D. RESULTS

I NEURONAL ACTIVITY DEPENDENT REGULATION OF CALEB

1. Activity dependent down regulation of cell surface CALEB

An initial screen with chick retina cultures incubated with 30 mM KCl to induce neuronal activity for 18-20 hr, followed by solubilization and Western blot with mAb 4/1 revealed a decrease in the level of the 80 kD CALEB band. Based on these findings four questions were asked: (1) Characterization of CALEB in chick; (2) Is the down regulation a discrete cell surface phenomenon; (3) What is the kinetic of the down regulation; (4) Is down regulation an action potential dependent process. In the following sections we have tried to answer these questions.

1.1 Characterization of CALEB in E11 chick retina

Synaptogenesis starts at E12 in the chick retina (Reiss et al., 1996;Hering and Kroger, 1996). Retina cultures were therefore prepared from E8 chick embryos and cultivated for 3 DIV in DMEM/N2. Incubation of chick retina cultures for 3 DIV might correspond to E11 chick retina *in-vivo*. Detergent extracts of 3 DIV chick retina cells were resolved by Western blot with mAb 4/1. Unfixed E8 chick retina cultures, 3 DIV were immunostained with mAb 4/1 to validate the CALEB expression in cultures. Layer specific expression of CALEB was analysed in E11 chick retina tissue and an overview of CALEB immunoreactivity was viewed in E11 chick brain to ascertain a plausible role during development of retina and parts of brain.

It has already been reported that CALEB is expressed in several molecular mass forms in the developing nervous system (Schumacher et al., 1997). The mAb 4/1 recognised three bands of chick CALEB, i.e., a 200 kD, followed by lower forms of 140 kD and 80 kD (Fig 1 (i)). The 200 kD band represents the glycosylated form of the 140 kD component, while the 80 kD band is derived from the 140 kD band (Schumacher et al., 1997). The 80 kD band is the most prominent band recognised by the mAb 4/1 and will be considered in all the following experiments for quantification although the effect observed by the 140 kD and 80 kD band was identical.

CALEB immunostaining in unfixed cultures revealed a punctate pattern on the extensions and surface of cell soma (Fig 1 (ii)). To further analyze CALEB expression cryosections were cut from brain and retina of E11 chick embryo. The sections of brain and retina were immunostained with mAb 4/1.

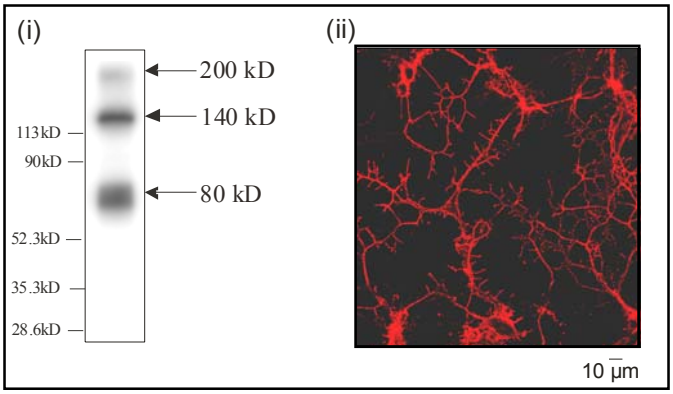


Fig 1:Chick CALEB detected in chick retina cultures. Retina cells from E8 chick embryos were cultivated on poly-l-lysine precoated culture dishes. **(i)** The cultures were solubilized with solubilization buffer after 3 DIV and Western blotted with mAb 4/1. Three bands were recognised at 200 kD, 140 kD and at 80 kD. **(ii)** After 3 DIV the chick retina cultures were stained with mAb 4/1 (3 µg/ml), followed by *Cy-3* tagged Goat α mouse (1:500), secondary antibody, staining the neurites and filopodia.

In E11 chick retina, immunoreactivity could be seen in the inner layers, such as ganglion cell layer (GCL), inner plexiform layer (IPL) and the nerve fiber layer (NFL). There is uniform staining in the IPL, and the GCL. In the NFL, the processes of glial cells are thicker and ramified as visualized by the staining. The outer layers such as the layer of rods and cones, i.e., the outer segment (OS) photoreceptor layer as well as retinal pigment epithelium (RPE) are stained intensely.

The E11 chick brain sections were stained with cresyl violet to get an overview of the regions (Fig 1 (a)). Staining of the sections with mAb 4/1 revealed some interesting regions.

CALEB immunoreactivity was visible in the upper and lower immature strata of the *stratum griseum et fibrosum superficiallies* (SGFS) of the optic tectum, of the mid brain (Fig 1 (b)). Strong staining could be observed in the pretectal region cell population in the primordium of the medial spiriform nucleus, which has a dorsalward extension with a swell in the terminal part known as dorsofrontal nucleus (Fig 1 (c)). In the telencephalic area, CALEB immunopositivity could be observed at the bottom, in the olfactory bulb (Fig 1 (d)).

The ventral pallium of the telencephalon is stained with the CALEB antibody. The lower limit is well delineated and is the pallio-subpallial boundary (Fig 1 (e)). The results suggest a role for CALEB in region formation and/or specification.

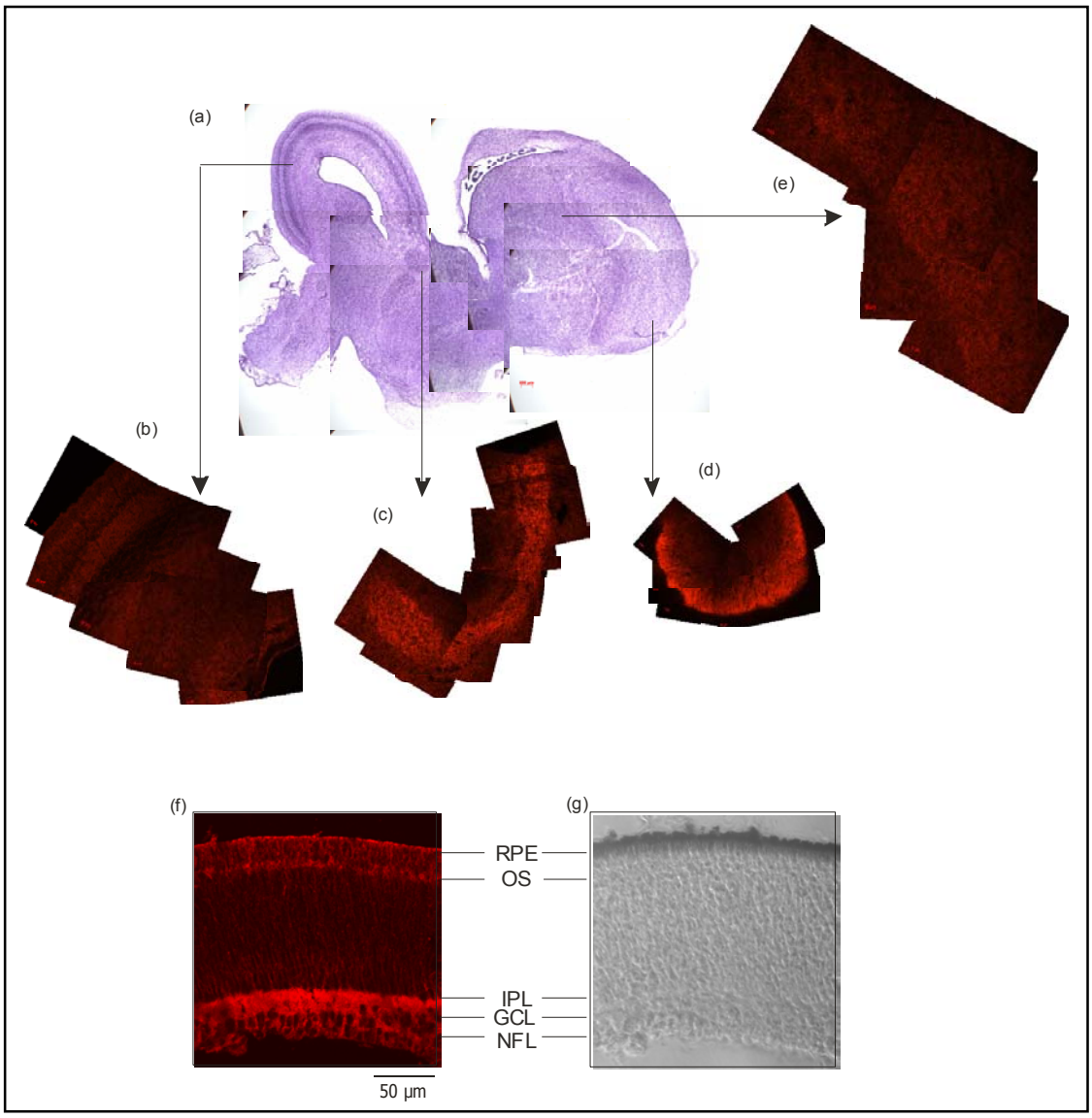


Fig 2:Chick CALEB immunohisto-staining. Stained sagittal sections of E11 chick brain (16 µm) and chick retina (18 µm); **(a)** Cresyl violet whole brain staining; the sections were stained with *Cy-3*- tagged secondary antibody. The stained regions are marked with arrows and have been depicted with a higher magnification. **(b)** SGFS of tectum**(c)** primordium of the medial spiriform nucleus **(d)** olfactory bulb **(e)** ventral pallium of telencephalon.

Retina sections of E11 chick were stained with **(f)** mAb 4/1, **(g)** unstained section visualized through phase contrast mode. (The regions of the chick brain were identified with the personal help of Prof. L. Puelles, University of Murcia, Spain and Prof. C. Redies, University of Jena, Germany).

In chick retina tissue CALEB is expressed in developmentally regulated pattern (Schumacher et al., 1997). This prompted us to look into the expression pattern of CALEB

in chick retina cultures from E8 embryos by incubating for various DIVs. The cells were solubilized and analyzed by SDS-PAGE. The Western blot results revealed a constant expression of CALEB in the chick retina cultures from DIV 3-8. For further experiments E8 chick retina cultures, 3 DIV were used.

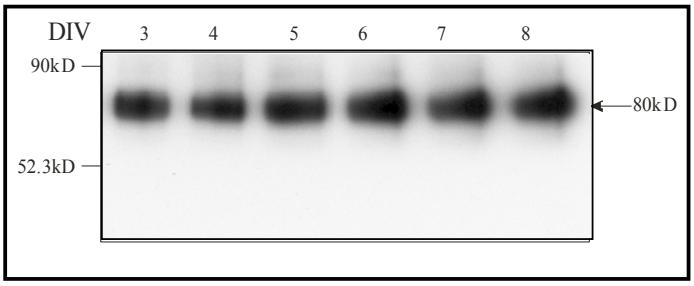


Fig 3:CALEB expression in the chick retina culture. Chick retina cultures were prepared from E8 chick embryo on poly-l-lysine precoated cell culture dishes. Cells were platted at a density of 1.5 x 10^6 cells/ml in a total volume of 1 ml and the cultures were incubated for various time periods in chick retina cell culture medium. The cells were solubilized in solubilization buffer and analysed by Western blot with mAb 4/1.

1.2 Cell surface CALEB down regulation with neuronal activity

Based on the initial results of CALEB down regulation with KCl incubation as observed by Prof. Rathjen, further experiments were performed to confirm the down regulation of CALEB; whether this is a cell surface phenomenon and to study the kinetics of the down regulation.

Chick retina cultures (3 DIV) were incubated with KCl to induce neuronal activity for 1-4 hr in order to determine the earliest time point of down regulation in CALEB expression. Detergent extracts of cells were immunoblotted with mAb 4/1 and the results revealed the down regulation of total CALEB only after incubation for a minimum of 4 hr (Fig 4). Detecting total CALEB had a couple of drawbacks one being that we were not able to differentiate between the effect of KCl on the internal pool and the cell surface expression of CALEB molecules. The next one was duration of exposing the cells with KCl, which was long and could be critical for the cells.

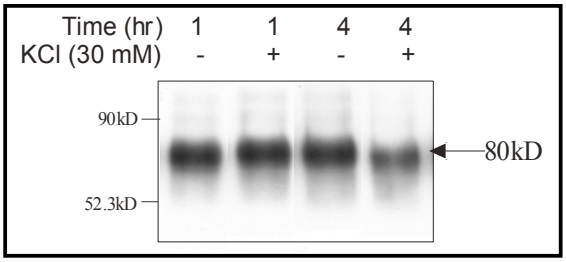
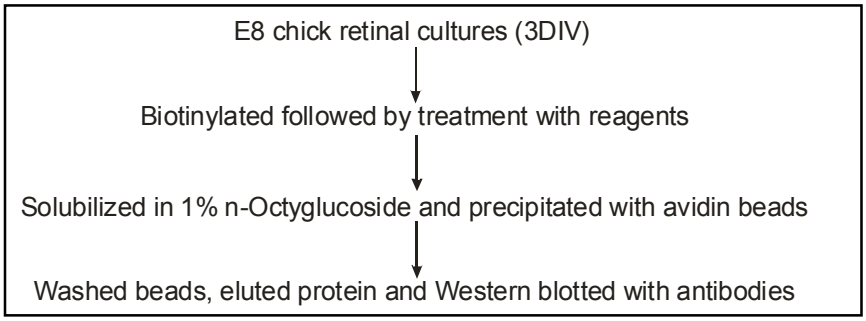


Fig 4:Total CALEB down regulation with KCl incubation. Chick retina cultures (3 DIV) were depolarized for different time periods (1 hr and 4 hr) with KCl (30 mM). The cells were solubilized and blotted with mAb 4/1.



Scheme 5: Cell surface biotinylation. Chick retina cultures (3 DIV) were biotinylated by Sulfo-NHS-LC-Biotin, followed by treatment with KCl (30 mM) for various time periods. The cultures were then solubilized and immunoblotted.

