E. DISCUSSION

Once, the initial neuronal connections are laid down by the activity-independent molecules, the final refinement and maturation of synapses is thought to be mediated by the activity-dependent mechanisms. The patterning of the electrical activity in neurons plays a crucial role not only in the establishment of synapses but also in the induction of neural plasticity by triggering molecular and structural changes.

Several families of proteins such as cadherins, semaphorins, and immunoglobulin super family members are known to play a role in laying the initial neuronal connections. Although, molecules modulated by neuronal activity are largely unknown, it has been widely accepted that proteins or genes which are regulated in an activity-dependent manner, might be candidates responsible for the refinement of initial neuronal connections. *cpg 15*, *c-Fos, c-Jun* and *Arg 3.1* are few of the genes which are modulated with neuronal activity (Naeve et al., 1997;Corriveau et al., 1999;Waltereit et al., 2001). We were interested in identifying proteins which might be involved in refinement and maturation of synapses. Membrane proteins were of special interest to us based, on their virtue of membrane anchorage. It can be assumed that the extracellular domain of the membrane protein can act as a receptor aiding in the refinement of neuronal connectivity.

A preliminary screen performed by Prof. Rathjen, using KCl (to induce neuronal activity) incubated chick retina cultures, detected down regulation of CALEB with respect to other unaffected proteins. Chicken acidic leucine-rich EGF-like domain containing brain protein (CALEB), had been earlier identified in our group by combining ELISA and immunological screens (Schumacher et al., 1997).

The mechanism of down regulation has been further investigated in the present work in relation to neuronal activity. I have also looked at the expression pattern during the development to presume a plausible role of CALEB in either regionalization or specification. Attempts on localizing CALEB gave evidence of a probable functional implication of the protein.

1. Characterization and distribution of CALEB

Initially we looked at the expression pattern of CALEB and its localization. Tissue expression profile of CALEB in mouse reaffirmed CALEB as a brain specific protein, which was in coherence with previous studies by our group as well as from the studies done on neuroglycan C, a rat homologue of CALEB, (Schumacher et al., 1997;Oohira et al., 2004). The core protein preparations generated by chondroitinase ABC digestion of whole brain lysates, revealed presence of glycosaminoglycan side chains attached to CALEB. Neuroglycan C undergoes glycosylations by *O*- and *N*-glycosylation as well as by chondroitin sulphate glycosylation (Watanabe et al., 1995). The presence of O- and N-glycosylations in CALEB has been shown earlier, but in contrast there are no chondroitin sulphate groups attached to CALEB ectodomain (Schumacher et al., 1997).

In order to determine the subcellular localization of CALEB, post-synaptic density fractions were prepared. Enrichment of CALEB in synaptic junctions of adult mouse brain indicated a probable role of CALEB either in the formation of synapses or its maintenance.

Immunostained unfixed chick retina cultures (3DIV), stained surface of cells and neurites, pointing to the epitope of mAb 4/1 to be on the cell surface. A similar pattern had been observed on mouse retinal ganglion cell cultures, by staining with antibodies to neuroglycan C (Inatani et al., 2000). In Western blots with mAb 4/1, chick CALEB was recognised appearing in three forms in retina tissue; 200 kD (glycosylated form), 140 kD, 80 kD. The 200 kD is the glycosylated form of the 140 kD component. The 80 kD form was derived from the 140 kD with a different N-terminal amino acid stretch and was the predominant band (Schumacher et al., 1997). The cytoplasmic antibody, mAb 1-2B-8, recognised two additional shorter bands of 44 kD and 36 kD, which were CALEB specific bands as recognised by protein sequencing. Western blot of E8 chick retina culture (3DIV) with mAb 4/1 detected three bands of CALEB similar to those found in the chick retina tissue, supporting the presence of similar forms in tissue as well as in culture conditions.

The stage of E8 chick retina culture followed by 3DIV, might correspond to E11 *in vivo* stage. I was interested in determining the regions which were stained positive by CALEB antibody. CALEB immunoreactivity was observed in inner layers of E11 chick retina such as, ganglion cell layer, inner plexiform layer and the nerve fiber layer with mAb 4/1. The outer layers such as the outer segment photoreceptor layer and retinal pigment epithelium of E11 chick retina expressed CALEB too.

CALEB immunopositivity could be noted in regions of E11 chick brain. In the embryonic chick brain (E11) CALEB was strongly expressed in the *stratum griseum et fibrosum superficiallies* (SGFS) of the optic tectum, which is a major optic nerve innervation layer (Ito et al., 1999a). The lateral magnocellular nucleus of the anterior neostriatum plays a vital role in initial learning of vocal patterns and has a defined class of neurons which project to the dorsal archistriatum. The archistriatum makes a large number of efferent projections from other regions including the medial spirifom nucleus (Bottjer et al., 2000). CALEB was strongly expressed in the pretectal region of the primordium of the medial spiriform nucleus ending in the dorsofrontal nucleus. The cells in the ventral pallium of telencephalic region expressed CALEB and are responsible for the development of the isocortex. These cells migrated from the ventral pallium in the telencephalon to the dorsal pallium for the isocortical development (Aboitiz et al., 2002).

