L3. **(D)** control has a very intricate glial membrane network, which is severely disrupted upon Cut knockdown **(E)** and not rescued upon P35 co-expression. **(G)** quantification of cortical glia nuclei (GFP+Repo+) in tVNC of L2 and L3 with control, upon Cut knockdown and along with P35 co-expression (total CNS counted per genotype>3) showing severe loss of nuclei upon cut knockdown and only slight rescue upon P35 co-expression. **(H)**Evaluation of the volume of cortex glia in L3 tVNC in control, Cut knockdown, and Cut knockdown co-expressing P35. The graph shows that Cut knockdown significantly reduces overall cortical glia volume, which is not rescued upon P35 co-expression. Statistical evaluation of significance $p<0.0001$, $p<0.001$, and $p<0.01$ based on unpaired t-test using GraphPad Prism 9 software.

4. Overexpression of Cut enhances the growth of cortical glial cell body and main branches at the cost of side extensions

During *Drosophila* development, Cut influences the complexity of the neuronal dendritic arbor in the peripheral nervous system, determines the identity of wrapping glia, and influences the formation of membrane protrusions (Bauke et al., 2015; Grueber et al., 2003). Furthermore, Cut protein level also regulates the branching pattern of dendrites in various neuronal classes in the peripheral nervous system differentially (Grueber et al., 2003). Therefore, we asked if increasing Cut levels in cortical glia would affect their growth and branching patterns.

We found that the Cut in cortical glia remarkably affects the cytoplasmic membrane extension in several ways. While Cut knockdown resulted in stunted growth of cortical glial membrane extension as noted above (Figs. 2, 3), its over-expression led to longer main cortical extensions but with little side branches (compare Fig. 4A, D, red arrows and yellow dotted lines marking main extensions). Note in control where one of the marked main extensions coming out of the cell body is shorter (Fig.4A, red arrow, yellow line); in Cut overexpressing glia it grows thicker and longer (Fig.4D, red arrow). To validate the visual interpretation, we measured the total volume of cortical glia and found an apparent increase in overall glial volume upon Cut overexpression (Fig. 4I). Since Cut overexpressing glia has more extended membrane projection, the cortical glia chambers are also larger and globular (compare Fig.4 B,C with E, F, graph H). However, they lack the thinner processes which enwrap the individual neural cell bodies typically seen in the control tVNC (compare Fig. 4E with B). Further, to check the extent of loss of side membrane extension upon Cut overexpression, we compare the density of main vs. side extensions through the surface and plot profile along a line. The loss of side extensions upon Cut overexpression is very evident as the profile of Cut overexpressing glia has broader peaks, away from each other, compared to the control, where the peaks are more uniform, sharp and close to each other (Fig. 4K-N, H).

Intriguingly, the cell bodies of Cut overexpressing cortical glia were considerably bigger and brighter but less in number compared to that in control (compare Fig. 4A with D, G, and J). Strikingly, a threefold increase in nuclei size was noted upon Cut overexpression (Fig. 4G). Thus, these observations all together propose that high Cut levels are sufficient to influence the overall growth of cortical glial cells. In addition, the Cut level affects the glial network's complexity by increasing the growth of main cortical branches and retard the growth of side protrusions.