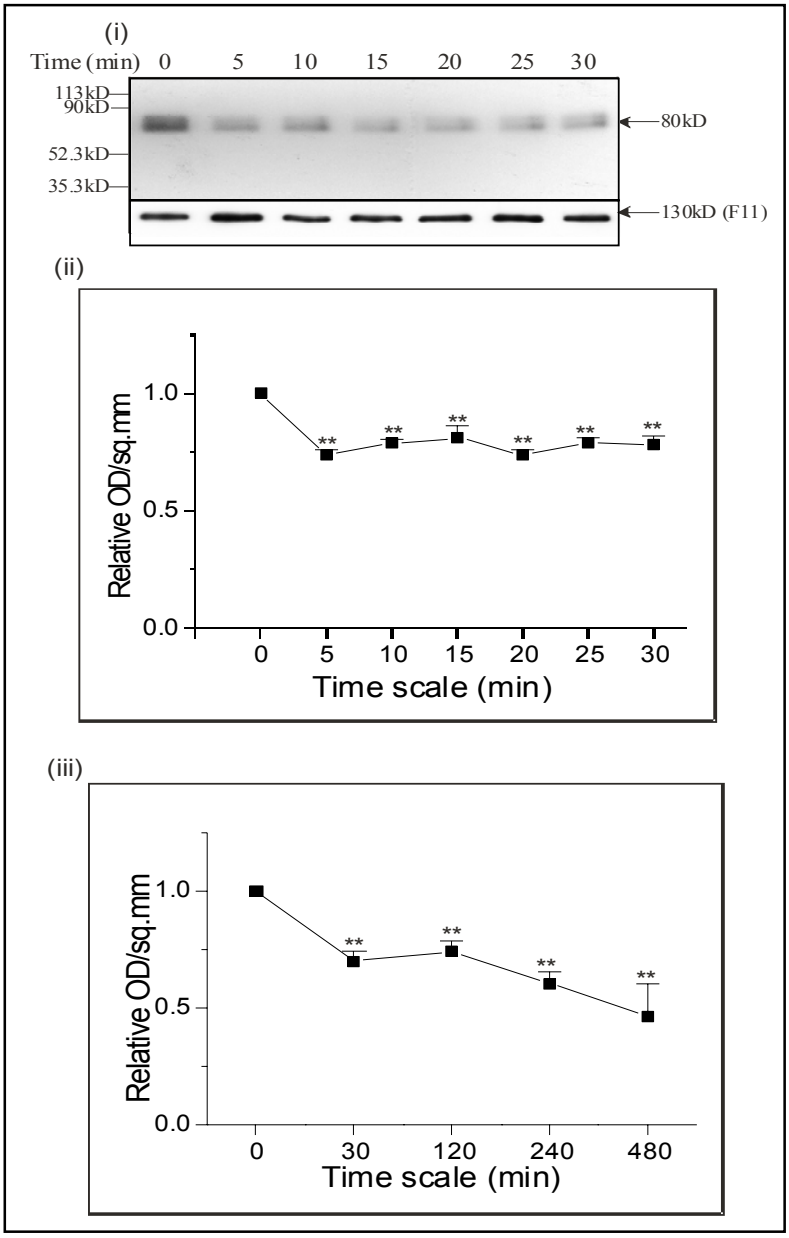


Fig 5:Time period for cell surface down regulation. **(i)** Chick retina cultures (3 DIV) of 1.5 x 10^6 cells/ml were biotinylated with EZ link-sulfo-NHS-LC-biotin. The cultures were then treated

with 30 mM KCl in chick retina culture medium. Cultures were solubilized in solubilization buffer after various durations of incubation with KCl. The solubilized samples were resolved in reducing conditions on a 10% SDS-PAGE and analysed by Western blot with mAb 4/1 and developed with chemiluminescence. mAb F11 was used as a control **(ii)** The bands obtained were scanned and quantified with Image J software. Statistical significance was calculated and data were represented graphically after normalization of values with respect to the untreated culture. (n=4; Mean ± S.E; $p < 0.05$) **(iii)** Graphical representation of 80 kD bands quantified from biotinylated chick retina cultures incubated with 30 mM KCl for durations longer than 30 min till 480 min (n=2; Mean ± S.E; $p < 0.05$)

The reason for observing the CALEB down regulation after 4 hr could be because of the masking effect of largely unaffected population of CALEB in comparison to the down regulated CALEB. In order to differentiate the cell surface from the internal pool of CALEB and to determine the specific population of CALEB molecules that undergo down regulation, chick retina cultures were biotinylated by NHS-LC-Biotin (Scheme 5). Incubation of the biotinylated cells with KCl for 5-30 min, revealed down regulation of CALEB within 5 min in Western blot, which was significant with respect to untreated cultures.

The 80 kD band was quantified by Image J software and graphically represented (Fig 5 (ii)). The decrease observed in the expression of cell surface CALEB was in the range of 30% in comparison with the untreated cultures. The down regulation occurred within 5 min of incubation with KCl, and remained constant for over 30 min (Fig 5 (i)) and decreased further till 12 hr post incubation.

These results show the down regulation of CALEB restricted to a cell surface phenomenon. The detection of cell surface CALEB down regulation within 5 min of depolarization, suggests the mechanism to be a fast kinetic event. Based on these findings all the further experiments were performed by incubating the chick retina cultures for 10 min with reagents for better handling and convenience.

1.3 Cell surface down regulation occurs with the activation of glutamate receptors

Incubation of cell cultures with KCl not only artificially induced neuronal activity but also it changed the osmolality of the medium. The down regulation observed previously in our experiments could be an outcome of either of the mentioned effects. To determine if cell surface CALEB down regulation could be observed on inducing neuronal activity, cultures were incubated with agonists of ionotropic glutamate receptors.

Biotinylated chick retina cultures when incubated with NMDA, an agonist of NMDA receptor or Kainate, an agonist of Kainate receptor revealed down regulation of cell surface CALEB in the range of 30 % (Fig 6 (i)) in Western blots with mAb 4/1. The extent of down regulation observed with agonists of glutamate receptors was similar to the down regulation detected with KCl incubation.

In order to confirm the findings obtained by using glutamate receptor agonists, biotinylated chick retina cultures were incubated with NMDA and APV (specific antagonists of NMDA receptor) and Kainate and DNQX (specific antagonists of Kainate receptor). Western blot of the solubilized cultures revealed that the specific receptor blockers did not have any effect on CALEB expression, when incubated alone in the chick retina cultures.

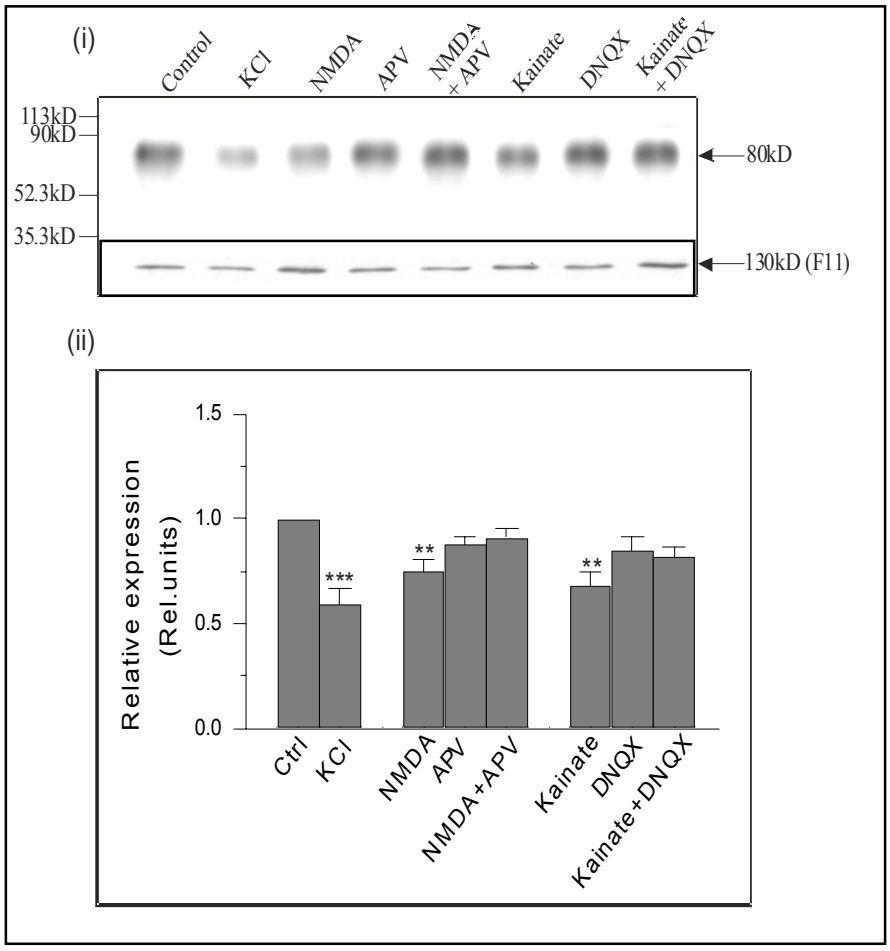


Fig 6:Down regulation of CALEB in the presence of glutamate receptor agonists. Chick retina cultures (3 DIV) were biotinylated with EZ link-sulfo-NHS-LC-Biotin followed by incubation with NMDA (10 μM) (Katsuki et al., 2001), Kainate (50 μM) (Ientile et al., 2001) alone or in combination with specific inhibitors APV (50 μM) (Meffert et al., 2003), DNQX (25 μM) (Mathews and Diamond, 2003) respectively for 10 min. **(i)** The cultures were solubilized,

biotinylated proteins captured with avidin beads, and finally eluted with lammeli buffer. The eluted samples were analyzed by Western blot with mAb 4/1 and mAb F11 (to confirm equal protein loading)**.** ***Lane 1***, the control culture; ***lane 2***, culture treated with 30 mM KCl; ***lane 3***, cultures incubated with NMDA; ***lane 4***, cultures incubated with NMDA+APV; ***lane 5*** cultures incubated with Kainate and ***lane 6***, cultures incubated with Kainate+ DNQX. The blots were developed by chemiluminescence technique. **(ii)** The bands obtained were quantified. The data were represented graphically by normalising values with respect to the untreated cultures. Statistical significance tests were done by Student's t-test. (n=4; Mean± S.E.; $p<0.05$).

In cultures incubated with the glutamate receptor activators (NMDA and Kainate) in combination with their respective blockers (APV and DNQX), cell surface down regulation of CALEB was prevented and quantification of the 80 kD band obtained values comparable to the untreated cultures (Fig 6 (ii)).

The results obtained in this experiment confirmed, activity-dependent down regulation of cell surface CALEB, which can be induced specifically by activating NMDA or kainate receptors with agonists.

1.4 Down regulation of CALEB is an action potential independent process

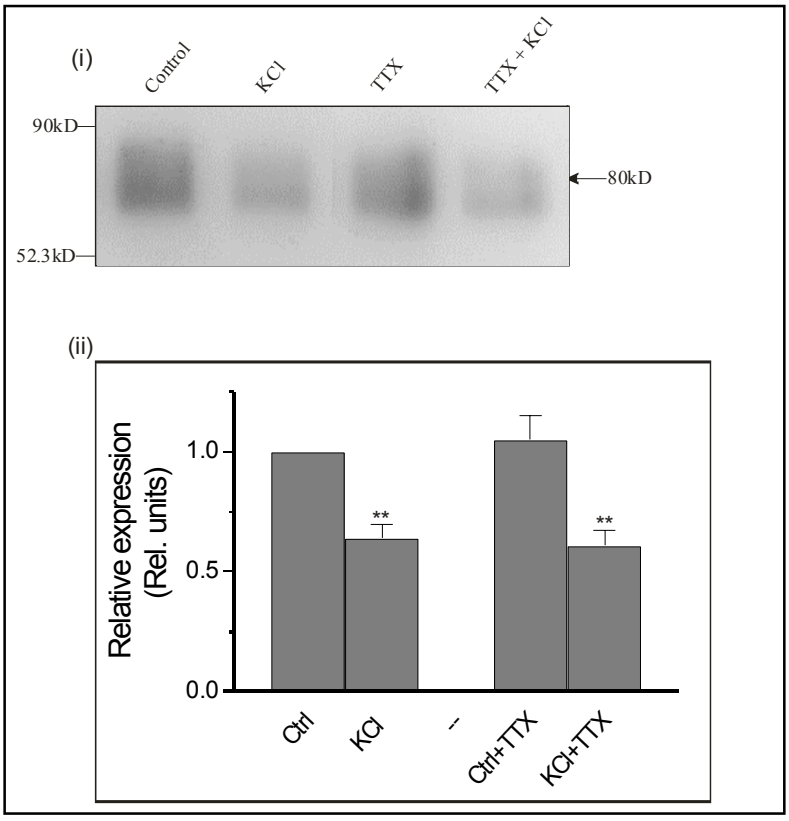


Fig 7:CALEB down regulation is an action potential independent process. Chick retina cultures (3 DIV) were biotinylated with EZ link-sulfo-NHS-LC-Biotin. The cells were then incubated with 30 mM KCl in combination with 1 μM TTX (Cadas et al., 1996)or were left untreated for 10 min. The biotinylated proteins were solubilized, precipitated with avidin

beads, eluted with lammeli buffer and analyzed by Western blotting with mAb 4/1. **(i)** ***Lane 1***, untreated culture; ***lane 2***, 30 mM KCl treated culture; ***lane 3***, cultures incubated with 1 μM TTX; ***lane 4***, cultures incubated with KCl+ TTX. The blots were developed by chemiluminescence method. **(ii)** The 80 kD band was quantified by Image J software. The graph was plotted by normalizing the values with respect to the untreated cultures. The statistical significance was calculated by Student's t-test. (n=3; Mean± S.E.; $p<0.05$)

Depolarization induces calcium influx and generation of action potential in a short exposure with KCl. Hence, we were interested in investigating the role of action potentials in causing the cell surface down regulation of CALEB.

Incubation with tetrodotoxin (TTX) in biotinylated chick retina cultures along with KCl did not have any effect on cell surface CALEB down regulation as detected in Western blot with mAb 4/1 (Fig 7- (i)). Quantification of 80 kD bands from cultures with or without TTX were alike, i.e., 30% of cell surface CALEB got reduced in comparison to untreated cultures (Fig 7 (ii)).

The results from the blots with TTX incubated cultures with/without KCl conclude that the cell surface down regulation of CALEB is an action potential independent phenomenon.

2. Role of Calcium in cell surface down regulation of CALEB

Calcium is known to be responsible for several signalling pathways acting either directly or as a secondary messenger. On depolarization with KCl there is an influx of calcium occurring in cells. Based on these facts, the role of calcium and calcium binding proteins was investigated in the down regulation of cell surface CALEB.

2.1 Calcium influx is necessary for the down regulation of CALEB

In order to investigate the role of calcium influx in down regulation of cell surface CALEB, biotinylated chick retina cultures (3 DIV) were incubated with (a series of) different concentrations of increasing amount of calcium in the medium in the presence of KCl. The health of cells in cultures was verified by phase contrast microscope before solubilization and Western blot with mAb 4/1.

There was no down regulation of CALEB expression when cells were treated with KCl in a calcium free medium, but there was a steady decrease in the cell surface CALEB with increasing concentration of calcium in the presence of KCl. A significant decrease was

detected in the cell surface CALEB at 2 mM and 3 mM calcium in the culture medium (Fig 8 (i)). The decrease at 2 mM calcium in the presence of KCl was comparable to the decrease in the CALEB from cell surface in KCl with routine cell culture medium, which contained 1.8 mM calcium. Higher concentrations than 3 mM were critical for cell survival and hence could not be used.

Calcium influx seems to be essential for the down regulation of CALEB. The importance of calcium was further confirmed by specifically blocking voltage gated calcium ion channels in the presence of KCl. Nimodipine, a L-type calcium channel blocker and ω-conotoxin MVIIC, a blocker of P/Q-type calcium channel blocker, were used to specifically block all the voltage gated calcium channels (Fujisawa et al., 1999;Dolmetsch et al., 2001).