The spatiotemporal expression patterns observed in the retina and in the whole brain might highlight role of CALEB, in either regionalization or development of the particular parts during development.

2. Activity dependent down regulation of CALEB.

Initial studies by Prof. Rathjen, had shown that CALEB undergoes a down regulation at the protein level, when cells were incubated with KCl for long time periods.

In order to explain the phenomenon of CALEB down regulation further, chick retina cultures were incubated with KCl for various durations and results analyzed by Western blots. It was well known that incubation of cultures with KCl (potassium chloride) induced neuronal activity, by depolarizing them (Ishida and Deguchi, 1983). There are several studies where cell cultures have been incubated with higher concentration of KCl (Ishida and Deguchi, 1983;Zhang et al., 1988;Schramm et al., 1990), but in our incubation experiments I used 30 mM KCl (Weiss, 1988;Riederer et al., 1992), since exposure to higher concentration of KCl resulted in higher calcium influx as an out come of depolarization, finally resulting in cell toxicity (Schramm et al., 1990;Fernandez et al., 1991). Depolarization with KCl also increased the osmolality from a base level of 340 m osmol to 381m osmol as recorded by me, which was close to threshold of cell starting to go into apoptotic pathway.

The shortest time period in which down regulation of total CALEB occurred was noted to be after an incubation of 4 hr with KCl. The duration of 4 hr with KCl could turn out to be toxic for cells, which was needed to be avoided. Furthermore, down regulation in the total CALEB was not significantly higher in KCl incubated cultures in comparison to untreated cells, which could be explained by the “masking effect”. If down regulation occurs only in a small fraction of CALEB which is restricted to the cell surface CALEB, it would be likely that the internal pool of CALEB masks the effect. The observed down regulation in total CALEB after 4 hr incubation with KCl also suggested the down regulation of CALEB to be a progressive phenomenon increasing with time.

In order to determine which part of cellular CALEB gets activity-dependent regulated, cell surface proteins were biotinylated with a cell impermeable biotin, EZ-link-NHS-LC-Biotin. Depolarization of these cells with KCl, followed by capturing the biotinylated proteins by streptavidin beads and immunoblots with mAb 4/1 revealed down regulation of CALEB as a cell surface phenomenon. The down regulation of the cell surface CALEB was in the range of approx. 30% with respect to the untreated cultures, which was highly significant as analyzed by the quantification of the 80 kD component. I was able to observe a similar effect on the 140 kD component, i.e., a decrease by 30 %, but quantification was done only for the 80 kD band since, this was the predominant form detected by mAb 4/1 at this stage of chick retina development. Cell surface down regulation occurred within 5 min of KCl incubation and remained constant for over 30 min, decreasing further till 4 hr. This increase in the cell surface down regulation of CALEB till 4 hr, justified the detection of decrease in total CALEB (without biotin labelling) with KCl incubation only after 4 hr in the initial experiments. This finding also confirmed my assumption that the cell surface down regulation is a progressive phenomenon. The results, confirmed that down regulation observed on cell surface CALEB was getting masked by the internal pool of CALEB which remained unaffected, when I detected total CALEB after KCl incubation.

I expected that there occurs a trafficking of internal pool of CALEB to the cell surface which gets further down regulated, keeping down regulation constant over 30 min. Hence, biotinylating the surface proteins before KCl incubation would eliminate detecting the new CALEB protein which gets incorporated to the plasma membrane. If this would be true then I would expect to observe a time dependent decrease of the cell surface CALEB in 5-

30 min time interval. Contradicting my thought, pre-labelling experiments (incubation with KCl followed by biotinylation) revealed a steady state decrease in the cell surface CALEB which remained constant from 5 min to 30 min, concluding the phenomenon of down regulation independent of trafficking of proteins to the plasma membrane. The stagnancy in the cell surface down regulation of CALEB which I observed could be clarified by three different assumptions, (i) A feedback mechanism activated in parallel to the one causing down regulation, might prevent the complete CALEB to get decreased, (ii) it could be that some molecules of CALEB are so called “tagged” which was making them accessible to the down regulating mechanism (iii) the other possibility would be that KCl induced a pathway for the inhibition of cell surface down regulation independent to the pathway for activation of down regulation and this resulted in 30 % decrease in the amount of cell surface CALEB.

Cell surface down regulation of CALEB could be observed by depolarizing cells with KCl, which was an artificial way to depolarize cells forcing them to survive at a higher osmolality. Incubation with KCl also has several other effects and I wanted to be sure that the cell surface down regulation of CALEB was the consequence of neuronal activity only. Hence, in order to be a closer to the *in vivo* or physiological situation or to induce neuronal activity, I investigated the effect of excitatory neurotransmitters on CALEB down regulation. Activation of ionotropic glutamate (excitatory) receptors i.e., NMDA receptors and kainate receptors are known to cause depolarization of cells (Nastrom et al., 1994;Brorson et al., 1994). NMDA when added exogenously, attaches to the NMDA receptor and there by opens the channel allowing calcium influx. The kainate channels open in the similar fashion like the NMDA receptors, permitting calcium influx, but to a lesser extent in comparison to the NMDA receptors.