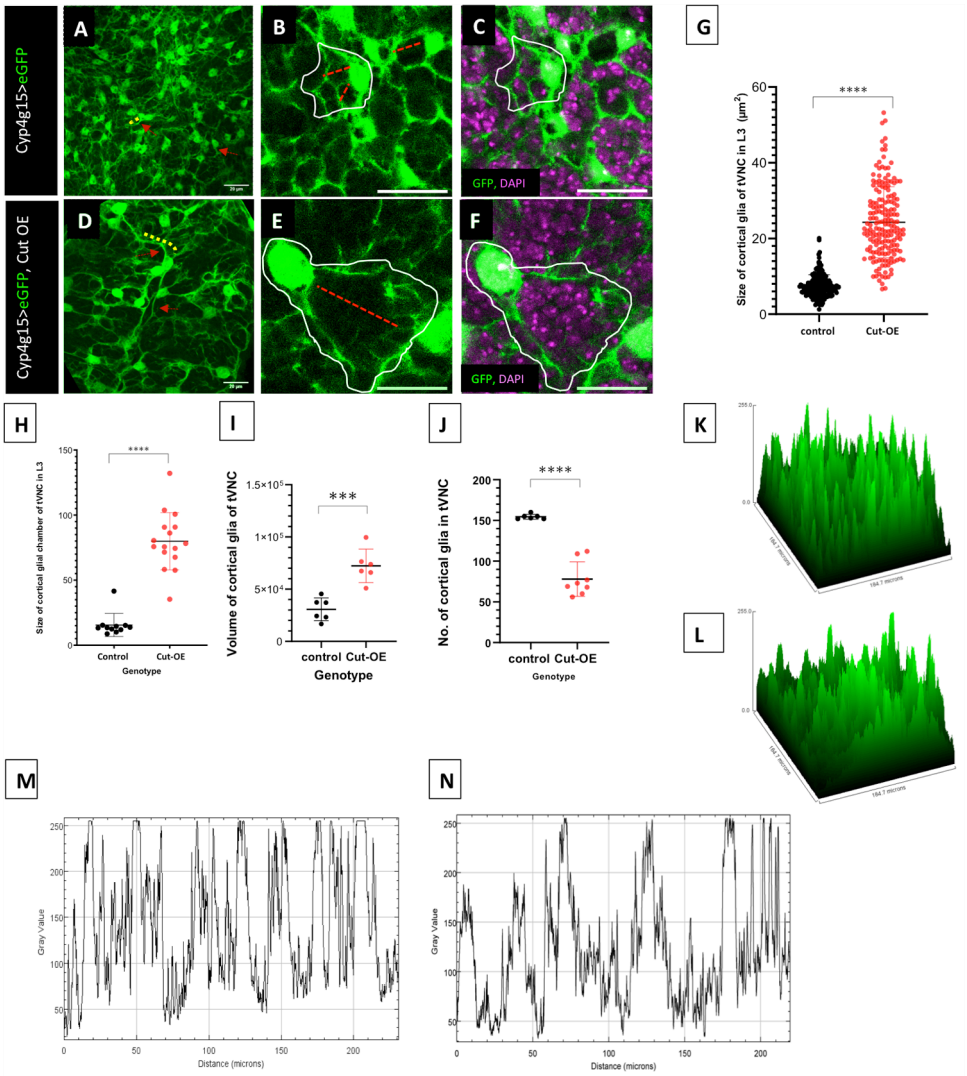


Figure 4: Overexpression of Cut in cortical glia enhances the growth of cell body and main branches at the cost of terminal extensions

(A,B,C) are control (Cyp4g15>eGFP,+); **(A)** cortical glial cell bodies (red arrows) and dense main extension (yellow lines) can be seen, **(B)** the cortical glia chambers (white encircle) are small and compact (red line to show the area covered), **(C)** same image in **B** with DAPI showing single DAPI neural nuclei in the encapsulation. **(D,E,F)** are Cut overexpressing cortical glial cells (Cyp4g15>eGFP, Cut-OE); Compare **D,E,F** with control **A,B,C** where cell bodies are less dense, big, and their cytoplasmic extensions are longer and thicker **(D)**, the main cortical branches are significantly longer, making bigger glial chambers **(E)**, and encapsulating several neural bodies marked with DAPI **(F).** Scale bar is 20µm. **(G-J)** quantifications in control (Cyp4g15>eGFP, R+) and Cut overexpression (Cyp4g15> eGFP, Cut-OE). **(G)** size of the cortical glial nuclei (Repo+, GFP+) was measured by counting 20 nuclei/tVNC (n=5). **(H)** size of the cortical glial chamber, total chambers counted 5/tVNC (n=3). **(I)** total cortical glia volume in tVNC (n>5). **(J)** number of cortical glia nuclei marked with Repo in L3 (n>5), which gets significantly reduced in Cut overexpression in tVNC. **K-L)** represents the surface plot of cortex glia in tVNC to compare the intensity and extensions of Cut overexpressing cortical glial cells with control. In Control **(K)** there are more peaks representing cell and their extensions. In contrast, upon Cut-overexpression the number of peaks is less and of variable intensities showing the loss of cortical glia side extensions. **(M, N)** is the plot profile along a line in the tVNC showing the peaks and their intensities in Control **(M)** and Cut overexpression**(N)**, again emphasizing the loss of fine glial network. Statistical evaluation of significance $p<0.0001$, $p<0.001$, and $p<0.01$ based on unpaired t-test using GraphPad Prism 9 software.