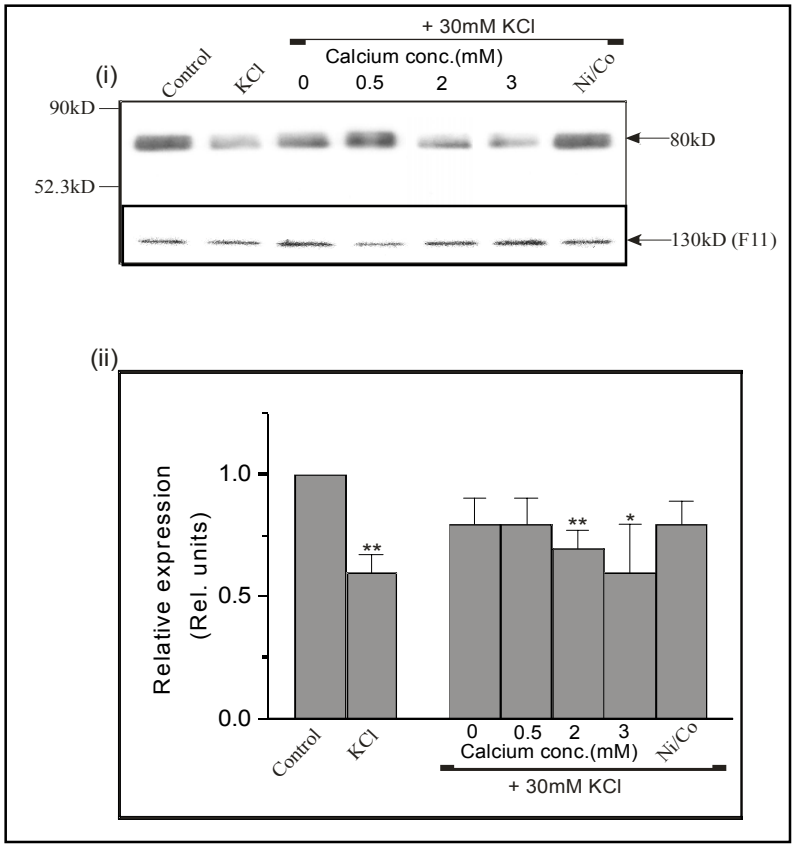


Fig 8:Calcium influx necessary for CALEB down regulation. **(i)** Dissociated chick retina cultures (3DIV) were biotinylated (EZ-link-NHS-LC-Biotin), followed by two washings and further pre-incubation in calcium free medium for 2-3 hr. The cultures were then incubated with increasing concentrations of calcium chloride (0, 0.5 mM, 2 mM, and 3 mM) in the presence of 30 mM KCl in a calcium free medium for 10 min. To confirm further the role of calcium in down regulation of CALEB, biotinylated chick retina cultures were incubated with 30 mM KCl in the presence of Nimodipine (10 μM) and ω-conotoxin MVIIC (1 μM) for 10 min. All the cultures were solubilized, avidin precipitated, eluted from beads with lammeli buffer and finally analyzed by Western blot with mAb 4/1 and mAb F11. The Western blot was developed by chemiluminescence. **(ii)** The 80 kD band was quantified and represented

graphically by normalizing the values with respect to the untreated cultures. Statistical significance was calculated by Student's t-test. (Ni/Co: Nimodipine/ω-conotoxin MVIIC; n=3; Mean±S.E.; $p<0.05$).

The results obtained by blocking the calcium channels with Nimodipine and ω-conotoxin MVIIC revealed that the decrease in the cell surface CALEB which was induced by incubation with KCl was blocked, bringing the value of cell surface CALEB to the levels of untreated cultures (Fig 8 (i)). The 80 kD band obtained was quantified and represented graphically (Fig 8 (ii)).

To summarise the results obtained, we observed a dose dependent decrease in the amount of 80 kD band recognised with mAb 4/1 with increasing concentration of calcium in the culture medium. This decrease in the 80 kD band in the presence of KCl could be prevented by using calcium channel blockers (Nimodipine, ω-conotoxin MVIIC) (Fig 8).

2.2 Down regulation is a calmodulin and calcineurin dependent process

The previous results indicated calcium influx to be an important event in the CALEB down regulation initiation step. To define the complete pathway, from calcium influx to the down regulation of CALEB, role of calcium binding protein calmodulin was analyzed. Calmodulin was my candidate because it has been implicated in the down regulation of HB-EGF and TGF-α down regulation, which are other members of EGF-family (Diaz-Rodriguez et al., 2000;Dong and Wiley, 2000). Calmodulin also acts as second messenger, via which calcium regulates several pathways involving molecules which cannot directly bind to calcium (Levitan, 1999).

Biotinylated chick retina cultures were preincubated with TFP (Trifluoperazine), an antagonist of calmodulin or left untreated (control) followed by depolarization with/without KCl. Avidin precipitated cells lysates were analyzed by Western blot with mAb 4/1 (Fig 9 (i)).

The results disclosed that in the presence of specific calmodulin blocker, TFP, the CALEB cell surface down regulation got prevented, and the amount of CALEB 80 kD fragment detected was alike the level of untreated cultures (Control). TFP, alone had no effect on CALEB expression (Fig 9 (ii)).

Therefore, based on the findings we assumed calmodulin to be most probably the first molecule that gets activated upon calcium influx resulting finally in CALEB down regulation. Calmodulin is known as one of the important calcium sensing proteins through which calcium plays a role in various cellular pathways. Calmodulin acts as a calcium presenting protein by binding to calcium, for molecules which cannot directly bind to calcium.

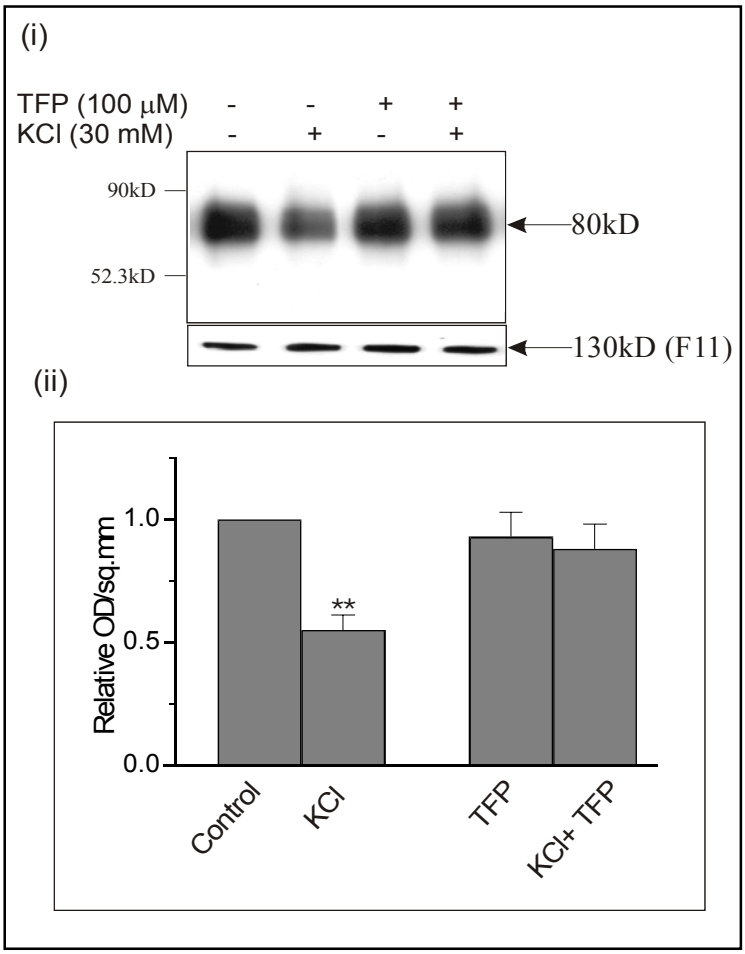


Fig 9:Calmodulin plays a role in the CALEB down regulation. Chick retina cultures (3DIV) were biotinylated, and then preincubated with TFP (100 μm) (Tanokura and Yamada, 1986) for 20 min or left untreated (Control). The untreated cultures were either incubated further with 30 mM KCl or left untreated. The TFP preincubated cultures were then either depolarised with 30 mM KCl or left untreated. **(i)** All the cultures were solubilized, precipitated and then the proteins were separated by Western blot with mAb 4/1 and mAb F11. **(ii)** The bands obtained were quantified. Statistical significance calculated by using Student's T-test and represented graphically in reference to the untreated cultures (Control). (n=3; Mean±S.E.; $p<0.05$)

Calcineurin, is a calmodulin dependent protein phosphatase 2B, known to regulate diverse proteins effecting neuronal function and excitability (Klee et al., 1998). It is enriched in neurons and regulates gene expression in an activity-dependent manner thereby, functioning in refinement (Shi et al., 2000). Calcineurin influences voltage gated calcium

channels as well as neurotransmitter receptors and enzymes (Norris et al., 2002). Calcineurin has been known to be activated via calmodulin in the presence of calcium (Yardin et al., 1998). These mentioned attributes to calcineurin prompted us to investigate the probable role of calcineurin in CALEB down regulation.

In order to define the role of calcineurin, a pharmacological calcineurin blocker, FK-506 was used (Liu et al., 1991;Fruman et al., 1992). Biotinylated chick retina cultures were preincubated with calcineurin blocker, FK-506 followed by depolarization with KCl. The cells were then solubilized and resolved by Western blotting with mAb 4/1 and mAb F11 (Fig 10 (i)).

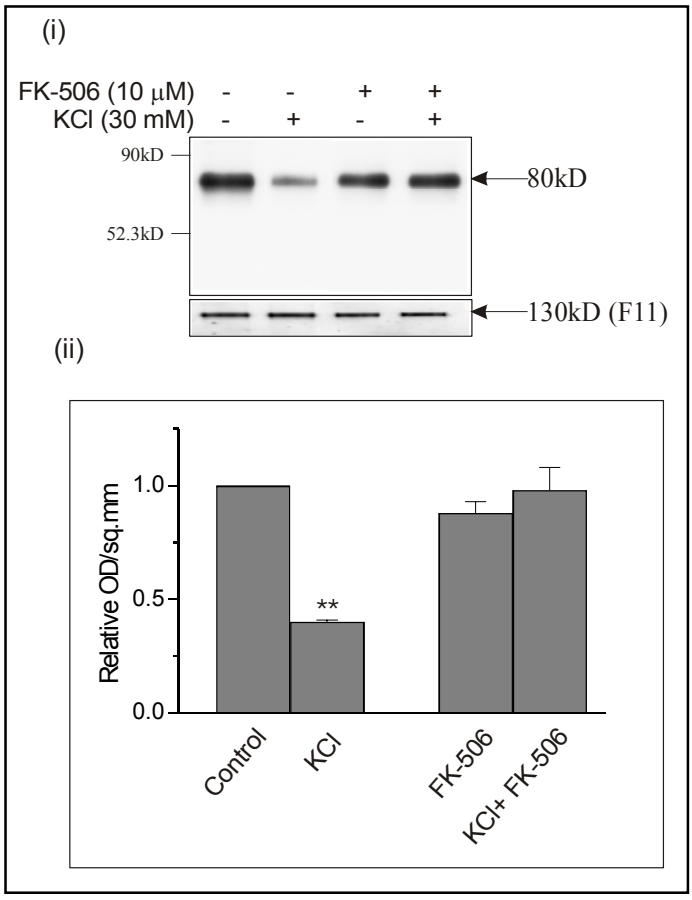


Fig 10:Activation of calcineurin important for CALEB down regulation. Chick retina cultures (3DIV) were biotinylated, and then preincubated with FK-506 (10 μm) (Sharma et al., 2003) for 30 min or left untreated (control). The untreated cultures were either incubated further with 30 mM KCl or left untreated. The FK-506 preincubated cultures were then either incubated with 30 mM KCl or left untreated. **(i)** All the cultures were solubilized, avidin precipitated and the proteins were separated and analyzed by Western blotting with mAb 4/1 and mAb F11 (loading control). **(ii)** The bands obtained were quantified. Statistical significance calculated by using Student's T-test and represented graphically in reference to the untreated cultures. (n=3; Mean±S.E.; $p<0.05$)

Quantification of the 80 kD band revealed that, cultures when preincubated with calcineurin blocker FK-506 followed by KCl incubation, prevented the proteolytic processing. FK-506 alone did not have any effect on the CALEB expression (Fig 10 (ii)). The control cultures were left untreated. The amount of 80 kD band of CALEB detected in the cultures, incubated with FK-506 and KCl was similar to the untreated cultures.

We summarised the above findings by stating that cell surface down regulation of CALEB with depolarization induced by KCl involves not only calcium influx but also the activation of calmodulin binding to calcium and further activating calcineurin as down stream molecules of calcium influx.

3. Shedding is the cause of down regulation of cell surface CALEB

Receptors as well as ligands are known to undergo internalization or proteolytic processing (Colledge et al., 2003;Shah et al., 2004). AMPA receptor internalization is a fast kinetic event, triggered by activation of NMDA receptors and is a calcium dependent process (Lin et al., 2000;Man et al., 2000;Ehlers, 2000). Whereas, E-cadherins undergo ectodomain shedding in a calcium dependent manner (Ito et al., 1999b). Our argument about the rapid down regulation of cell surface exposed CALEB in Western blots as an outcome of mechanisms such as ectodomain shedding and/or regulation of endocytosis/exocytosis rested on the basis of the findings of mentioned AMPA receptors or E-cadherins. To support our argument the mechanism of CALEB down regulation was investigated.

3.1 Down regulation of cell surface CALEB is an outcome of ectodomain shedding

In order to demonstrate the validity of my hypothesis, chick retina cultures were biotinylated with cell impermeant cleavable biotin (NHS-SS-Biotin), followed by depolarization with KCl for 10 min or left untreated. The cells were then solubilized, avidin precipitated and immunoblotted against mAb 4/1 (Fig 11 (ii) (a)) and pAb GluR 2/3 (Fig 11 (i) (a)) (for GluR 2/3 subunits of AMPA receptors).

AMPA receptors undergo internalization and hence, we expected to detect no difference between cultures untreated and treated with 30 mM KCl. The results obtained substantiated our expectation by showing no difference in the total amount of AMPA

receptors between the treated and untreated cells. Whereas, a decrease in the amount of CALEB detected by mAb 4/1 in cultures incubated with KCl in comparison to untreated cultures, gave an indication of the loss of epitope of mAb 4/1 in treated cultures. The decrease in the cell surface CALEB was approximately in the range of 30% with respect to untreated cultures. In the following part of the experiment, biotinylated (NHS-SS-Biotin) chick retina cultures were incubated with KCl for 10 min. Then the covalently bound biotin was cleaved using glutathione containing cleavage buffer following which, the cells were solubilized, avidin precipitated and resolved by SDS-PAGE with mAb 4/1 and pAb GluR2/3.