Incubation of cultures with NMDA and kainate, resulted in the down regulation of the cell surface expressed CALEB. The down regulation of 80 kD CALEB band with the glutamate receptor agonists was significant and was comparable to the down regulation observed when cells were incubated with KCl. These results were again reaffirmed by using specific blockers of NMDA and kainate i.e., APV and DNQX respectively. Chick retina cultures when incubated with NMDA in combination with APV or kainate in combination with DNQX, completely inhibited the down regulation. The results suggested that the mechanism or the cascade of pathway responsible for cell surface down regulation of

CALEB in the presence of KCl or glutamate receptor activators must be same, starting with a calcium influx.

CALEB down regulation occurred by the depolarization of the cells. Depolarization causes opening of the voltage gated Na+ ion channels from the closed state. This opening occurs for 4-6 milliseconds after which the Na+ channels are inactivated and need a state of repolarization to be able to go to the closed state again. When the cultures were depolarized with KCl incubation there must have been a burst of opened Na+ channels, taking the membrane from a resting membrane potential of around -120 mV to around -30 mV (Hammond, 2001). I asked whether the down regulation of CALEB occurred in the interval when the Na^+ channels are open during cell depolarization. Hence, I blocked the voltage gated Na^+ channels with tetrodotoxin (TTX) and simultaneously depolarized the cells with KCl. In the case that the opening of the voltage gated Na^+ channels play a role in the down regulation of the cell surface CALEB, blocking with TTX would be able to inhibit the down regulation. But in contrary the results showed that TTX did not have any effect on CALEB down regulation suggesting thus, CALEB down regulation induced by depolarization is independent of the opening of the voltage gated Na^+ channels.

3. CALEB down regulation is a Calcium dependent process

Depolarization of cells opens the voltage gated calcium channels. This results in an influx of calcium which plays vital role by acting directly or as a secondary messenger in various cellular processes. Calcium is known to regulate processes such as transcription, translation and post translational modifications of proteins (Ghosh and Greenberg, 1995;Zhang and Poo, 2001).

Since depolarization leads to opening of the voltage gated calcium channels, I was interested to define the role of calcium in cell surface CALEB down regulation. Biotinylated chick retina cultures were incubated with increasing concentrations of calcium (0.5-3 mM) in the presence of KCl. The results obtained from these experiments showed the occurrence of a dose dependent decrease in the cell surface CALEB expression. Calcium concentration in the medium was directly proportional to the decrease in CALEB on cell surface. The effect observed at 2 mM calcium concentration in the extracellular medium was similar to the effect observed in the regular DMEM/N2 medium, since the regular medium contains 1.8 mM of calcium.

These experiments suggested that down regulation of CALEB required calcium influx. If this was true, I assumed then blocking the voltage dependent calcium channels would result in blocking the cell surface down regulation. There are three major types of voltage gated calcium channels known as the high threshold- activated (HVA) calcium channels: L- type channel, the N-type channel and the P-type channel. The L- type channels undergo slow inactivation during depolarization. The N- type channel open bursts and then inactivates with time and voltage, where as the P- type voltage gated calcium channel shows low time dependent inactivation (Hammond, 2001). The L- type channels generate large calcium currents and reaches a half inactivation at -40 mV, N and P- type channels generate smaller calcium currents and reach a half inactivation at around -60 mV whereas the resting membrane potential is -70 mV To prove our assumption, I used specific blockers of L-type calcium channels as well as N/P- type voltage gated calcium channels. The L-type channel was blocked by Nimodipine and the N/P –type channels were blocked by ω-Conotoxin MVIIC (Fujisawa et al., 1999;Dolmetsch et al., 2001). Blocking of the calcium channels in the presence of KCl resulted in complete inhibition of the cell surface down regulation of CALEB. Blocking the L-type channel alone also inhibited the KCl induced down regulation where as, blocking of N/P type calcium channels alone did not have any significant effect on KCl induced down regulation (data not shown).

The results concluded that the down regulation of CALEB induced with KCl depolarization is by the calcium influx through the voltage gated calcium channels. Calcium influx is the initiation step of cell surface down regulation of CALEB. By using glutamate receptor activators, the glutamate receptors channels opened, allowing calcium to enter the cells and then open the voltage gated calcium channels during the depolarization effect.

Calcium after entering the cell can bind to calmodulin, which acts as one of the intermediate molecule for the pathways which are regulated by calcium. Calmodulin is a calcium sensing protein which binds four calcium ions and has been found to be activating signalling pathways in the cell, such as during muscle contraction, secretion (Levitan, 1999). This binding of four ions of calcium to calmodulin induces a conformational change, allowing other proteins such as calmodulin dependent kinase to interact with calmodulin (Hammond, 2001). Antagonists of calmodulin stimulate down regulation of EGF and HB-EGF, which are members of EGF-family of proteins (Dong and Wiley, 2000). Based on the

mentioned facts we investigated the involvement of calmodulin in down regulation of cell surface CALEB.

TFP, trifluoperazine dimaleate is a small molecule which acts as calmodulin inhibitor. It blocks the hydrophobic sites of the calcium/calmodulin complex resulting in the inhibition of other target proteins to bind to the calcium/calmodulin complex. TFP, at higher concentrations binds to calmodulin alone (Tanokura and Yamada, 1986).