5. Constitutive activation of Cut increases DNA content in cortical glial cells

The number of cortical glial nuclei in the tVNC progressively increases from the L1 to L3 stages (Fig. 5A-C,(Coutinho-Budd et al., 2017). It is proposed that this increase in the number of cortical glial nuclei is due to endomitosis (Coutinho-Budd et al., 2017; Rujano et al., 2022; Unhavaithaya and Orr-Weaver, 2012). We note that in control tVNC 25% of cortical glia nuclei are very close (almost juxtaposed), indicating that they might be getting separated and undergoing endomitosis (Fig. 6G-I). As shown before, Cut overexpression increases the nuclei size significantly (Fig 4G); we set out to check their number and DNA content to evaluate if the process of endomitosis is affected.

Cut overexpressing cortical glia in tVNC increases their number from the L1-L2 stage similar to control (compare Fig. 5D,E with 5A, B graph H). On the contrary, while this number increases further in control tVNC from the L2-L3 stage (Fig.5B-C, graph H); the rise is not seen in the Cut overexpressing cortical glial number (Fig.5E-F, Graph H). In control tVNC, the number of cortical glial nuclei increases 1.4 fold from L2-L3, while for Cut overexpressing glia, instead of an increase in the nuclei number; their size increases (compare Fig 5E and F, Graph H). Furthermore, we observed that the distribution pattern of cortical glial nuclei in L3 tVNC of Cut overexpressing CNS is more similar to the control L1/L2 tVNC instead of L3 (compare Fig 5F and 5A-B). For example, in Control L2 larval tVNC, the glial cells are arranged in a linear pattern on both sides of the midline and 2-3 cells per hemisegment on the lateral side (Fig 5B, G). Strikingly, the L3 larval tVNC of Cut overexpressing glia also have a similar arrangement and number, although the pattern is deformed due to unexpected loss of cells (Fig 5F).

Based on these observations, we hypothesize that excess Cut protein may interfere with the splitting of nuclei during endomitosis of cortical glia leading to increased nuclear size and content. To further confirm, we checked the DNA content measuring the integrated density of DAPI of each cortical glial nuclei labeled with GFP and Repo (DAPI+, GFP+, Repo+) through image J. Noticeably, the DNA content in each nucleus showed a 2-3 fold increase upon Cut overexpression when compared with control (Fig.6 compare C with F, Graph G). Together these observations indicate that constantly high Cut levels inhibit nuclei splitting during endomitosis, and these nuclei remain endoreplicated instead of separating. Therefore, we propose that the homeodomain protein Cut is required for the cortical glial cells to increase the DNA content. Conversely, the nuclei divide when its level goes down, and cells undergo endomitosis. The continued high Cut levels lead to endoreplicated nuclei remaining as a single nucleus in a cell.