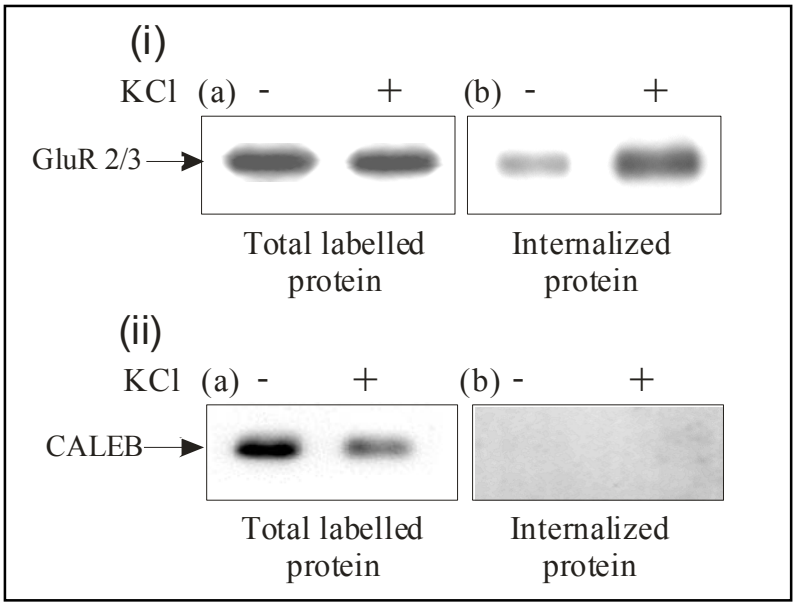


Fig 11:Internalization is not the cause of down regulation of cell surface CALEB. Chick retina cultures (3 DIV) were biotinylated with EZ-link-NHS-SS-Biotin (cleavable biotin). **(i)** ***(a)*** The cells were treated with 30 mM KCl for 10 min or left untreated. The cells were then solubilized in the solubilization buffer. The biotinylated proteins were extracted from the cell lysates by avidin precipitation, the proteins were then resolved in reducing conditions on a 10% SDS-PAGE. The proteins were analyzed by Western blotting with pAb GluR 2/3 (1:500). ***(b)*** The cells were treated with 30 mM KCl for 10 min, followed by cleavage of the biotin from the cell surface proteins with a non-reducing buffer containing glutathione. The cells were then neutralised, solubilized and precipitated with avidin beads to capture the biotinylated proteins which were internalised. The proteins were eluted from beads by boiling in lammeli buffer and were further analyzed by Western blot with pAb GluR 2/3 (1:500) **(ii)** ***(a)*** The samples prepared as mentioned in (i) (a), were again resolved in SDS-PAGE and immunoblotted with mAb 4/1. ***(b)*** The samples prepared as mentioned in (i) (b), were separated again and Western blotted with mAb 4/1 All the blots were developed by chemiluminescence method . The blot for (ii) (b) was exposed a period twice longer as compared to the other blots. (n=2)

In the blot only the internalized biotinylated proteins could be detected. Western blot analysis revealed internalized AMPA receptors upon depolarization of chick retina cells. Whereas, blot results with mAb 4/1 could not detect any internalized CALEB. These results ruled out the possibility of internalization as mechanism leading to cell surface

down regulation of CALEB in chick retina cultures. Lack of internalized CALEB, supported the idea of protein ectodomain shedding as a plausible mechanism responsible for the cell surface CALEB down regulation.

3.2 Presence of the shed ectodomain fragment of CALEB protein

The results of the previous experiments imparted the hypothesis, “ectodomain shedding as a cause for the cell surface down regulation of CALEB”. Hence, in order to validate our hypothesis, experiments were done to isolate the released fragment from the cell culture supernatant and the remaining membrane attached stump.

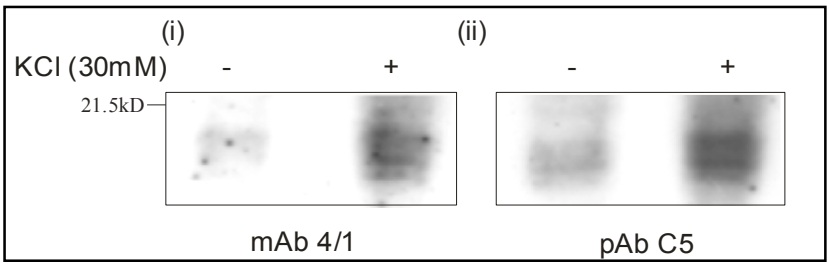


Fig 12:Released ectodomain fragment of CALEB. Dissociated chick retina cultures were made from E-8 embryos by platting 3 x 10^6 cells/ml on poly-l-lysine precoated dishes. Chick retina cultures (3 DIV) were treated with 30 mM KCl or left untreated for 10 min. The supernatants collected and protein precipitated with 10% trichloro acetic acid in the presence of protease inhibitors. The precipitates were washed and pellets dissolved in lammeli buffer and resolved on a 10% SDS-PAGE, followed by Western blotting with **(i)** mAb 4/1, **(ii)** pAb C5. The blots were developed by chemiluminescence. (n=3)

Supernatants of KCl treated and untreated dense chick retina cultures were collected and precipitated with 10% trichloro acetic acid (Phong et al., 2003;Tsakadze et al., 2004) in the presence of protease inhibitors. Precipitates were washed and pellet dissolved and boiled in Laemmli buffer. The samples were separated on a 15% SDS-PAGE gel by Western blotting with mAb 4/1 (Fig 12 (i)) and pAb C5 (Fig 12 (ii)).

Western blot results of both the antibodies detected a small fragment of approximately 18 kD which got enriched in supernatants of KCl treated cultures.

The pAb C5 and mAb 4/1 were generated to the full CALEB but they recognized only the N-terminal part and residues 280-285 of CALEB, respectively. Based on the size of CALEB and the epitope of mAb 4/1, the expected size of the released fragment of CALEB with KCl incubation was of higher molecular weight. The presence of incoherence between the predicted size of the band and the observed size of the band suggested among others

that most likely the extracellular part of CALEB undergoes further degradation after the KCl depolarization which is not detected on protein precipitation. Also, the inappropriate mobility because of the presence of the acidic box (charged entity) can result in the difference observed between the expected and observed size of bands. This 18 kD fragment could not be further characterized.

The enrichment of a fragment recognised by antibodies against CALEB in KCl treated chick retina cultures corroborates the idea of proteolytic processing causative of cell surface down regulation of CALEB.

3.4 Presence of the membrane attached part of CALEB after shedding

The presence of a soluble released fragment of CALEB implied the presence of a membrane attached stump. Experiments were done to detect enrichment in the plasma membrane attached component of CALEB after shedding as, to support the evidences confirming proteolytic processing to cause the decrease in cell surface CALEB.

We expected to observe an increase of the membrane attached part of CALEB upon KCl incubation. Results from chick retina cultures revealed a lower membrane attached component of CALEB without any observable difference in the lower fragment between KCl treated and untreated cultures. One explanation for this discrepancy between the hypothesis and the result could be that the membrane attached component of CALEB undergoes further degradation together with the fact that the extracellular part is cleaved with KCl incubation. In order to prove degradation of the membrane attached CALEB subsequent to the release of the extracellular domain, chick retina cultures (3 DIV) were preincubated with MG132 (Carbobenzoxy-L-leucyl-L-leucyl-L-Leucinal), a potent cell permeable proteolysis inhibitor, for 8 hr (Jung et al., 2003) or left untreated before incubating with KCl. This would prevent the proteasome mechanism to further degrade the membrane remaining stump of CALEB. Cells were homogenized in hypotonic solution and crude membrane fractions isolated. Different membrane fractions were resolved on Western blot with mAb 8-1B-8 as well as mAb 3-2G-10 (data not shown).

The blots revealed several bands among which there was a band at approximately 38 kD, which was enriched in cells incubated with KCl in the presence of MG132 in comparison to cells incubated with KCl without preincubation with MG132 (Fig 13 (i)). The 38 kD band

was recognised by both the antibodies, mAb 3-2G-10 and mAb 8-1B-8. Quantification disclosed an increase of approximately twice of the 38 kD band isolated from the KCl treated cultures in comparison to untreated cultures (Fig 13 (ii)). The presence of a significant increase in the lower component (38 kD) in the cells which were incubated with MG132 along with KCl as compared to cultures which were treated with KCl alone confirmed intracellular proteolytic degradation of the membrane attached stump.

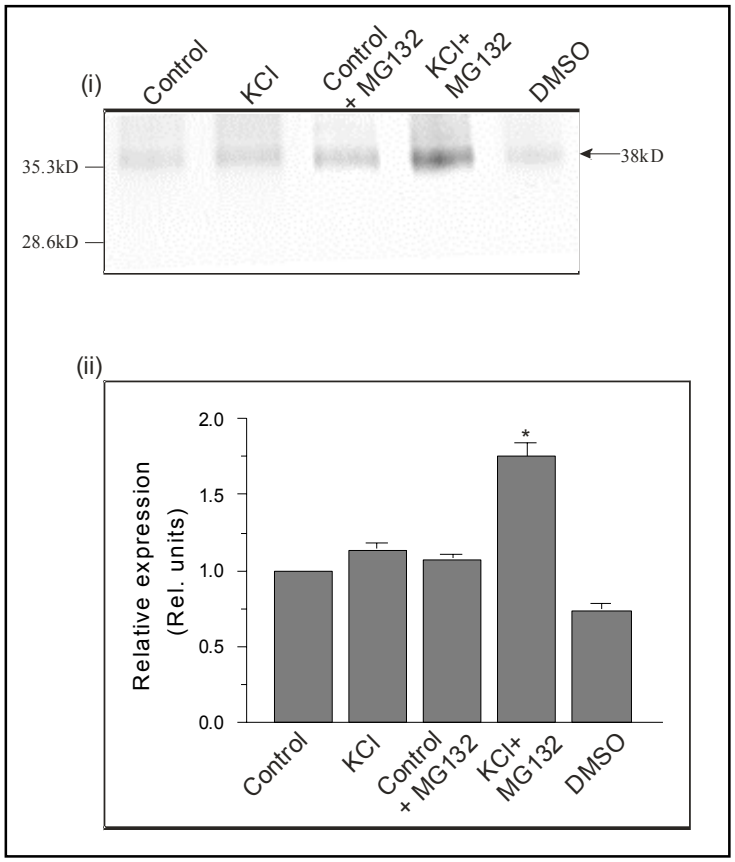


Fig 13:Membrane attached part of CALEB after shedding. **(i)** Dissociated chick retina cultures (3 DIV) were left untreated or were treated with 10 μM of MG132 for 8 hr. The untreated cultures were incubated with 30 mM KCl or left untreated for 10 min. The MG132 preincubated cultures were left untreated or were incubated with 30 mM KCl in the presence of MG132 for further 10 min. The cells were then scraped in hypotonic solution, homogenised and membrane fractions isolated. The membrane proteins were boiled and analyzed by Western blot with mAb 8-1B-8. The blot was developed by chemiluminescence. DMSO treated cultures were used as control, to rule out the effect due to DMSO. **(ii)** The lower band of 36 kD was quantified with Image J software. The values obtained were normalised with respect to the untreated cultures and represented graphically. The statistical significance was calculated by Student's t-test. (n=3; Mean± S.E.; $p<0.05$)

Detection of a releasable component and remaining membrane attached stump in KCl incubated chick retina cultures strengthened hypothesis of cell surface down regulation of CALEB as an outcome of ectodomain shedding.

3.5 Presence of lower fragment in the developmental stages of chick

Neuronal activity patterns the development of the chick brain. The detection of the membrane attached fragment in the culture prompted to look for the membrane attached fragment in the tissue. Hence, attempts were made to determine at what developmental stage the smaller component appears and if this component gets regulated during development.

Chick retina, tectum and telencephalon were collected from E 7-17 stages. These samples were resolved by Western blot with after homogenisation and protein estimation, with the antibody to mAb 4/1 and mAb 8-1B-8.

CALEB expression had been shown to be regulated during the development of the chick retina using Western blot with mAb 4/1 (Schumacher et al., 1997). This antibody recognizes the extracellular region as mentioned in the previous section. Initial results confirmed the presence of a regulation in CALEB expression in the developing chick retina. The results reasserted the earlier findings (Schumacher et al., 1997) i.e., a weak expression of 140 kD was observed at E-7 and the 80 kD form started appearing at E-11. The 80 kD is expressed in a developmentally regulated manner, with a peak expression at around E-13 to E-14 followed by a down regulation at later stages, whereas the expression of the 140 kD increases till birth (Fig 14 (i)).

Lower components of CALEB could be recognized by immunoblotting retina lysates from developmental stages with mAb 3-2G-10. Three major lower components were visible of 44 kD, 38 kD and 28 kD among the 140 kD, 80 kD bands.

The lower bands appeared at E-11 in the retina with a steady increase till E-17 retina. The blot reveals that at least in the retina, the appearance of lower components of CALEB is independent of the 140 kD and rather depends on the 80 kD component of CALEB (Fig 14 (ii)).

Contrastingly, in chick tectum the 140 kD band appears at E-7 and stays till E-17, whereas a 90 kD band appears at E-11 which is detected by the mAb-1-2B-8. A lower component of approximately 60 kD is recognised, which undergoes down regulation during

development. No components lower than 60 kD were identified in chick tectum (Fig 14 (iii)).

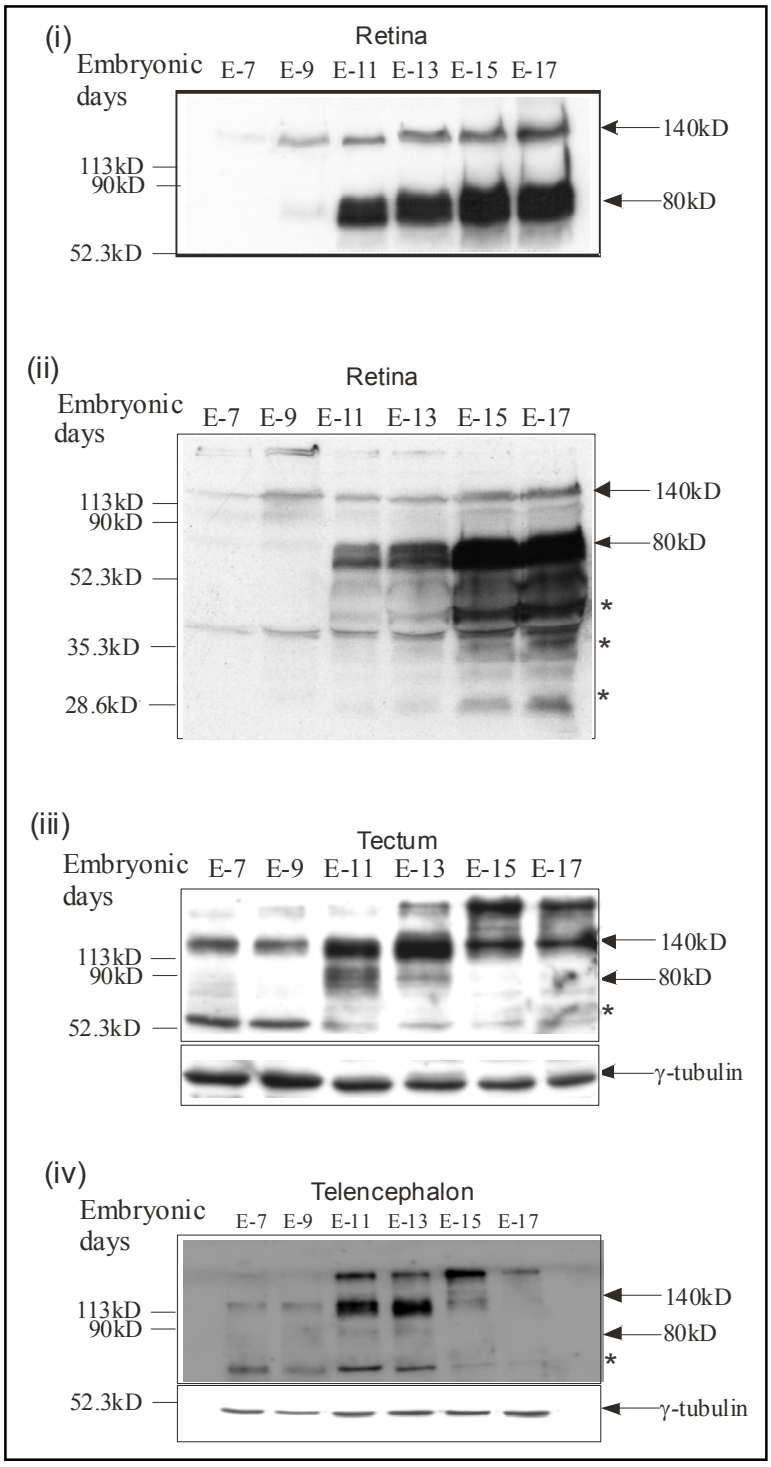


Fig 14:**CALEB expression in the chick retina tissue**. Chick retina, tectum and telencephalon were collected from various embryonic stages (E-7, E-9, E-11, E-13, E-15, and E-17). Tissues were homogenised in solubilization buffer, followed by protein estimation using Lowry estimation protocol. Equal amounts of proteins from chick retina were resolved on a 10% SDS-PAGE and analyzed by Western blot with **(i)** mAb 4/1; The bands of interest (140 kD and 80 kD) are noted on the right hand side of the blot, **(ii)** mAb 3-2G-10; the lower molecular weight bands ranging from 44 kD to 38 kD and 28 kD are recognised, **(iii)** Equal

amounts of chick tectal proteins were resolved by Western blot with mAb 8-1B-8 and mAb γ-tubulin; revealed the lower components of CALEB in the range of 60 kD, **(iv)** Equal amounts of chick telencephalon proteins were separated by Western blot with mAb 8-1B-8 and mAb γ-tubulin; revealed the lower components of CALEB in the range of 60 kD. The lower components are denoted with '*****'. The molecular mass standards are marked on the left side of the gel and the most prominent bands of CALEB as well as γ-tubulin are marked on the right of the gel.