Pre-treatment of chick retina cultures with TFP, resulted in inhibition of the cell surface CALEB down regulation. TFP inhibited the activation of the target protein of calcium / calmodulin complex. The results suggested that calcium most likely binds to calmodulin once calcium enters the cell, resulting in the formation of calcium/calmodulin complex which further activates another target protein down stream for CALEB down regulation. These results come up in disagree to the results obtained by using TFP for the shedding of L-selectin, the cell adhesion molecule, where calmodulin inhibitors induce ectodomain shedding (Diaz-Rodriguez et al., 2000).

The down stream molecule of the calcium/calmodulin complex that I investigated was calcineurin since it is present in high amounts in neurons and regulates genes in an activity-dependent manner. Calcineurin is a calcium dependent phosphatase (also called protein phosphatase 2B) involved in several neuronal cell processes such as inhibition of GABAergic and glutamatergic neurotransmitters, it also regulates the voltage gated calcium channels (Klee et al., 1998;Norris et al., 2002). Calcineurin has been known to be activated via calmodulin in the presence of calcium (Yardin et al., 1998). Pharmacological calcineurin blocker, FK-506 (Sharma et al., 2003) when preincubated in chick retina cultures followed by KCl incubation, resulted in inhibition of CALEB down regulation at cell surface. The amount of CALEB 80 kD component detected in the cultures incubated with FK-506 and KCl were comparable to the untreated cultures.

Based on the findings till now, I could conclude, chick retina cultures when incubated with KCl, resulted in an influx of calcium through voltage gated calcium channels. These calcium ions, bind to calmodulin intracellularly and thereby activate calcineurin. Activated calcineurin initiates a cascade of molecular pathways culminating finally with cell surface down regulation of CALEB.

4. Down regulation as a consequence of CALEB ectodomain shedding.

Underlying mechanism of the down regulation of cell surface molecules could be a consequence of internalization or protein ectodomain shedding. Calcium influx has been known to cause ectodomain shedding as well as internalization of cell surface proteins such as TGF-α, HB-EGF, E-cadherins and AMPA receptors respectively (Pandiella and Massague, 1991;Ito et al., 1999b;Ehlers, 2000). I was curious to find out the mechanism responsible for CALEB cell surface down regulation in chick retina cultures incubated with KCl.

Biotinylated (NHS-SS-Biotin) chick retina cultures were incubated with KCl in the presence of leupeptin, followed by cleavage of the cell surface biotin by cleavage buffer containing reduced glutathione. In the presence of leupeptin the lysosomal degradation of the internalized receptor or protein was prevented (Man et al., 2000). The cleavable property along with the ability to force the impermeant biotin to be cell permeable when bound to protein made NHS-SS-Biotin, ideal to study internalization of proteins. I expected that in case, KCl incubation resulted in internalization of the CALEB, then we would detect the internalized CALEB once the cell surface biotin was cleaved. In case, there occurred a cell surface ectodomain shedding we would not be able to detect any internalized CALEB. For experimental control I used pAb GluR 2/3 antibody against AMPA receptor 2/3. These receptors undergo internalization on KCl incubation (Ehlers, 2000).

The results revealed internalized GluR 2/3 receptors upon KCl incubation, whereas CALEB could not be detected after cleaving the cell surface biotin, based on the Western blots with mAb 4/1. This suggested that CALEB down regulation as an outcome of ectodomain shedding from the cell surface.

To confirm our results further, the epitope of the mAb 4/1 was mapped. Mapping of the epitope by transfecting COS7 cells with different constructs of chick CALEB with a GPI-anchor, narrowed down the epitope to be within residues 280-286, which lied in the extracellular domain of CALEB distal of the EGF domain (data from Mechthild). The presence of the epitope in the extracellular domain and the absence of the internalised chick CALEB confirmed that CALEB undergoes ectodomain cleavage with incubation with KCl.

I searched for fragments which were released and remained on the membrane after the ectodomain shedding of CALEB. A released fragment of 18 kD was enriched from TCA precipitated supernatants of KCl treated cultures in comparison to the untreated cultures. This was detected by both mAb 4/1 and pAb C5. The pAb C5 binds to the N-terminal region of CALEB whereas mAb 4/1 binds to residues 280-286. The presence of weak amounts of the 18 kD band in the untreated cultures suggested that ectodomain shedding of chick CALEB occurred constitutively and with KCl incubation the ectodomain shedding was only enhanced. The 18 kD component could not be further characterized, because of low amounts.

Membrane attached fragments were detected in chick retina cultures which were incubated with KCl in the presence of MG132 by immunoblotting with mAb 1-2B-8. MG132 was a blocker of intracellular proteolysis involving the proteasome pathway, calpains or by lysosomal cysteine proteases. Hence, the results compromised that most probably the short cytoplasmic part of remaining membrane-tethered CALEB undergoes proteolysis after the extracellular domain has been shedded. This process was similar to the degradation of the cytoplasmic domains of E-cadherin after shedding of the ectodomain or to the p75 receptor after undergoing internalization, where the degradation could be inhibited in the presence of MG132 (Ito et al., 1999b;Jung et al., 2003). In the KCl untreated chick retina cultures, which were incubated with MG132, we could detect a weak band expressed at 38 kD, suggesting again that presence of KCl only enhances the proteolytic processing which is a constitutive process.