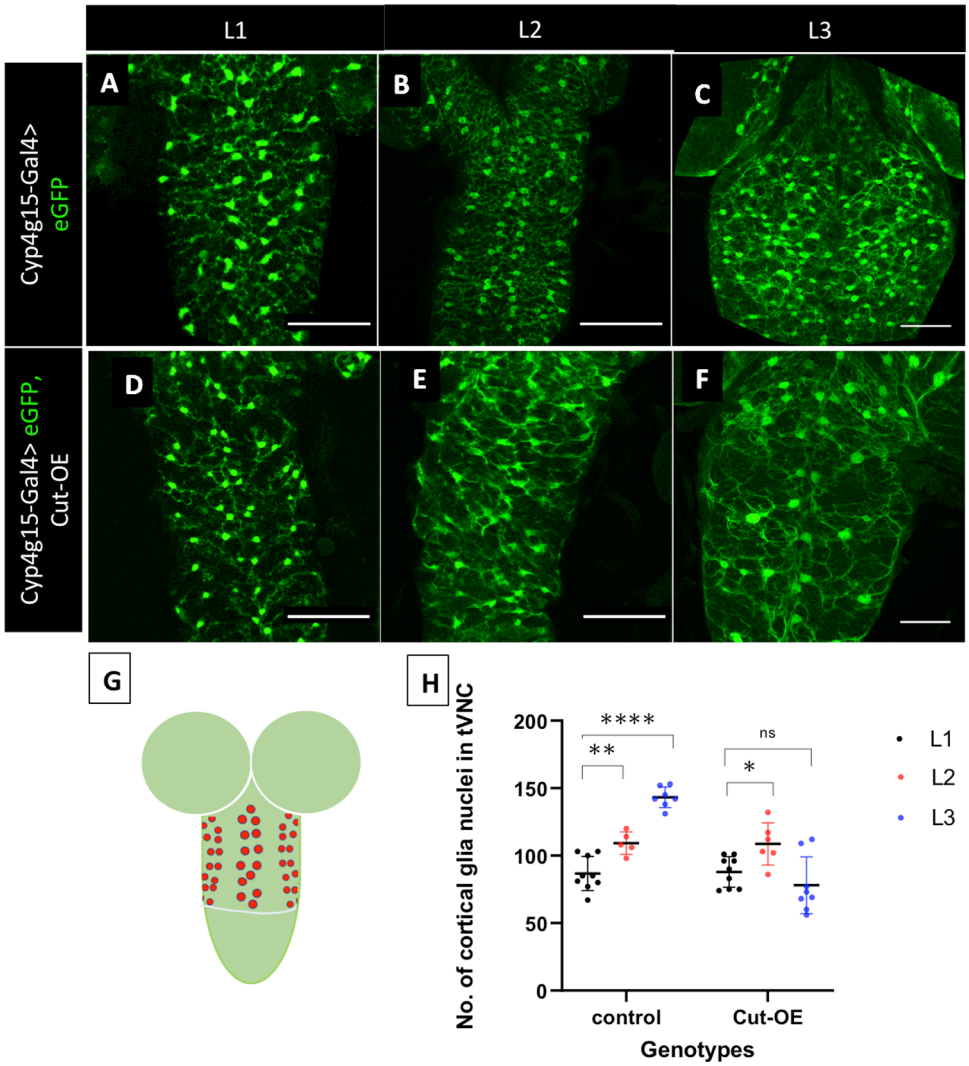
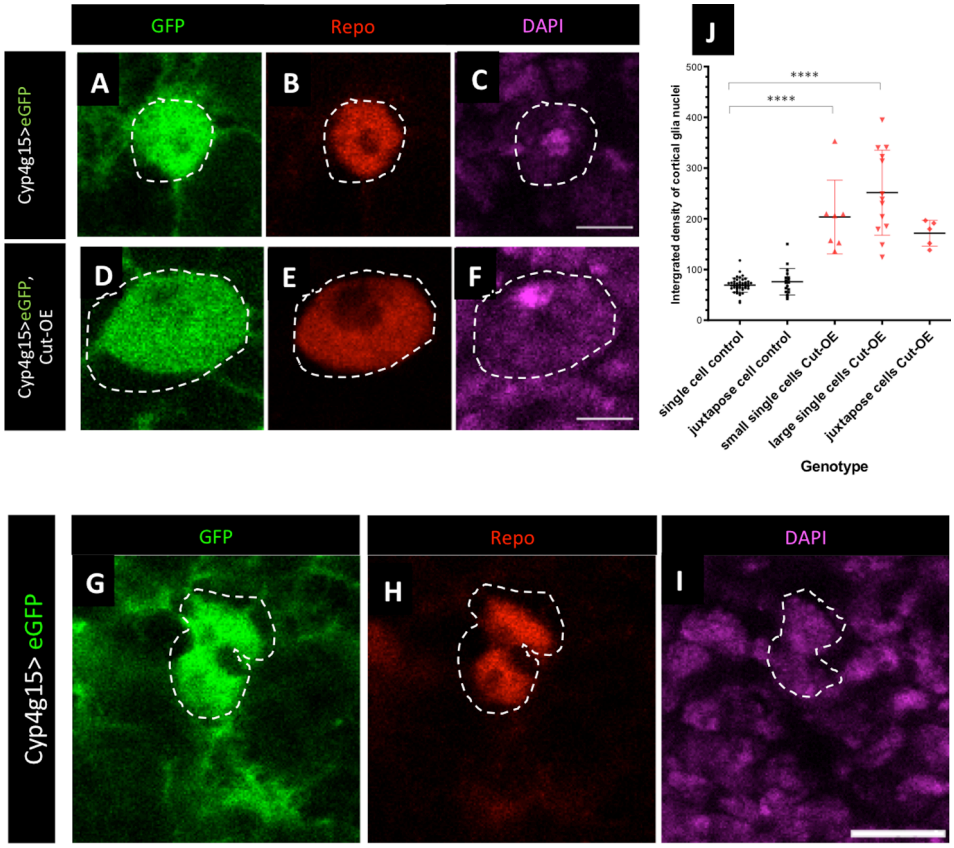