The pattern of CALEB expression in chick telencephalon was unlike the pattern seen in tectum, i.e., the 140 kD appears at E-7 peaking at E-13. A band of 60 kD is seen to be increase steadily reaching peak at E11 and then decreasing till E17 during the development (Fig 14 (iv)).

From my observations, differential expression pattern of CALEB in different tissues could be concluded. There are lower components generated of different sizes, which are in a way tissue specific regulated. The amounts of lower bands increase at later stages of development though the number of lower bands remains constant. The lower bands seem to be regulated with respect to the higher band of 80 kD. The band of interest i.e., of 38 kD seems to appear along with the appearance of the 80 kD fragment, but is independent of the presence of the 140 kD in chick retina.

4. Metalloprotease inhibition, inhibits shedding

Proteolytic processing is a post-translational modification known to impede functionality to proteins (Loeb and Fischbach, 1995). Most of the EGF family members are known to undergo this kind of post-translational modification, to act as receptor or ligands for either juxtacrine or paracrine signalling during growth and differentiation (Sunnarborg et al., 2002). Among several members of metalloprotease family, the proteins containing a disintegrin and metalloprotease domain (ADAMs) are the most prominent participants for the proteolytic processing of proteins (Moss and Lambert, 2002;White, 2003). The ADAMs are formed in a pro-protein form, truncated to the active form and can be activated by calcium influx (Pandiella and Massague, 1991;Srour et al., 2003). In the CNS various amounts of ADAMs have been detected, a major contribution being those of ADAM10 and ADAM17. (Arribas et al., 1996;Robache-Gallea et al., 1997;Codony-Servat et al., 1999;Le Gall et al., 2003;Peiretti et al., 2003;Myhre et al., 2004)

The results described above conveyed proteolytic processing to be the probable process leading to the CALEB down regulation. Based on the previous findings and the

background information, we asked the following questions: (1) Is the CALEB ectodomain shedding a membrane protease dependent process; (2) Are ADAM groups of enzymes responsible for the CALEB proteolytic processing; (3) Which is/are the most probable ADAM enzyme (s) responsible for the cleavage of extracellular domain of CALEB.

4.1 Shedding of the ectodomain occurs by membrane associated proteases

Initially to determine the role of membrane metalloproteases in the ectodomain shedding of CALEB, we prepared membrane fractions of chick retina cultures and incubated them at 37^0C.

We anticipated that the membrane bound enzyme (s) and CALEB are co-isolated during the membrane preparation. If CALEB ectodomain was cleaved by membrane bound enzymes, then incubation of the membrane fractions would enhance ectodomain shedding of CALEB resulting in appearance of more cleavage products, in comparison to membrane fractions without incubation or membrane fractions incubated in the presence of metalloprotease inhibitor.

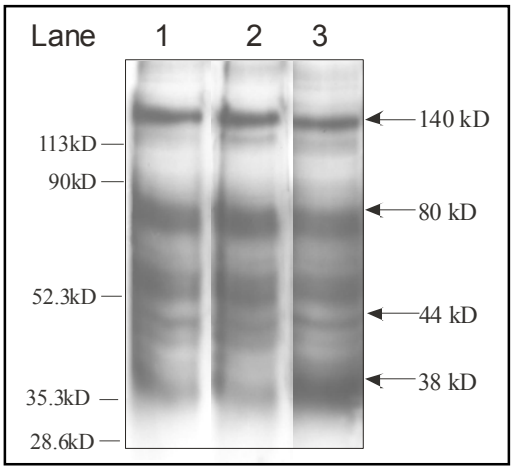


Fig 15: Membrane attached protease responsible for releasing CALEB. Chick retina cells were cultivated (3DIV) and membrane fractions isolated (Materials and methods). The membrane fractions were either left unincubated or incubated for 2 hr at 37^0C ± TAPI-1. The fractions were then lysed and resolved under reducing conditions followed by analysis with Western blot with mAb 8-1B-8. ***Lane 1***, membrane fractions which were not incubated; ***lane 2***, membrane fractions incubated for 2 hr in the presence of TAPI-1 (100 μM); ***lane 3***, membrane fractions incubated for 2 hr. (n=2)

The results of immunoblots with mAb 8-1B-8 revealed that when the membrane fractions were directly loaded on the gel without any prior incubation, there was less amount of 38 kD component. Whereas in samples, when loaded after 2 hr incubation at 37^0C, higher

amounts of 38 kD band appeared. The membrane fractions when incubated for 2 hr in the presence of TAPI-1 (broad spectrum metalloprotease inhibitor), reduced the level of 38 kD component of CALEB to the levels detected in the unincubated membrane fractions (Fig 15).

The mentioned findings confirmed that the protease responsible for CALEB proteolytic shedding was captured along with CALEB during the membrane preparation finally resulted in the appearance of lower CALEB components.

The molecular size of the lower component matched to the molecular weight of the lower component that was increased in the cultures depolarized with KCl i.e., 38 kD, as expected. The inhibitory effect of TAPI-1 suggested the involvement of membrane bound metalloprotease in release of CALEB ectodomain.

4.2 ADAM group of metalloproteases is responsible for shedding

I demonstrated in the previous experiment that the protease cleaving ectodomain of CALEB is most likely a membrane bound metalloprotease.

To reaffirm the results various broad range pharmaceutical inhibitors were used to test for their effectiveness in preventing the ectodomain release of CALEB which could be initiated by KCl incubation.

Hence, biotinylated chick retina cultures were incubated with broad spectrum metalloprotease inhibitors, TAPI-1; TAPI-2; Ilomastat; 1,10 Phenanthroline; RU36156 in combination with or without KCl (Arribas et al., 1996;Gallea-Robache et al., 1997;Kang et al., 2002;Borrell-Pages et al., 2003;Lin et al., 2003;Myhre et al., 2004). Biotin labelled proteins were captured with streptavidin beads and results analyzed by Western blot with mAb 4/1 on a 10% reducing SDS-PAGE.

The results revealed in the presence of the broad spectrum metalloprotease inhibitors, that KCl induced ectodomain shedding of membrane CALEB was blocked. Among all the broad spectrum metalloprotease inhibitors, inhibitory effect of TAPI-1 was significantly higher than the untreated cultures (control) (Fig 16 (ii)). TAPI-1 when incubated without KCl had a strong inhibitory effect; the intensity of the band obtained was significantly

higher than the untreated control (Fig 16 (iii)). The rest of the broad spectrum inhibitors except TAPI-1 did not have any effect when used alone in comparison to the untreated cultures.

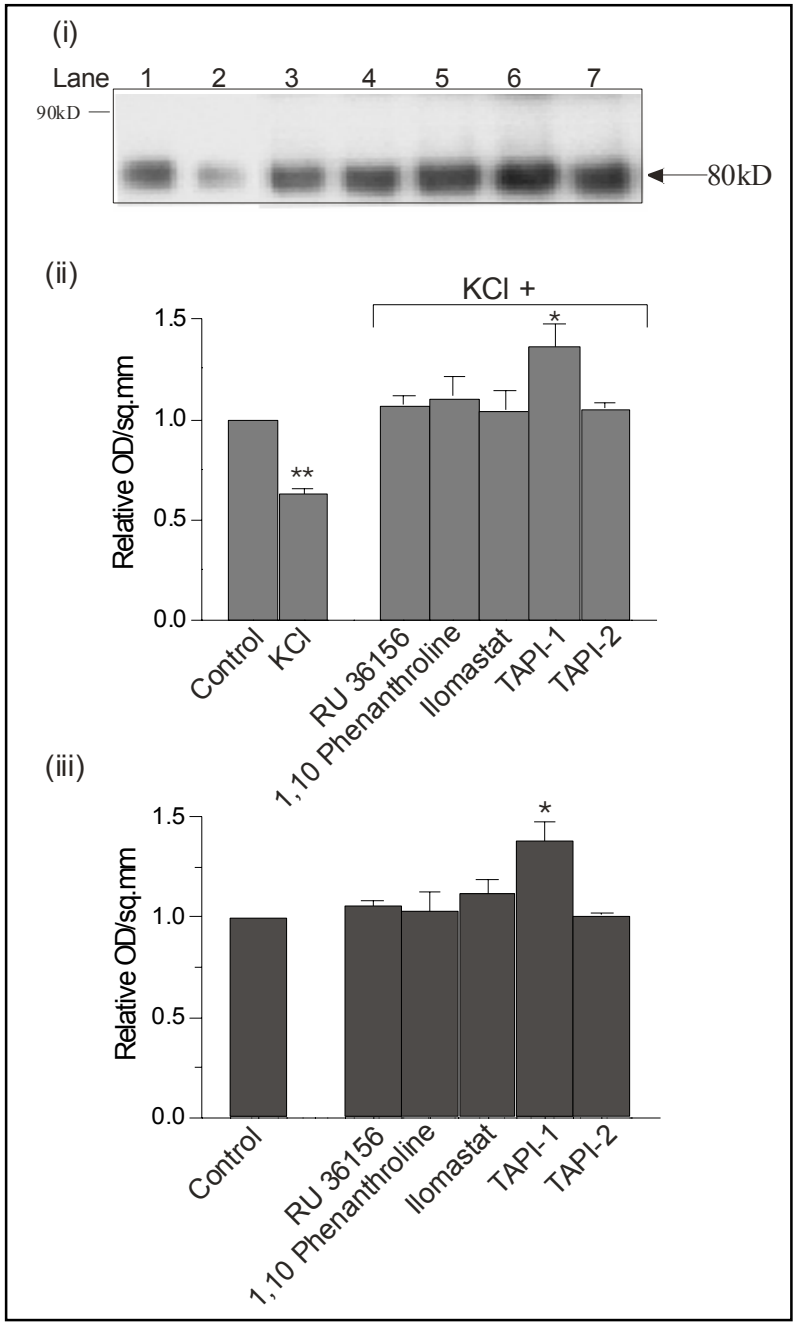


Fig 16:Metalloprotease inhibitors block KCl induced shedding. Biotinylated chick retina cultures were left untreated or incubated with KCl (30 mM) alone or in combination with metalloprotease inhibitors for 10 min. Solubilized cells were analysed by Western blot with mAb 4/1. **(i)** Representative Western blot ***lane 1***, Untreated; ***lane 2***, KCl (30 mM); ***lane 3***, KCl (30 mM)+ RU 36156 (10 μM); ***lane 4***, KCl (30 mM)+ 1,10 Phenanthroline (10 mM); ***lane 5***, KCl (30 mM)+ Ilomastat (5 μM); ***lane 6***, KCl (30 mM)+ TAPI-1 (100 μM); ***lane 7***, KCl (30 mM)+ TAPI-2 (100 μM). **(ii)** The 80 kD band was quantified and graphically represented with reference to the untreated cultures, after calculating the significance by Student's t-test. **(iii)** Graphical representation of quantified 80 kD band obtained by Western blot with

mAb 4/1 of cultures incubated with metalloprotease inhibitors without KCl (n=4; S.E ± Mean)

The summarised results substantiated the earlier role of membrane bound metalloprotease, by inhibition of ectodomain shedding in the presence of several broad spectrum hydroxamate metalloprotease inhibitors.

4.3 ADAM 10 and ADAM 17 are responsible for causing the ectodomain cleavage of CALEB

In order to narrow the search for the responsible protease, ADAM proteases which are predominantly present in the CNS and cleave other members of the EGF-family were considered as potential candidates. ADAM 10 and ADAM 17 (TACE) were prominent among other ADAM proteases, since they have been implicated in proteolytic processing of EGF family members (Le Gall et al., 2003;Borrell-Pages et al., 2003). The idea was to use specific inhibitors of ADAM 10 and ADAM 17 in the chick retina cultures in order to asses their outcome.

Biotinylated chick retina cultures were preincubated with specific hydroxamate inhibitors GW 280264X (TACE and ADAM 10 inhibitor) and GI 254023X (preferentially ADAM 10 inhibitor) (Hundhausen et al., 2003). This was followed by KCl induced depolarization for 10 min in the presence of GW 280264X and GI 254023X inhibitors. The cell surface proteins were avidin precipitated, eluted, resolved on a 10% SDS-PAGE and analyzed by Western blot with mAb 4/1.

The results showed, the ADAM 10 specific inhibitor when incubated along with KCl, prevented the proteolytic processing. Incubation with GW 280264X alone which inhibits ADAM 17 and ADAM 10 simultaneously did not have any effect on the cell surface CALEB as had been observed in the case of GI 254023X (ADAM 10 inhibitor). GW 280264X when used in combination with KCl prevented the cell surface CALEB proteolytic processing to levels more than the untreated cultures (Fig 17 (i)). The 80 kD band obtained was quantified and the increase in the cell surface CALEB in treated cultures to untreated cultures was significantly higher (Fig 17 (ii)).