The 38 kD represents the cytoplasmic domain, transmembrane stalk region and the EGF-domain of CALEB. The bands recognised by mAb 8-1B-8 from the affinity purified whole eye lysates were sequenced. The results revealed presence of EGF-domain in the 38 kD fragment (results from Prof. Rathjen). Results of transfecting CALEB construct containing cytoplasmic domain, transmembrane stalk region and EGF domain with a small stretch of aminoacid overhang, revealed the appearance of a 38 kD band in Western blot (results from Petra). The similarity in the mass of the membrane attached fragment after ectodomain shedding of CALEB and the results of transfecting CALEB construct containing cytoplasmic domain, transmembrane stalk, EGF domain suggests an identical confirmation. The stalk region between the EGF domain of CALEB and the

transmembrane region is a short stretch of around 4 aa, which makes it difficult for the enzyme to be accessible in the juxtamembrane region for cleaving the EGF-domain away from the transmembrane stalk. The length of the stalk region plays a vital role in causing cleavage of the cell surface proteins (Mullberg et al., 1994). A minimum of at least 10-13 aa stretch is necessary between the transmembrane region and the site of cleavage for the enzyme to be able to cleave. The ectodomain release of CALEB hence, most probably makes the EGF domain accessible by other ligands for activating signalling pathway.

Isolation of 38 kD membrane attached fragment from KCl treated retina cultures and expression of CALEB in a developmentally regulated manner in the retina, prompted me to look for the presence of this fragment during the chick brain development.

Three major lower components were visible of 44 kD, 38 kD and 28 kD in the chick retina. The lower bands appeared at E11 in the retina and steadily increased till E17 retina. The appearance of the lower components coincided with the appearance of the 80 kD component, suggesting the lower components to be a derivative of the 80 kD form. The lower components of CALEB in retina seem to be independent of the 140 kD. Contrastingly, in chick tectum a lower component of approximately 60 kD is recognised, which decreases during development. In telencephalon, the 60 kD component reaches a peak at E11 and then goes down during development. No components lower to 60 kD were identified in chick tectum or telencephalon. 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.

I was interested in finding out the signalling pathway that leads to the ectodomain shedding of CALEB. It was known that activation of the Erk kinase pathway leads to cleavage of HB-EGF and TGF-α (Fan and Derynck, 1999;Umata et al., 2001).

Inhibition of the Erk kinase by using specific blockers inhibited the ectodomain cleavage of chick CALEB in the chick retina cultures, suggesting a mechanism similar to the one observed in shedding of HB-EGF and TGF- α. Extracellular domain shedding of CALEB most likely did not need new protein synthesis as this was a fast kinetic process. This assumption of non-involvement of protein synthesis was confirmed by using cycloheximide (a protein synthesis blocker) (Fan and Derynck, 1999), where presence and absence of

cycloheximide did not affect the cleavage of chick CALEB from the cell surface. Erk kinase activation in neurons has been reported to occur by calcium influx occurring through either NMDA receptor or L-type calcium channels (Paul et al., 2003). Increase in the intracellular calcium via calcium influx through NMDA channels or L-type calcium channel activates calcineurin in neurons (Graef et al., 1999;Paul et al., 2003).

Studies have shown that ectodomain shedding can be initiated by the activation of protein kinase C pathway, which is a pathway different from the calcium influx pathway (Arribas et al., 1997;Fan and Derynck, 1999;Thabard et al., 2001). I investigated the role of protein kinase C pathway in ectodomain shedding of CALEB. Results of incubating biotinylated chick retina cultures with or without PMA (phorbol 12-myristate 13-acetate, an activator of protein kinase C) in the presence of KCl had not effect. Inhibition of ectodomain shedding of CALEB or activation of ectodomain shedding was not initiated in the presence of PMA (data not shown). These findings supported the concept that there are independent pathways activating ectodomain shedding of proteins, one is a calcium dependent pathway (Ito et al., 1999b) and another a calcium independent pathway (Le Gall et al., 2003).

Ectodomain shedding has been implicated in several functional consequences, such as exchange of growth and differentiation signal through releasable growth factors, receptors released by cleavage of the ectodomain can modulate the function of the ligands in forming active signalling complexes, ectodomain shedding of cell adhesion molecules represent a crucial point in the dynamic regulation of cell-cell and cell-extracellular matrix interactions (Arribas and Borroto, 2002). Hence, the results obtained may hint for the plausible role of CALEB either acting as a receptor or a ligand activating paracrine signalling pathways.

The findings can be summarised, as depolarization of chick CALEB with KCl leads to calcium influx, which binds to calmodulin thereby activating calcineurin. The activated calcineurin most likely activates Erk kinase pathway and in another way, calcium can directly activate Erk kinase pathway. Both of the above pathways lead to ectodomain release of CALEB, leaving behind the EGF-domain attached to the membrane for mediating signalling cascades leading to growth and differentiation.