Figure 5. Cut regulates endomitosis of cortical glial Cells

(A-F) Confocal projections of the top few sections are shown for clarity. Compare the pattern of cortical glia distribution from L1-L3 in tVNC of control (Cyp4g15> eGFP, +, **A-C**) with Cut overexpressing (Cyp4g15> eGFP, Cut-OE **D-F**). L1 and L2 control tVNC **(A, B** cartoon in **G),** the nuclei are arranged more or less in a vertical line pattern (depicted as); nuclei number drastically increased in L3 with brighter cell bodies. The distribution of cortical glial in L1 of Cut overexpression is more or less similar to control (compare **A** with **D**), whereas in L2, it gets a bit distorted (compare **B** with **E)**. A striking difference is seen in Cut overexpressing L3 **(F)** from L3 control **(C)**. The pattern of GFP-positive cortical glia upon Cut overexpression appears more like L1/L2 of controls (compare **D** with **A, B**). No increase in the GFP+ cells is observed in the Cut overexpressing tVNC (compare **F** with **C**). **(G)** Model of L2 CNS showing cortical glia pattern in tVNC. (**H)** Further, quantification of cortical glial nuclei from L1-L3 in control and Cut-OE clearly shows that in Cut overexpression, the nuclei number does not increase from L2 to L3 (n>5). Statistical evaluation of significance $p<0.0001$, $p<0.001$, and $p<0.01$ based on unpaired t-test using GraphPad Prism 9 software.



6. Constitutive activation of Cut increases DNA content in cortical glia.

(A-I) cortical glia nuclei of L3 are marked with GFP (green), Repo (Red), and DAPI (magenta). **(A-C)** control cortical glia (Cyp4g15>eGFP,+) nuclei of L3 tVNC **(D-F)** overexpression of Cut (Cyp4g15>eGFP, UAS-Cut) results in a significant increase in the size of cortical glia nuclei (compare control **A-C** with **D-F** Cut-OE). **(G-I)** juxtaposed control cortical glia appears to be splitting during endomitosis.