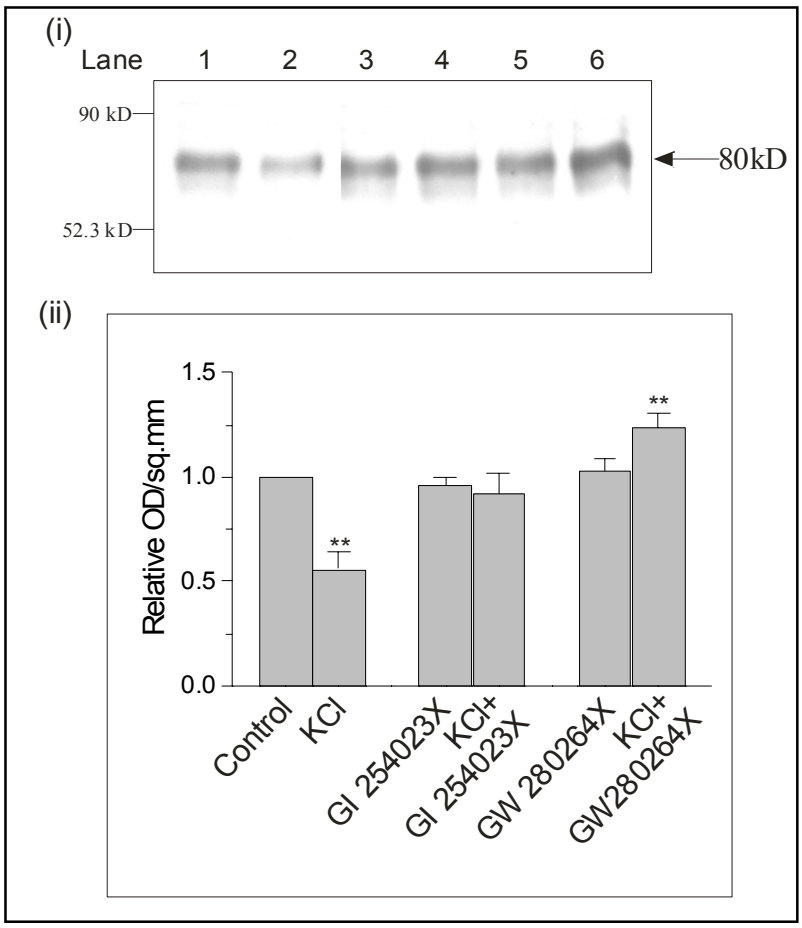


Fig 17:Pharmacological blockers of ADAM-10 and ADAM-17 inhibit CALEB ectodomain shedding. Biotinylated chick retina cultures were left untreated or preincubated with GI 254023X or GW 280264X for 30 min. The untreated as well as the preincubated cultures were either left untreated or incubated with KCl for 10 min, followed by solubilization with solubilization buffer, avidin precipitation, analyzed by Western blot with mAb 4/1. **(i)** A representative blot, ***lane 1***, Untreated culture; ***lane 2***, KCl (30 mM) incubated for 10 min; ***lane 3***, GI 254023X (10 μM) incubated for 30 min; ***lane 4***, preincubated with GI 254023X (10 μM) for 30 min, followed by KCl (30 mM) incubation for 10 min; ***lane 5***, GW 280264X (10 μM) incubated for 30 min; ***lane 6***, preincubated with GW 280264X (10 μM) for 30 min, followed by KCl (30 mM) incubation for 10 min. **(ii)** The 80 kD band was quantified and graphically represented with reference to the untreated cultures, after calculating the significance by Student's t-test. (n=4; S.E ± Mean; **$p \leq 0.01$)

The prevention in the decrease of 80 kD band by inhibitors of ADAM 10 and ADAM 17 suggested that both enzymes might be implicated in the proteolytic processing of CALEB. To confirm the results obtained by using specific inhibitors of ADAM 10 in chick retina cultures, the active as well as the inactive form of the ADAM 10 enzyme cDNA with chick CALEB cDNA (clone 12; 275-595 aa) were transfected in COS-7 cells (Fig 18).

In the co-transfection experiments fixed amount of chick CALEB cDNA (0.5 μg) was transfected with increasing amounts of wild type ADAM 10 (Flag-tagged) cDNA (0.7 μg,

1.4 μg, 2.8 μg and 4 μg) or dominant negative ADAM 10 (HA-tagged) cDNA (4 μg). In the control cells, the vector alone was transfected.

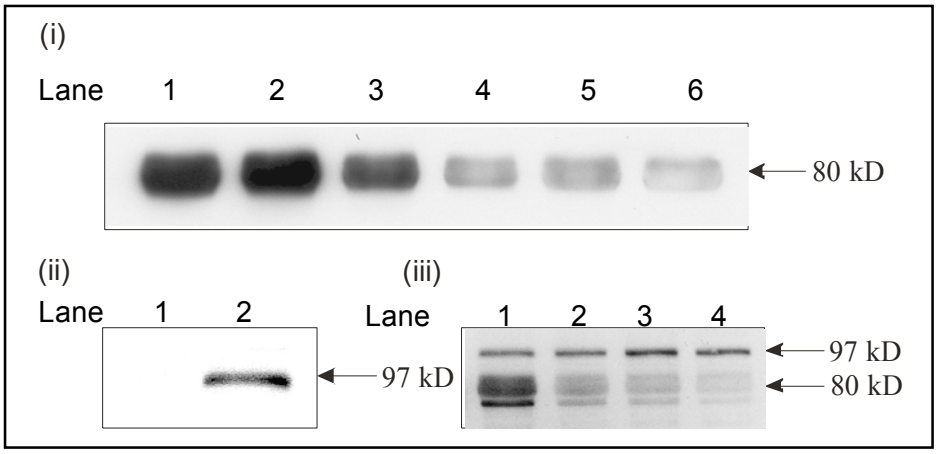


Fig 18:Chick CALEB in the presence of ADAM 10 undergoes down regulation in COS-7 cells. COS-7 cells were co-transfected with chick CALEB (pcDNA6-preprotrypsin/Flag/ch.CALEB/Myc/His) and bovine ADAM 10 cDNA (active or dominant negative form). Transfection was done by Lipofectamine with chick CALEB cDNA (0.5 μg) in combination with bovine wild type (WT) ADAM 10-Flag tagged (0.5 μg, 1 μg, 2 μg and 3 μg) or bovine dominant negative (DN-ADAM) 10-HA tagged (3μg). The cells were solubilized in the solubilization buffer and blotted with different antibodies. **(i)** Equal amount of samples were resolved in reducing condition and immunoblotted with mAb 4/1. ***Lane 1***, ch.CALEB (0.5 μg); ***lane 2***, ch. CALEB (0.5 μg) + bovine DN-ADAM-10-HA tagged (3μg); ***lane 3***, ch. CALEB (0.5 μg) + bovine WT-ADAM 10-Flag tagged (0.5 μg); ***lane 4***, , ch. CALEB (0.5 μg) + bovine WT-ADAM 10- Flag tagged (1 μg); ***lane 5***, ch. CALEB (0.5 μg) + bovine WT-ADAM 10- Flag tagged (2 μg); ***lane 6***, , ch. CALEB (0.5 μg) + bovine WT-ADAM 10- Flag tagged (3 μg). **(ii)** Equal amount of proteins were blotted with mAb HA. ***Lane 1***, ch. CALEB (0.5 μg); ***lane 2***, ch. CALEB (0.5 μg) + bovine DN-ADAM 10-HA tagged (3 μg). **(iii)** Equal amount proteins were separated by Western blotting with mAb Flag. ***Lane 1***, ch. CALEB (0.5 μg) + bovine WT-ADAM 10- Flag tagged (0.5 μg); ***lane 2***, ch. CALEB (0.5 μg) + bovine WT-ADAM 10- Flag tagged (1 μg); ***lane 3***, ch. CALEB (0.5 μg) + bovine WT-ADAM 10- Flag tagged (2 μg); ***lane 4***, ch. CALEB (0.5 μg) + bovine WT-ADAM 10- Flag tagged (3 μg).

A dose dependent decrease of chick CALEB with increasing amounts of wild type ADAM 10 (Fig 18 (iii)) was observed. When cells were cotransfected with dominant negative ADAM 10 and chick CALEB, the results revealed no change in the amount of CALEB with respect to the single transfection of chick CALEB alone.

5. Shedding of CALEB in the presence of pharmacological reagents that affect intracellular signalling pathways

Ectodomain shedding of EGF-family members requires the activation of various downstream molecules such as calmodulin, MAPK kinase pathway or in some cases activation of protein kinase C pathway (Fan and Derynck, 1999;Diaz-Rodriguez et al.,

2000;Thabard et al., 2001;Montero et al., 2002). CALEB undergoes proteolytic cleavage upon depolarization, i.e., with the involvement of calcium channels. Calcium influx plays an important role in CALEB processing, and has been cited to be vital for the processing of other proteins (Ito et al., 1999b). Calcium, after influx binds to calmodulin (Levitan, 1999). Calcium influx is also known to result in regulation of protein synthesis (West et al., 2001) and activate MAPK kinase pathway via the Raf kinase which is elicited for various complex cellular processes (Dolmetsch et al., 2001). Here, based on the mentioned knowledge, we asked: (1) Does MAP kinase pathway play a role in cleavage of CALEB ectodomain; (2) Is protein synthesis necessary for shedding of CALEB ectodomain.

5.1 Involvement of Erk kinase pathway in shedding of CALEB ectodomain

Calcium is known to have several functions in the cell. The previous results indicated that calcium after moving into the cell through the voltage gated calcium channels (VGCC) binds to the calcium binding proteins, like calmodulin.

Activated calmodulin in the presence of calcium activates calcineurin. Involvement of MAP kinase pathway was investigated in the cascade of signalling molecules leading finally to ectodomain shedding of CALEB, since it has been implicated for the proteolytic processing of other EGF-family members.

Pharmacological blockers were used to determine the involvement of MAP kinase pathway. The candidate molecule to be investigated was Erk kinase, as it is known to be involved in the shedding of HB-EGF and TGF-α (Gechtman et al., 1999;Fan and Derynck, 1999;Diaz-Rodriguez et al., 2002).

Biotinylated chick retina cultures were depolarized with KCl in the presence of U 0126, an Erk kinase blocker (Diaz-Rodriguez et al., 2002;Weskamp et al., 2004). Detergent extracts were immunoblotted with mAb 4/1. The results revealed that U 0126 itself did not have any effect on the 80 kD band of CALEB recognised by mAb 4/1 (Fig 19 (i)). The intensity of the 80 kD band was decreased by around 30% in cultures treated with KCl with respect to the untreated cultures as had been observed previously. The decrease of cell surface CALEB by 30% was completely inhibited when the cultures were treated with KCl in the presence

of U 0126. The values of the bands after quantification for cultures treated with KCl in the presence of U 0126 were comparable to untreated cultures (Fig 19 (ii)).

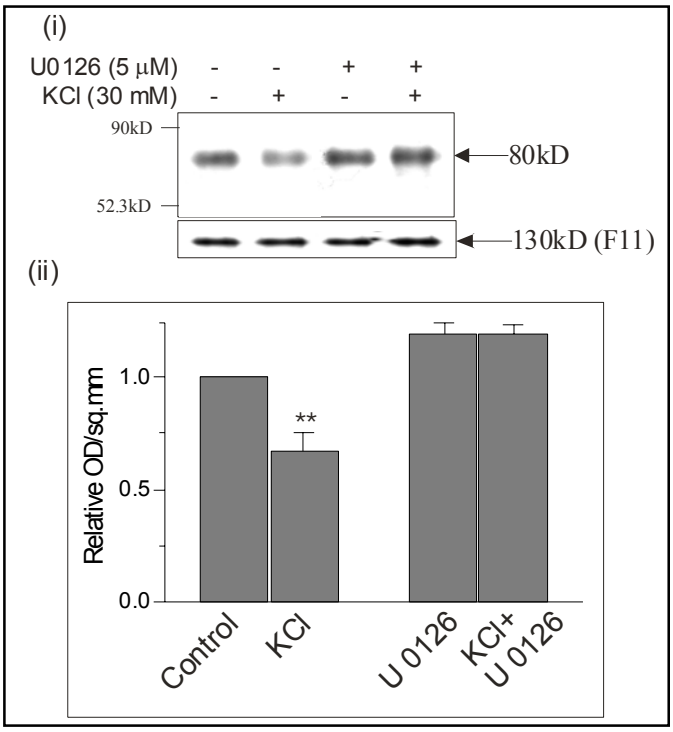


Fig 19: Erk kinase involved in CALEB down regulation. Chick retina cultures (3 DIV) were biotinylated, and then preincubated with U 0126 (5 μM) for 20 min or left untreated. The untreated as well as U0126 preincubated cultures were either incubated further with 30 mM KCl or left untreated. **(i)** All the cultures were solubilized, avidin precipitated and then the proteins were separated by SDS-PAGE and Western blotted with mAb 4/1 and mAb F11. **(ii)** The bands obtained were quantified. Statistical significance calculated by using Student's T-test and represented graphically in reference to the untreated cultures. (n=3; Mean± S.E.; $p<0.05$)

The results can be summarized, stating that activation of the Erk kinase pathway is necessary for the proteolytic processing of CALEB.

5.2 Shedding is independent of protein synthesis

Calcium influx can lead to a regulation of protein synthesis (Tao et al., 2002). CALEB proteolytic processing is activated with the influx of calcium as an initiation step. Although the time in which CALEB undergoes ectodomain shedding is too short for new proteins to be synthesized, I investigated this aspect by looking at the transcription level of protein synthesis.

In order to analyze the effect of protein synthesis the pharmacological blocker of protein synthesis cycloheximide (Fan and Derynck, 1999) were used in biotinylated chick retina

cultures. Cultures were either preincubated with cycloheximide or left untreated in the presence of absence of KCl. Detergent extracts of the cultures were avidin precipitated and analyzed by Western blot with mAb 4/1. Quantification of the Western blot bands showed no difference between the cultures which were preincubated with cycloheximide and cultures which were left untreated. The results between chick cultures depolarised after preincubation with cycloheximide and those cultures left without preincubation were comparable and there was no significant difference (Fig 20).

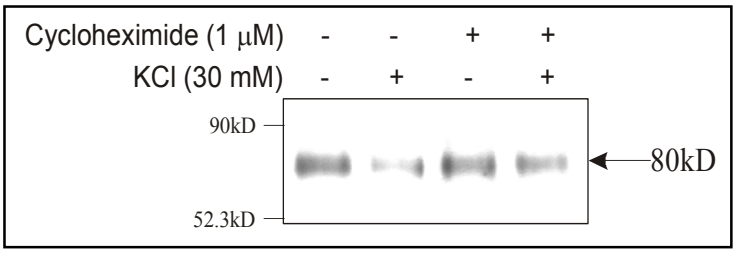


Fig 20:Protein synthesis is not necessary for CALEB shedding. Chick retina cultures (3DIV) were biotinylated, and then preincubated with cycloheximide (1 μM) for 30 min or left untreated. The untreated cultures and the cycloheximide preincubated were either incubated further with 30mM KCl for 10min or left untreated. All the cultures were solubilized, precipitated and then analyzed by Western blot with mAb 4/1.

The results obtained suggested ectodomain processing of CALEB, a protein synthesis independent phenomenon.

6. Calcium dependent upregulation of total CALEB

The previous results show that CALEB undergoes ectodomain processing upon KCl induced depolarization. This ectodomain processing is initiated with the influx of calcium followed by a cascade of molecular reactions, finally resulting in the release of CALEB ectodomain. Calcium is known to affect many cellular processes either directly or by acting as a secondary messenger (Ghosh and Greenberg, 1995). It is also known that calcium affects proteins at their transcription as well as translational levels (Sheng et al., 1990;West et al., 2001;Li et al., 2004) resulting in a regulation at protein expression levels. Cells have many feedback mechanisms that help a cell to maintain a homeostasis. In a feedback inhibition the end product of a pathway controls the rate of its own synthesis, resulting in more synthesis if there is less end product whereas, less synthesis in conditions with accumulation of more end products. This kind of mechanism works mostly in enzymes (Hascilowicz et al., 2002). I asked the following questions; (1) Is there an accumulation of CALEB in the presence of KCl, if ectodomain processing from the cell

surface is prevented; (2) If there occurs an increase in the total CALEB, is it an outcome of transcription or translation.

6.1 Total CALEB protein upregulation upon neuronal activity

We assumed that inhibition of CALEB ectodomain shedding for longer duration in the presence of KCl would prevent accumulation of CALEB, based on a feed back mechanism if the end product is the unprocessed CALEB. In case the end product of CALEB synthesis is the cleaved form of CALEB, then inhibition of ectodomain shedding would lead to accumulation of more uncleaved CALEB.