5. Metalloproteases cause the ectodomain shedding in the chick retina cultures

Metalloproteases are a group of membrane proteases which are responsible for cleaving the ectodomain of several protein families ranging from cytokines, to growth factors receptors, prions and amyloid precursor proteins (Arribas et al., 1997;Gallea-Robache et al., 1997;Mechtersheimer et al., 2001;Sahin et al., 2004). The name metalloprotease, gives an indication of the involvement of a metal ion in the function and structure of these proteases, and this metal ion is zinc (Hooper, 1994;Schlondorff and Blobel, 1999). There are two broadly classified classes of metalloproteases i.e., matrix metalloprotease (MMP) and a disintegrin and metalloprotease (ADAM). There are approximately 34 members of the family of ADAMs, which is the predominant class of protease responsible for ectodomain shedding of membrane proteins. The ADAMs are membrane proteases, which are formed in a pro-protein form and then truncated to the active form and can be activated by calcium (Pandiella and Massague, 1991;Srour et al., 2003).

To identify the protease responsible, I initially inquired into the property of protease involved i.e., a membrane bound protease or a secreted protease. Membrane preparations were made from the chick retina cultures, which were then incubated for 2 hr without or with TAPI-1 (broad spectrum hydroxamate metalloprotease inhibitor) and resolved by immunoblots with mAb 8-1B-8, antibody to the cytoplasmic domain of CALEB. If the protease was a membrane bound protease, I expected to detect the 38 kD band increased in membranes which were incubated without TAPI-1 in comparison to membranes which were left unincubated or membranes which were incubated with TAPI-1. I observed an increased amount of the 38 kD band in membranes which were incubated without TAPI-1, when compared to the membrane fractions not incubated or incubated with TAPI-1. Results were similar as had been observed in case of the proteolytic processing of E-cadherins (Ito et al., 1999b), and indicated the protease responsible for CALEB shedding to be a membrane protease.

To reaffirm the results chick retina cultures were incubated with KCl in the presence of membrane impermeable broad spectrum hydroxamate metalloprotease inhibitors TAPI-1, TAPI-2, RU 36156, 1,10 phenanthroline and ilomastat. Western blotting of the streptavidin precipitated samples with mAb 4/1 unravelled the prevented ectodomain shedding of chick CALEB with KCl incubation in the presence of the metalloprotease inhibitor. A stronger

inhibition was observed in the presence of TAPI-1 alone or in combination with KCl with respect to untreated culture. This ascertained the likely inhibition of shedding of CALEB by TAPI-1.

The results obtained with the incubation of metalloprotease inhibitor narrowed down the search from the proteases to the membrane bound protease. The membrane bound proteases are either MMPs or ADAMs (Hooper, 1994).

In the adult CNS ADAM 9, 10, 11, 12, 15, 17, 19, 22 and 23 are expressed widely with variable amounts of each present in different regions of the brain (Karkkainen et al., 2000;Moro et al., 2003). Among the ADAMs present in the CNS, I looked at ADAM 10 and ADAM 17 (or TACE) which have been implicated for causing ectodomain shedding of HB-EGF, TGF-α, amphiregulin, betacellulin and epiregulin (Brown et al., 1998;Lemjabbar et al., 2003;Sahin et al., 2004).

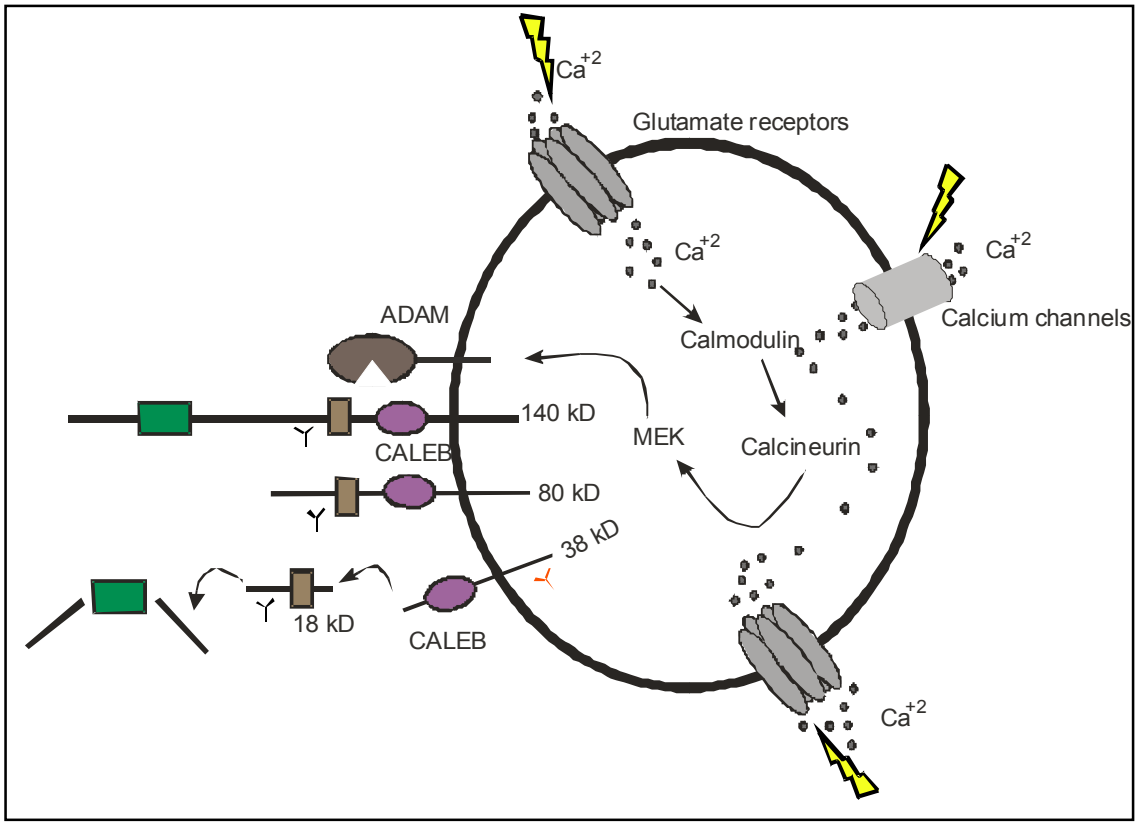
In order to analyze for the specific roles in cleaving the CALEB ectodomain, I used specific blockers of ADAM 10 and ADAM 17, GI 254023X and GW 280264X respectively. GW 280264X preferentially blocks the activity of ADAM 17 with a higher sensitivity in comparison to ADAM 10 (Hundhausen et al., 2003).