(J) DNA quantification by the integrated density of DAPI in each cortical glia nuclei in single and juxtaposed cells (more than 20 nuclei counted per CNS (n=3) showing a significant increase in DNA content upon Cut overexpression. Statistical evaluation of significance **** $p<0.0001$, $^{ns}p<0.183$ based on unpaired t-test using GraphPad Prism 9 software.

Discussion

In this study, we report a novel role of the homeodomain transcription factor, Cut, in the growth and development of cortical glial cells, which form a complex reticular network around the neural cells. Continued high Cut levels in these cells interfere with their normal endomitosis process and affect glial growth. The loss of Cut in the cortical glial cells hampers the development of cytoplasmic processes. Likewise, overexpression of Cut enhances the growth of their primary extensions although at the cost of finer networks that cover individual neural cells. Moreover, a continued high level of Cut also inhibits the splitting of the cortical glial nuclei resulting in a significant increase in the DNA content in these cells. Thus, Cut is a novel player which autonomously regulates the dynamic growth of cortical glial niche around neural cells by controlling the complexity of membrane growth and endomitosis.

Cut a novel regulator of cortical glial morphogenesis

The transcription factor Cut is known to play several roles in context of cell type and tissue-dependent manner. Acting as a selector gene, it regulates the identity of cells and their growth during development (Krupp et al., 2005). The differential level of Cut and its mammalian homolog, CUX1/2, controls the complexity of dendritic branching, the number of dendritic spines, and tracheal development (Cubelos et al., 2010; Grueber et al., 2003; Pitsouli and Perrimon, 2013). Similarly, Cut instructs the growth of membrane protrusions of the wrapping glia in the peripheral nervous system and acts downstream of FGF signaling (Bauke et al., 2015). In the case of cortical glia, we see that Cut activation enhances the overall growth and thickness of main cortical glial extensions but at the cost of side branching. Our data adds that Cut controls the growth of cortical glial cells by regulating endomitosis.

It is significant that when cortical glial cells are ablated in a restricted area, the neighboring cortical glial membrane extend their processes and fill the gaps (Coutinho-Budd et al., 2017; Hirase et al., 2022). However, Cut defective cortical glia does not show such compensatory growth, resulting in visible wide gaps in the glial trophospongium in late third instar CNS. Thus, we conclude that Cut is required and sufficient for the growth of cortical glial membrane processes (Coutinho-Budd et al., 2017). Although the signaling pathways and the domain through Cut functions await further investigation.

Cortical glial membrane extensions start growing around the NSC and neurons of the larval nervous system after receiving the nutritional signals via activation of PI3K/Akt signaling (Spé Der and Brand, 2018; Yuan et al., 2020). Since Cut defective cortical glial cells show severely hampered growth of cytoplasmic extension even in early L2, we think that Cut might act upstream of the PI3K/Akt signaling. Other known regulators of cortical glia growth are FGF signaling, components of the membrane fusion machinery, and secreted neurotrophin Spätzle 3 (Avet-Rochex et al., 2012; Coutinho-Budd et al., 2017; Dong et al., 2020; Read, 2018; Read et al., 2009; Reddy B. V. V. G. and Irvine Kenneth, 2011; Spé Der and Brand, 2018; Witte et al., 2009). However, the broad phenotype of Cut activation differs from FGF activation in the cortical glia. Ectopic activation of FGF signaling induces cortical glia overgrowth with a robust increase in nuclei number and overall membrane surface area (Avet-Rochex et al., 2012). We found that overexpression of Cut does not enhance the nuclei number of cortical glia. Instead, it resulted in giant nuclei with higher DNA content. Furthermore, overexpression of FGF receptor htlACT in cortical glia makes a heavy network of finer cortical glia extensions (Avet-Rochex et al., 2012). In contrast, Cut overexpression cause a widespread reduction in the

finer extensions. Thus, the cytoplasmic growth of fine extension in overexpression of Cut vs. FGF signaling in cortical glial cells also has different outcomes and may act through distinct mechanisms.