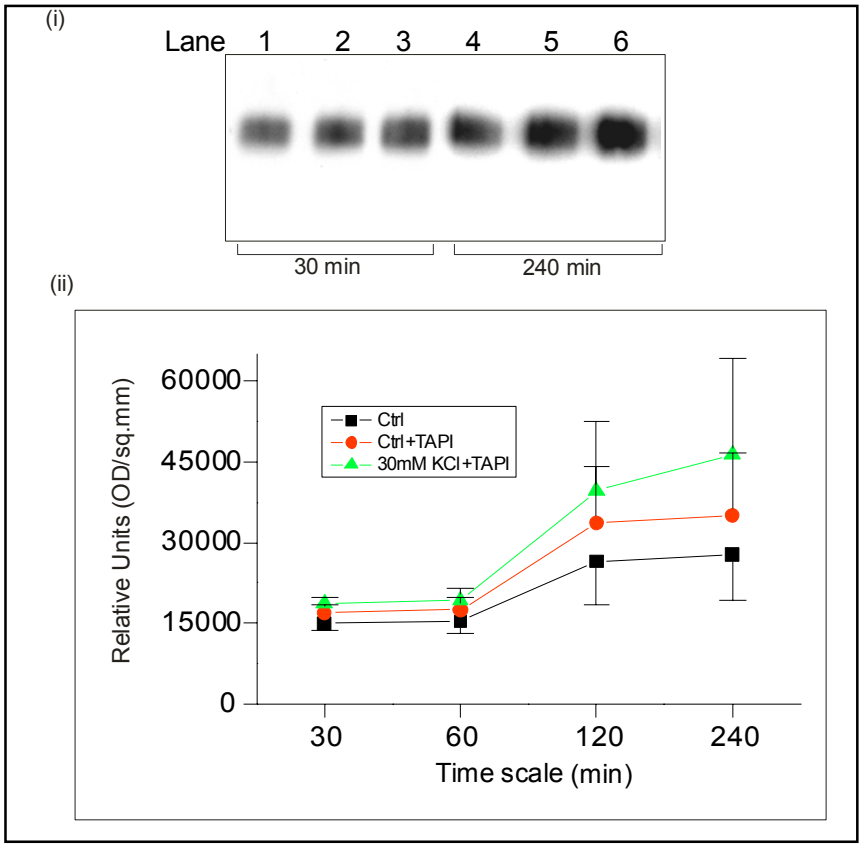


Fig 21:Upregulation of total CALEB with KCl incubation. Chick retina cultures (3 DIV) were incubated with TAPI-2 (100 μM), or KCl (30 mM) in combination with TAPI-2 (100 μM) or were left untreated for 30 min, 60 min, 120 min and 240 min. The cells were solubilized, and proteins were analyzed by Western blot with mAb 4/1. **(i)** Representative Western blot for combination of reagents in different time periods. Chick retina cultures were incubated with ***Lane 1***, Untreated (30 min); ***lane 2***, TAPI-2 (30 min); ***lane 3***, TAPI-2 + KCl (30 min); ***lane 4***, Untreated (240 min); ***lane 5***, TAPI-2 (240 min); ***lane 6***, TAPI-2 + KCl (240 min). **(ii)** The 80 kD bands were quantified, and represented graphically with the real values (OD/sq.mm). (S.E ± Mean; n=3).

In order to prove one of our assumption chick retina cultures were incubated with KCl in the presence of TAPI-2 for various durations. The cultures were incubated 30-240 min,

followed by solubilization and Western blotting with mAb 4/1. The 80 kD bands obtained in the Western blots were quantified and graphically plotted with respect to the untreated cultures. Analysis of the results from cultures treated with TAPI-2 alone showed an increase of CALEB expression in comparison to the untreated cultures. The increase observed in cultures which were simultaneously incubated with KCl and TAPI-2 was significantly higher than the cultures which were incubated with TAPI-2 alone as well as the untreated cultures.

The increase was observed in a time dependent manner, with the maximum expression after 4 hr incubation. The results suggested that most likely the end product of CALEB synthesis is the ectodomain processing of CALEB, releasing the extracellular soluble part and a remaining membrane attached stump. In the untreated cultures we observed an increase in the amount of total CALEB after 4 hr in comparison to 30 min which could be due to protein turn over.

6.2 Total CALEB increased because of more translation

The total CALEB protein upregulation could be due to calcium regulation at the level of transcription or translation. In order to determine the mentioned cause, chick retina cultures (3 DIV), were preincubated with transcription blocker (Actinomycin D) (data not shown) or translation blocker (cycloheximide).

This was followed by treatment with KCl and TAPI-II. The incubation was done for various time durations (30 min, 1 hr, 2 hr, and 4 hr). The cells were solubilized, loaded on a 10% SDS-PAGE and results analyzed by Western blot with mAb 4/1.

The presence of Actinomycin D, did not have any effect on the CALEB expression suggesting that the up regulation observed by incubating cells with KCl along with TAPI-2 was not an outcome of transcription (data not shown). In the presence of cycloheximide along with KCl and TAPI-2, the upregulation that was observed following the incubation with KCl and TAPI-2 could be prevented. The 80 kD bands were quantified with Image J software and plotted graphically.

These results suggest that CALEB upregulation observed after 4 hr incubation was a consequence of translation and not transcription.

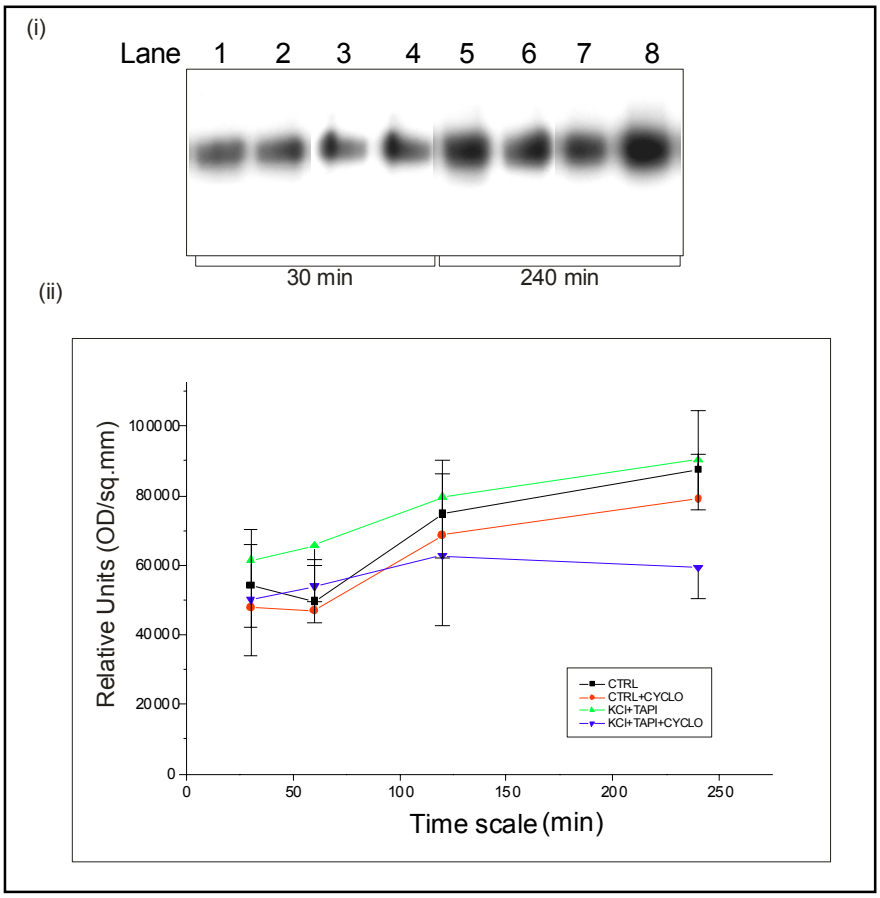


Fig 22:Upregulation of total CALEB with KCl incubation. Chick retina cultures (3 DIV) were preincubated with cycloheximide (1 μM) for 30 min. This was followed by incubation with TAPI-2 (100 μM), or KCl (30 mM) in combination with TAPI-2 (100 μM) or was left untreated for 30 min, 60 min, 120 min and 240 min in the presence of cycloheximide. The cells were solubilized, and proteins analyzed by Western blot in reducing conditions and blotted with mAb 4/1. **(i)** Representative Western blot of combination of reagents for time periods. Chick retina cultures were incubated with ***Lane 1***, Untreated (30 min); ***lane 2***, Cycloheximide (30 min); ***lane 3***, Cycloheximide + TAPI-2 + KCl (30 min); ***lane 4***, TAPI-2 + KCl (30 min); ***lane 5***, Untreated (240 min); ***lane 6***, Cycloheximide (240 min); ***lane 7***, Cycloheximide + TAPI-2+ KCl (240 min); ***lane 8***, TAPI-2 + KCl (240 min). **(ii)** The 80 kD bands were quantified, and represented graphically with the real values (OD/sq.mm). (S.E ± Mean; n=3).

II. CHARACTERIZATION OF CALEB IN MOUSE NEURAL TISSUE

1. Expression of mouse CALEB

1.1. Tissue specific expression of mouse CALEB

Expression of CALEB in the wild type mouse tissues was inquired, with the background knowledge from the expression of CALEB in chick where it is described as a brain specific protein, expressed in a developmentally regulated manner in the retina.

Wild type (C57Bl/6J) adult mouse tissues (brain, heart, lungs, liver, kidney, spleen) and the whole brain of CALEB knock-out mouse were collected in ice cold PBS, homogenised, protein content estimated by Lowry's protein estimation protocol. Western blot of tissue lysates with pAb 462 and mAb γ-tubulin revealed, CALEB as a brain specific protein. The appearance of the smear in the brain suggests that CALEB is present in a highly glycosylated form.

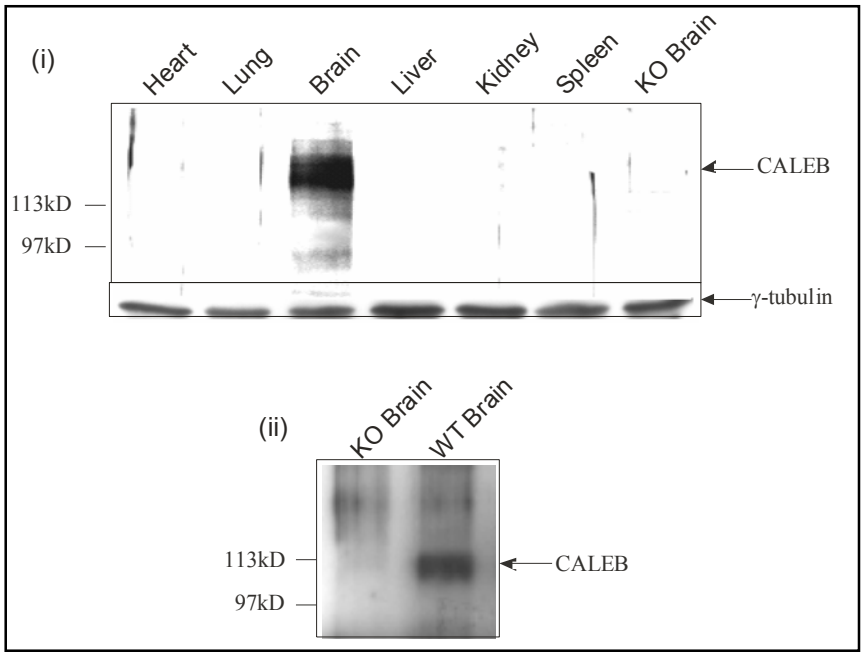


Fig 23:Tissue specific expression of CALEB. **(i)** Tissues were collected from adult, C57Bl/6J strain mice. The tissues were collected in ice cold PBS and homogenized in solubilization buffer. Protein content was estimated with Lowry's protocol and equal amounts of protein were boiled with lammeli buffer. The protein samples were resolved in reducing condition on a 10% SDS-PAGE. The gel was Western blotted with primary antibodies, pAb 462 and mAb γ-tubulin. Equal amount of protein loading was confirmed with mAb γ-tubulin. **(ii)** Brain homogenates of wild type (C57Bl/6J) mice and CALEB knock-out mice were deglycosylated with chondroitinase ABC, and analyzed by Western blot with pAb 462.

In order to characterize the glycosylations on CALEB in brain, homogenates of brain were deglycosylated with chondroitinase ABC, followed by Western blotting with pAb 462. The results revealed a shift in CALEB band to 110 kD approximately in deglycosylated wild type mouse brain from around 200 kD in the undeglycosylated brain. The CALEB band also appeared much sharper and distinct after deglycosylation as compared to undeglycosylated brain. The CALEB knock-out brain tissue did not reveal any band and was the negative control.

1.2 Mouse CALEB enrichment in the synaptic junctions

In order to study the subcellular localization of post-synaptic density (PSD) fractions were prepared from whole brains of C57Bl/6J adult mice. Fractions were collected at different stages comprising of synaptosomes, synaptic vesicles, synaptic junctions and post-synaptic densities.

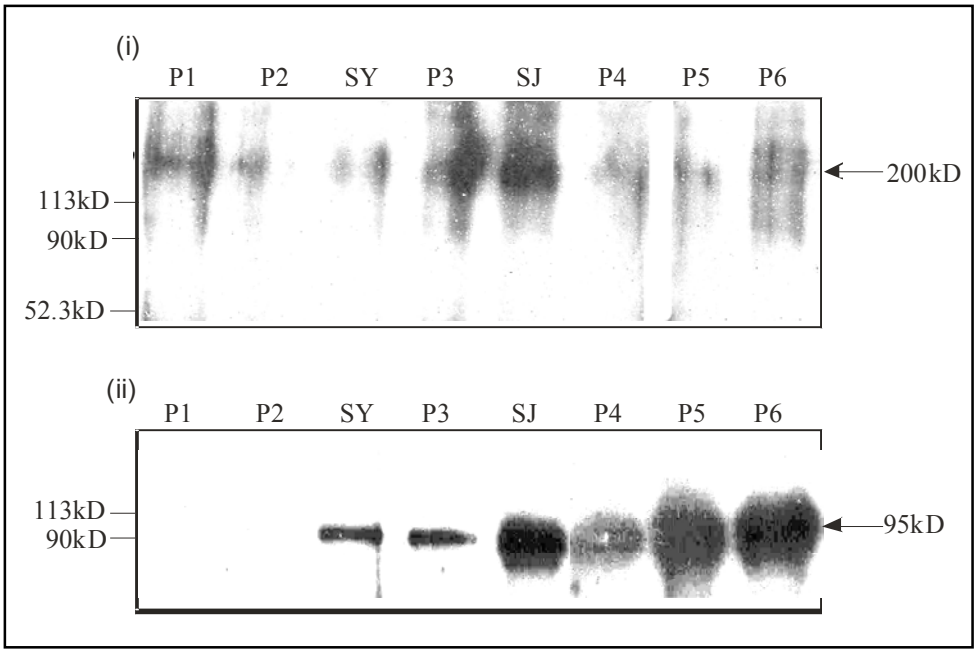


Fig 24:CALEB enrichment in the synaptic junctions. Post synaptic density fractions were prepared from brain of adult (C57Bl/6J) wild type mice. All fractions were collected and equal amounts of protein were resolved on a 10% SDS-PAGE, followed by Western blotting with **(i)** pAb 462 **(ii)** mAb PSD-95. The blots were developed by chemiluminescence method. Lanes in the blots are abbreviated as follows, P1- homogenate, P2-pellet 2, SY-synaptosomes, P3- pellet 3, SJ- synaptic junction, P4-post synaptic density 1, P5- post synaptic density 2 and P6- post synaptic density 3. CALEB seemed to be enriched in the synaptic junction (SJ) fraction. Western blot with PSD-95 revealed a steady enrichment of PSD-95 till the isolation of PSD in fractions P4-P6.