In the presence of the blocker GI 254023X, KCl induced cleavage of cell surface chick CALEB was blocked and the levels were alike the control levels. GI 254023X alone did not have any effect on the cell surface CALEB expression. In the presence of GW 280264X when the cells were incubated with KCl, the cell surface down regulation of CALEB was inhibited. The levels were significantly higher than the untreated cultures. GW 280264X alone slightly increased the level of the CALEB expression in the absence of KCl.

The results could be interpreted that the ectodomain cleavage of CALEB takes place by the catalytic activity of both ADAM 10 and ADAM 17, stayed in consistent with other EGF-family members such as HB-EGF and TGF-α that are cleaved by ADAM 10 as well as ADAM 17 (Sahin et al., 2004).

The role of ADAM 10 in the ectodomain cleavage of CALEB was investigated further. Transfected COS 7 cells with chick CALEB in the presence of wild type and dominant

negative ADAM 10, displayed a dose dependent decrease of chick CALEB expression of chick CALEB in the presence of the wild type ADAM 10 and in contrary there was no effect on CALEB expression in the presence of dominant negative ADAM 10. It is known that processing of tumour necrosis factor-α (TNF- α), occurs in a dose dependent decrease of the cell surface TNF- α expressed in the presence of TACE (ADAM 17) (Itai et al., 2001).



Scheme 6:Molecular mechanism of CALEB ectodomain shedding. Schematic representation of the probable cascade of molecular pathways involved with the initiation of the calcium influx and the final out come being the ectodomain shedding of chick CALEB. There are two forms of CALEB i.e., the 140 kD and the 80 kD. The 38 kD is the ectodomain cleaved CALEB. ADAM represents the matrix metalloprotease. MEK denotes the Erk kinase, electric activity is denoted by , represents calcium ions, is the LP motif of CALEBis represented by ....., is the acidic box, represents the EGF domain of CALEB. is the epitope of mAb 4/1 and is the epitope of mAb 8-1B-8 and 3-2G-10.

The findings can be summed up stating, KCl and agonists of glutamate receptors induced depolarization in chick retina cultures that consequently resulted in an influx of calcium. The entering calcium binds to calmodulin and actuated calcineurin, followed by Erk kinase activation. The activated Erk kinase lead to ectodomain shedding of CALEB by triggering membrane metalloprotease (s), one of which is ADAM 10.

7. Upregulation of chick CALEB by neuronal activity

Calcium is known to regulate protein expression (West et al., 2001). I tested therefore whether KCl has an effect on CALEB expression if ectodomain shedding is prevented.

I assumed that inhibition of CALEB shedding for longer duration would not lead to any upregulation of CALEB, if unprocessed CALEB was supposed to be the end product of CALEB synthesis. This would occur because of a probable feedback mechanism preventing accumulation of unprocessed CALEB. Whereas, in case ectodomain shedding of CALEB is the final step of synthesis, then inhibition of ectodomain shedding would lead to accumulation of unprocessed CALEB. My assumption was based on the fact that feedback inhibition mechanism occurs when there is an accumulation of the end product. This accumulated end product inhibits the initiation step. The results from the experiment could hint of disclosing the probable active and the pro-active form of CALEB, depending on the end product.

Experiments on inhibition of CALEB shedding in chick retina cultures disclosed results bringing out unprocessed CALEB not as the end product. An upregulation of chick CALEB was observed after 4 hr of KCl incubation in the presence of TAPI-1 in comparison to cultures incubated with TAPI-1 alone or left untreated. The increase in the CALEB accumulation on inhibition of shedding suggested cleaved form of CALEB to be the supposedly end product of CALEB synthesis, and this observation can lead to the speculation that most likely ectodomain shedded form of CALEB is the active form. I speculated this based on the assumption that the end product is the shedded CALEB and not the unprocessed form. This significant increase in the chick CALEB upon KCl and TAPI-1 incubation could be a result of increased protein synthesis due to transcription or translation.