Regulation of ploidy is essential in the glial cells to control their growth

The size of a cell is important and plays a crucial role during development. Several differentiated cells undergo ploidy changes to increase their size or metabolic output. For example, germline nurse cells, salivary glands, fat bodies, gut, and trachea show varying levels of endopolyploidy (Lilly and Spradling, 1996). Interestingly, neurons and glia in invertebrate and vertebrate adult brains compensate for aging-related cell loss or other damages by undergoing polyploidy (Cohen et al., 2018; Losick et al., 2013; Losick et al., 2016; Nandakumar et al., 2020; Tamori and Deng, 2013). On the other hand, endomitosis is also a way to increase the cell size through which cells undergo incomplete mitosis and increase the number of nuclei per cell. A few examples of the same are known in *Drosophila* and mammalian systems (Britton and Edgar, 1998; Edgar and Orr-Weaver, 2001; Rios et al., 2016; Taniguchi and Kokuryo, 2012; von Stetina et al., 2018; Windmueller et al., 2020). Several reports from independent labs strongly indicate that cortical glial cells increase their nuclei number by undergoing endomitosis (Coutinho-Budd et al., 2017; Rujano et al., 2022; Unhavaithaya and Orr-Weaver, 2012; Yuan et al., 2020). A very recent study clearly shows that cortical glial cells grow by undergoing endocycling and endomitosis and make syncytia and complex membrane networks around neural cells (Rujano et al., 2022). Cut regulates the growth of cortical glia by regulating the complexity of cytoplasmic membrane extension and interferes with endomitosis if present in excess. The cortical glial cells overexpressing Cut are clearly stuck in an endocycling phase and unable to split their nuclei leading to increased DNA content in these cells. Thus, in cortical glial cells, over-expression of Cut does not show the routine increase of nuclei number from the L2 to L3 stage as seen in control tVNC; instead, it increases the ploidy in nuclei.

Aneuploidy is one of the most common phenomena seen in most tumors (Rajagopalan and Lengauer, 2004), and Cut overexpression is also (Sansregret and Nepveu, 2008) reported in a diverse classes of cancer. There are several tumors, including glioma, where CUX1 overexpression has been reported and considered among driver mutation (Feng et al., 2021; Griesmann et al., 2021; Sansregret and Nepveu, 2008; Xu et al., 2021). A *in vitro* study shows that mammalian CUX1 has the potential to cause chromosomal instability (Sansregret et al., 2011). Our findings highlight that Cut enhances the DNA content of glial cells. It would be

interesting to see if the genetic combination of Cut with any other tumor driver identified in glioma could make the nuclei unstable and lead to cancer in the brain. In conclusion, our finding that Cut regulates cortical glial growth and endomitosis is of significant importance for development and disease prospect.

Materials and methods

Fly stocks and genotypes

Drosophila melanogaster were reared at 22^0C and RNAi crosses were done at 29°C, on standard food medium containing sugar, agar, maize powder, and yeast. Appropriate fly crosses were set up following standard methods to obtain progeny of desired genotypes. The following fly stocks are used in the experiments: Oregon R+ as wild-type, repo-Gal4 (BL 7415), Cut-RNAi (BL 33967, strong), Cut-RNAi (BL29625, weak), UAS-Cut (II, referred in text as Cut-OE) (Norbert Perrimon, Harvard Medical School, MA, USA), cyp4g15Gal4 (39103), UAS-eGFP(II) (BL-5431), R54H02-Gal4(BL- 45784), R46H12-Gal4 (BL- 50285), NP577-Gal4 (Awasaki et al., 2008), UAS-p35(BL-5072). The following genotype combinations are generated in the lab for the study: UAS-p35; Cut-RNAi, UAS-eGFP; cyp4g15Gal4.