Synaptic junctions were obtained by rupturing synaptic vesicles with a cold shock. The quality of the various fractions isolated from the mouse brain homogenate was verified by equal loading of protein on SDS-PAGE followed by Western blot with antibodies against PSD-95 (for post-synaptic enrichment fractions). In order to localize CALEB, the fractions were Western blotted with pAb 462. The results showed an enrichment of CALEB in the synaptic junctions. The band was observed at 200 kD which is the highly glycosylated form.

2. Developmental expression profile of mouse CALEB

CALEB in the chick was found to be developmentally regulated in the retina (Schumacher et al., 1997). To clarify the effects observed in the CALEB knock out and also in order to verify the role of CALEB in the visual system, the developmental expression profile of CALEB in superior colliculus and retina was analyzed.

2.1 Superior colliculus

Visual system has been studied in detail and it is a simple pathway where activity-dependent regulation has been studied with relation to *cpg 15* (Corriveau et al., 1999). The retina is directly connected to the superior colliculus and activation of the retina with visual input activity has consequential effect on the superior colliculus.

Hence, we looked initially to the superior colliculus, for the expression of CALEB. Tissues were collected from different stages of mouse development from C57Bl/6J. Tissues were homogenised in the presence of protease inhibitors, followed by protein estimation by Lowry's estimation protocol. Equal amounts proteins were resolved in SDS-PAGE and Western blotted with pAb 462.

The antibody recognised three bands in the superior colliculus of wild type mice which were absent in the CALEB knockout. The three bands appeared smeary in various amounts. These three bands could be a consequence of the presence of three isoforms of CALEB or it could be that the bands differ in the mobility because of different patterns of glycosylations. The bands that appeared were of 140 kD, 113 kD and 108 kD in their molecular mass. The bands were quantified using the NIH Image, and represented graphically (Fig 26).

The quantifications revealed appearance of a peak expression during the superior colliculus development at P10-12. In order to rule out the possibility of the various glycolsylation forms, the extracts of superior colliculus were incubated with chondroitinase ABC for deglycosylation. Immunoblotting of the deglycosylated superior colliculus with pAb 462 unveiled a single band of 108 kD.

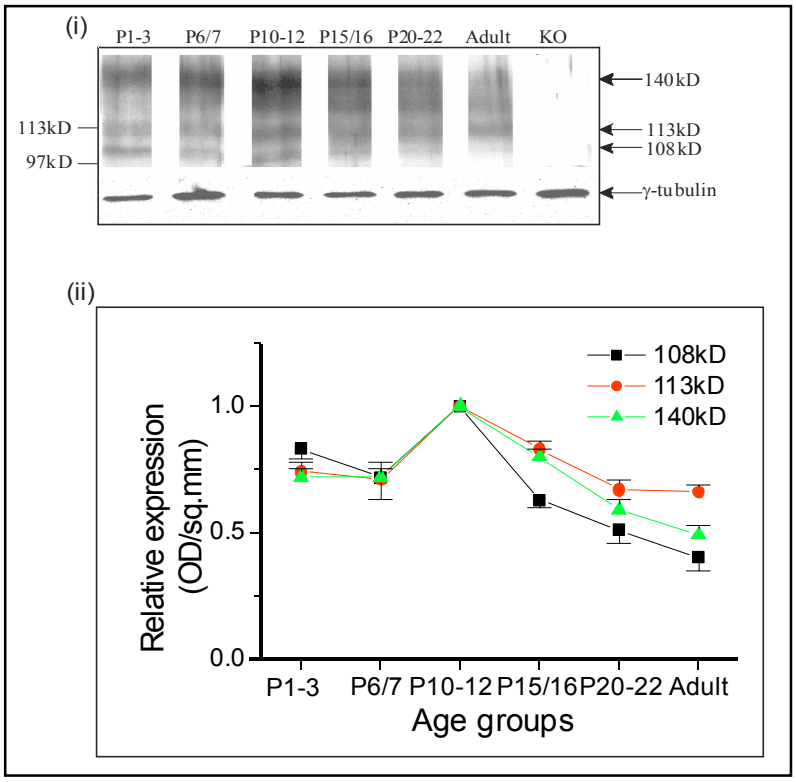


Fig 25:CALEB expression in mouse superior colliculus. Superior colliculus were collected from developmental stages of C57Bl/6J mice (KO-denotes the adult brain of CALEB knock-out) and homogenized in solubilization buffer. Protein estimated by Lowry method and equal amounts of protein were loaded on a 10% SDS-PAGE after boiling with lammeli buffer. Western blot analysis was done by pAb 462 and mAb γ-tubulin. **(i)** Mouse developmental stages were grouped into 6 stages, which have been noted as P 1-3, P 6/7, P10-12, P 15/16, P20-22 and adult. **(ii)** The bands obtained were quantified by Image J and the ratio calculated with respect to the γ-tubulin. Graphical representation was done of the ratio in relation to the developmental stage. (n=4; Mean ± S.E).

The Western blot results displayed a smear of other bands, pointing that CALEB is a highly glycosylated protein with glycosylations apart from Chondroitinase ABC sensitive ones. The 108 kD band obtained from the deglycosylated superior colliculus was quantified.

Equal protein loading for all stages was confirmed by Western blotting with mAb-γ-tubulin (Fig 26). These results could be summarized as presence of a peak CALEB expression in

the superior colliculus at P10-12, i.e., before eye opening. We next were interested to test for the expression of CALEB in retina to illuminate the role of CALEB in the visual system

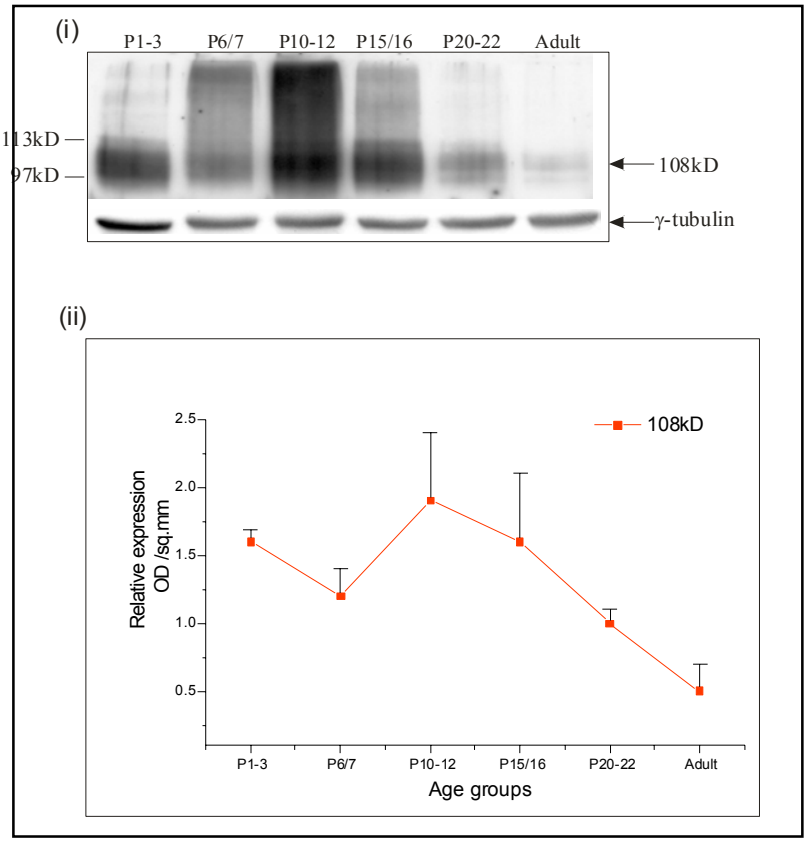


Fig 26:CALEB expression in the deglycosylated mouse superior colliculus. Superior colliculus was collected from various stages of C57Bl/6J mice and homogenized in solubilization buffer. Protein estimation was done by Lowry method and equal amounts of proteins were deglycosylated with Chondroitinase ABC as mentioned in the "Materials & methods". The samples were boiled in the Lammeli buffer and analyzed by Western blot with pAb 462 and mAb γ-tubulin. **(i)** Representative blot with the various stages of development. **(ii)** The prominent band of 108 kD was detected by pAb 462 and was quantified with Image J. The graph plotted was a representation of the ratio of 108 kD band and the quantification value of the γ-tubulin band. (n=3; Mean ± S.E)

2.2 Mouse Retina

Retina tissues of C57Bl/6J wild type mice were collected from different stages and homogenized. Protein estimated by Lowry method and equal amount of proteins were separated by Western blotting.

Immunoblotting with pAb 462 reveals two bands of 113 kD and 108 kD. It was very different as had been observed in the CALEB expression of superior colliculus. The quantification of both the bands revealed a steep decrease in the amount of CALEB expression at P10-12 age group i.e., just before the eye opening stage. We next asked if

the bands recognised by pAb 462 were the glycosylated forms of CALEB. In order to answer this question the tissue homogenate was deglycosylated with chondroitinase ABC and Western blotted against pAb 462. The results showed that the 113 kD band disappeared and only a single band at 108 kD was visualized. The band was quantified and represented graphically with respect to the band recognised by γ-tubulin

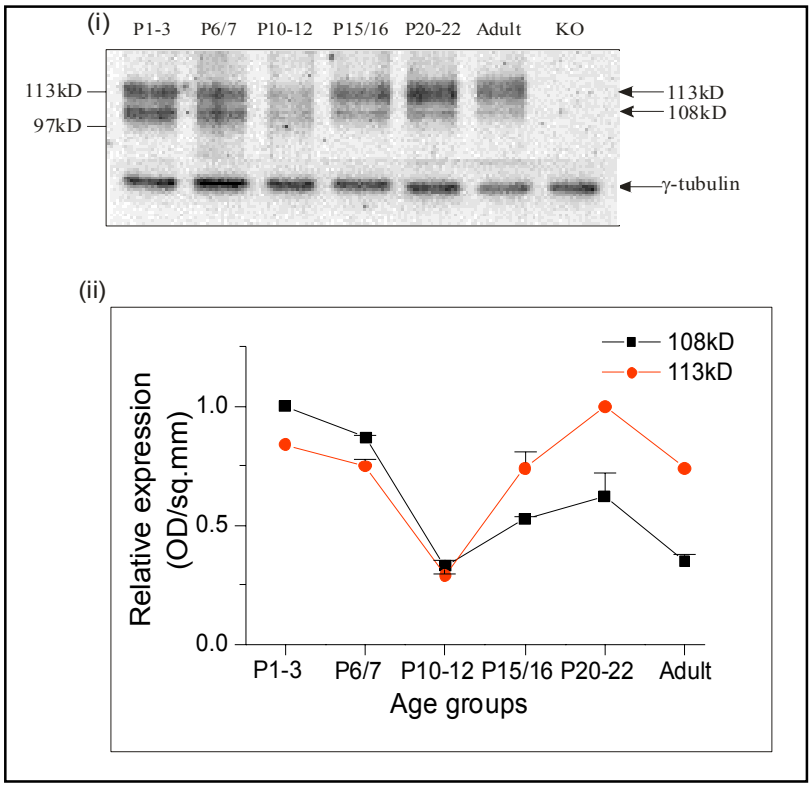


Fig 27:CALEB expression in the developmental stages of mouse retina. **(i)** Mouse retina was collected from developmental stages (P1-3, P6/7, P10-12, P15/16, P20-22, adult and CALEB Knock out (P10)) of C57Bl/6J-schoen mice. The retinas were homogenized in solubilization buffer, estimated the protein content and the proteins analyzed by Western blot with pAb 462 and mAb γ-tubulin. The bands were quantified and graphically represented in a ratio of γ-tubulin. **(ii)** The bands obtained in the Western blot was quantified and graphically represented with standard deviation. The graph represents the relative expression with respect to the band of γ-tubulin. (n=3; Mean ± S.E)

The results of deglycosylated samples showed a steep decrease in the 108 kD band as had been observed previously in undeglycosylated retina samples which were resolved by Western blotting. After a decrease in the expression just before the eye opening, the CALEB expression increases and then again comes down at the adult stages. The strong decrease in the unprocessed form of CALEB at the critical period before the eye opening suggest neuronal activity dependence, as in this stage there occurs a burst of neuronal activity.

The developmental expression shows that glycosylated forms of CALEB in the mouse retina and the superior colliculus were both sensitive to chondroitinase ABC and the core protein was of 108 kD. (Fig 28)

3. Activity-dependent regulation of mouse CALEB in superior colliculus

The previous results from the chick retina culture studies indicated CALEB as a neuronal activity-dependent down regulated molecule. We were interested in confirming the same in the mouse. For this purpose we used mouse superior colliculus, which has a very well define structure and functions as an input target region of the retinal ganglion cells.

In order to prove our hypothesis in mouse superior colliculus tissues, the tissues were incubated with various agonists of glutamate receptors or with KCl. This was followed by homogenisation of the tissue, protein estimation and Western blotting with pAb 462.

In the results we observed, an increase of the total CALEB upon incubation with KCl and bicucullin (GABA receptor blocker). There was observed a decrease in the total CALEB content when the tissue was incubated in APV and DNQX solution in comparison to the tissue which was incubated only in the physiological solution. The pAb 462 antibody detects only the unprocessed form of CALEB, and hence gave a hint that full form of CALEB undergoes a down regulation upon neuronal activity.

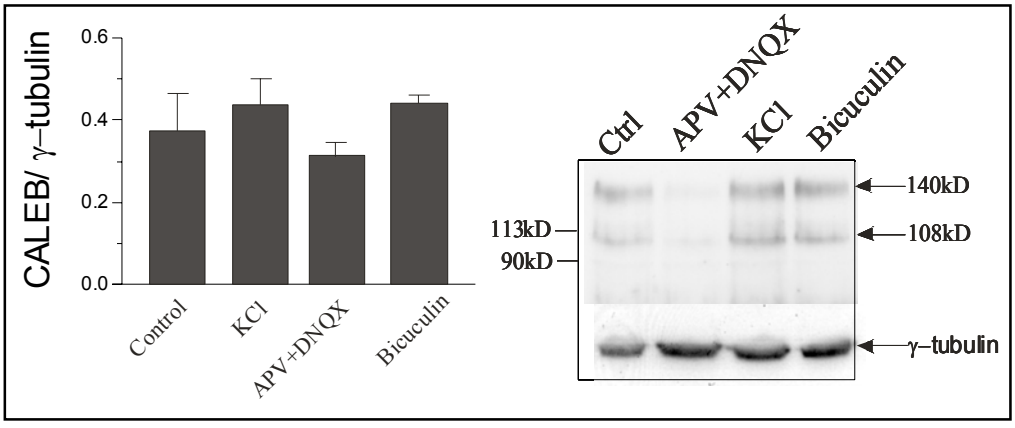


Fig 28:Mouse CALEB regulation in the presence of blockers of GABA and NMDA receptors. Superior colliculus of P10, C57Bl/6J mice were dissected and incubated in a solution containing, KCl (10 mM), APV/DNQX (50 μM/ 10 μM), Bicucullin (20 μM) or in physiological solution. The tissues were incubated for 2 hr, with gasing in a mixture of CO_2 and O_2. The tissues were then homogenized in solubilization buffer, protein estimated with Lowry protein estimation protocol. The proteins were Western blotted with pAb 462 and mAb γ-tubulin. The bands obtained were quantified and plotted graphically with reference to the band of γ-tubulin.