In order to prove one or the other I used specific blockers of transcription, actinomycin D in the presence of KCl and TAPI-1. The results revealed that the upregulation observed was independent of transcription. Incoming calcium due to depolarization binds to calmodulin, which in the presence of calcium activates calcineurin. The activated calcineurin regulates expression of immediate early genes, at the transcription level by controlling the translocation of the NF-ATc family of transcription factors to the nucleus in neurons as well as in cardiovascular system (Graef et al., 1999;Molkentin, 2004) The non-participation of

transcription for upregulation of unprocessed CALEB, justifies CALEB as not an immediate early gene, but instead the regulation occurring only at the protein level.

The presence of translation blocker, cycloheximide incubated with KCl and TAPI-1 resulted in inhibition of CALEB upregulation. Treatment with cycloheximide alone for the same duration of time did not decrease the levels of any CALEB, implying a slow turn over of these proteins (Tartaglia et al., 2001). Independent studies from our lab have also indicated that at the end of 4 hr, there was no escalation of CALEB specific mRNA level based on real-time PCR (data from Eva), which backed up my observation of the involvement of translation for upregulation of total CALEB in the presence of KCl and TAPI-1.

8. Mouse CALEB expression and regulation by activity

CALEB was expressed in mouse brain in a highly glycosylated form. I investigated the CALEB expression pattern in the mouse visual pathway, which is a well studied system. CALEB was expressed in a developmentally regulated manner with a peak expression in P10-12 in superior colliculus, during which there occurred an increase in the number of the functional α1 subunits of $GABA_AR$ resulting in an increase of the ratio of α1/ α3 receptors of $GABA_AR$ (Dunning et al., 1999;Okada et al., 2000). It could be that CALEB aids in the upregulation of the α1 receptors of the $GABA_AR$ mediated by the NMDAR. NMDAR activity has been implicated as a decisive force of $GABA_AR$ maturation (Facchinetti et al., 1993;Zhu et al., 1995;Mathews and Diamond, 2003). At this age group the NMDAR-mediated charge transfer displayed a peak, presumably by regulating the α1/α3 ratio. Among many possibilities, one may consider the involvement of immediately early genes (Harris et al., 1995), altered calcineurin expression (Shi et al., 2000) or NO activity (Contestabile, 2000) for the NMDAR mediated change in expression levels of the α1/ α3 subunits of $GABA_AR$. Eye opening marks the onset of patterned vision in rodents and other mammals. Based on studies on the cat visual cortex a 'critical period' has been defined as a period of enhanced susceptibility to visual deprivation. Interestingly, the peak of visual cortical plasticity coincided with a steep increase of α1/α3 (Chen et al., 2001). During the development of the visual pathway, a balance develops between the glutamatergic synaptic input from the retina and inhibitory GABAergic synapses of the superior colliculus, gets perturbed by the maturation of the cortico-tectal glutamatergic inputs (Henneberger et al., 2004).

In the retina, in the P10-12 mice, there occurred a steep fall in the CALEB expression, that is just before the eye opening stage. The retinal neurons during P10-12 fire short bursts of spontaneous action potentials with a strong correlation index, suggesting a role in establishing the refined retinotopic map. Until P10 stages the spontaneous waves are generated with the help of β2 subunit of acetylcholine receptors. These retinal waves during the P10-12 stages are mediated by ionotropic glutamate receptors with the appearance of the ionotropic glutamate receptors (McLaughlin et al., 2003). During this critical period the important events of the retinotopic map formation occurs. After the formation of the diffuse map in the early postnatal stages in mice, the large scale remodelling occurs before the visually evoked activity that emerges at around P10 (Tian and Copenhagen, 2003). The remodelling occurs by spontaneous retinal waves generated by a network of cholinergic amacrine cells which interconnect retinal ganglion cells.

There occurred a transient decrease in CALEB expression at P10-12 stages, which could be because of either less CALEB at this stage, or a higher ectodomain processing of CALEB at P10-12. The antibody that I used for developmental expression profiling recognizes unprocessed CALEB only (pAb 462). The decrease in the mouse CALEB expression in P10-12 stages could be speculated as a secondary effect of the appearance of retinal waves mediated by the ionotropic glutamate receptors, or CALEB could be playing crucial in establishment of the retinotopic maps.

In order to check for a similar property of the mouse CALEB as had been observed for chick CALEB that it undergoes an activity dependent down regulation we incubated superior colliculus in agonists and antagonists of neuronal activity. The samples were then detected by immunoblots with pAb 462. The results depicted that upon higher neuronal activity induced by KCl or in the presence of bicucullin, higher levels of CALEB were detected. In the presence of APV and DNQX there occurred decrease in the level of mouse CALEB. Though the results were not significantly higher or lower to the untreated superior colliculus, there was a clear tendency observed suggesting an activity-dependent regulatory property of CALEB.