Immunostaining, confocal microscopy and documentation

Larvae of the required age from F1 progeny of respective crosses were selected and CNS were dissected in Phosphate Buffer (PBS 1X containing- NaCl, KCL, NA2HPO4, KH2PO4, pH-7.4), fixed in 4% Paraformaldehyde for 30 min., rinsed in 0.1% PBST (1XPBS,0.1%TritonX-100), and incubated in blocking solution (0.1% TritonX-100, 0.1%BSA, 10%FCS, 0.1% deoxycholate, 0.02% thiomersal) for 30min., at room temperature. Samples were incubated in the required primary antibody at 4 °C overnight. Antibodies used are: mouse anti-Cut (1:10, Developmental Studies Hybridoma Bank, 2B10), mouse, Anti-repo (1:50, Developmental Studies Hybridoma Bank, 8D12), rabbit anti-GFP antibody (1:200, Invitrogen, A-11122). For rat anti-Dpn antibody (1:150, ab195173, Abcam) the samples were kept for 2 consecutive overnights for better signal. The following day, samples were rinsed thrice in 0.1% PBST and incubated with appropriate secondary antibody at 1:200 dilution either overnight or two hours at room temperature. secondary antibodies used are: donkey anti-rat Alexa 488 (Invitrogen, A-21208), Goat anti-Mouse Alexa Fluor 568 (cat. No. A11004), Chicken Anti-Rabbit Alexa Fluor 488 (Invitrogen, A-21441). Following incubation with secondary antibodies, the samples were washed thrice in 0.1%PBST, counterstained with DAPI (1mg/ml, Invitrogen, D1306) at 4

°C overnight whenever needed. After final washes in 0.1%PBST, the samples were mounted in DABCO (Sigma, D27802) for further analysis.

Images acquired at flolowing confocal microscopes Zeiss LSM-510 Meta at the Department of Zoology, Zeiss LSM-510 at ISLS BHU, and Leica SP8 STED facility at CDC, BHU. All the images were quantified using Fiji/ImageJ software (NIH, USA).

Image analysis using Fiji/ImageJ application

Confocal sections of cortical glial cells of tVNC were analyzed using Fiji/ImageJ software (NIH, USA) to measure number, area, and volume. For integrated intensity measurements in a region of interest (ROI) freehand selection tool was used to mark the area manually.

The number of Glial cells and NSCs were counted manually based on Gal4-driven GFP and Repo stainings. To measure the cell size of cortical glial cells (GFP+, Repo+) multipoint selection tool was used to outline the area. The area outlining was done using the freehand selection tool around repo staining, followed by the analyze-measure option. For volume analysis of cortical glia one specific threshold was chosen that fits best for the actual staining and was kept constant throughout the analysis. The thresholding procedure is used in image processing to select pixels of interest based on the intensity of the pixel values. Thereafter Measure stack plugin was used to find the fluorescent area of each cortical glial section of tVNC through analyze-measure option where Area, Mean gray value, Stack position, and Limit to Threshold checkboxes was selected and the area obtained was thus multiplied with the number of stack interval (=2) to find out the volume of cortical glia. Finally, the area covered was estimated by manually outlining the area occupied by tVNC.

The density of cortical glial extension was calculated through the plot profile and the surface profile options. The tools measure the signal intensities of cortical glial processes along the selected lines. The gray values indicate the intensities of cortical glial cells along a line and the number of peaks denotes the number of cortical glial extensions.

Integrated density for DAPI in the cortical glial nuclei was measured by manually outlining each GFP+, Repo+ DAPI+ cell. The integrated density of DAPI was measured in each section of tVNC using the analyze-measure option. The confocal scanning parameters for each experimental setup were kept constant for all intensity quantifications. Controls were analysed in parallel to each experimental set.

Statistical analysis was done using Graphpad prism 8.4.2 software, with $P < 0.05$ considered statistically significant. Two-tailed unpaired t-test was used to evaluate the statistical significance between the mean values of the two groups.

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Author's contribution

All authors contributed to the article. VY performed the animal experiments, image acquisition, design of the quantification workflow, and statistical analysis and made the figure panel under the supervision of RA. PD has re-analyzed the quantification of fig. 4G, 6G under the supervision of VY and RA. Part of the quantification of fig. 1J, 4G and 3H are done by RKM under VY, PD and RA supervision.

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