



Research Paper

Induction of α CaMKII Expression by KCl in Cultured Primary Cerebellar Granule Neurons



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Running Title Cultured Cerebellar Granule Cells Express α CaMKII

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ABSTRACT

Background: The cerebellum specializes in functions, such as motor learning. One of the abundant types of neuronal cells present in the cerebellum is the cerebellar granule (CG) cell. While CG cells exhibit synaptic plasticity, it has been found that CaMKinase II alpha (α CaMKII), an enzyme important in multiple forms of plasticity in other regions of the brain, is not expressed in CG cells in vivo, as observed by immunohistochemical analysis of the rat cerebellum. However, we observed that α CaMKII is expressed in primary cultures of rat CG cells, although the expression profile of several other proteins involved in calcium-based signaling mimics their expression pattern in vivo. KCl in culture is known to induce membrane depolarization in a concentration-dependent manner, which is also influenced by the age of the culture in vitro.

Objectives: We assessed the factors responsible for the expression of α CaMKII in primary CG cells.

Materials & Methods: The presence of α CaMKII in primary CG cells in vitro was analyzed by both mRNA transcript and protein analysis. To study the effect of different concentrations of KCl on primary CG cells, Western blot analysis and immunocytochemistry were employed.

Results: The results show that α CaMKII in primary CG cells in vitro is induced by KCl, which is supplemented in the medium to promote the survival of the cells.

Conclusion: The study reports for the first time that α CaMKII, which is absent in CG cells in vivo, is induced by KCl used to grow the primary CG cells in vitro. The expression increases with enhanced concentrations of KCl.

Keywords: Cerebellum, Cultured cells, CaMKinase II alpha (α CaMKII), Depolarisation, Potassium chloride

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Highlights

- CG cells do not express the memory molecule Ca^{2+} /Calmodulin-dependent kinase type II α (αCaMKII) in vivo.
- The growth of primary cultures of CG cells requires KCl to maintain membrane depolarization for the survival of these cells.
- However, the presence of KCl in the cells induces the expression of αCaMKII in vitro, as reported in this study.

Introduction

The cerebellum is the seat of motor learning and memory in the brain [1]. It mainly consists of two major cell types: Cerebellar granule (CG) cells and Purkinje cells, both of which are excitatory in nature. The CG cells constitute the most abundant cell type in the mammalian brain. These cells receive innervation from deeper parts of the cerebellum through mossy fibres, and their output is modulated through parallel-fibre Purkinje neurons [2]. The projections from Purkinje cells are the sole output from the cerebellum.

Calcium/calmodulin-dependent protein kinase type II (CaMKII) is important for inducing and maintaining synaptic plasticity in the hippocampus and is known as the 'memory molecule' [3]. Calcium entry through the N-methyl-D-aspartate receptors (NMDARs) activates CaMKII [4]. CaMKII comprises a family of isoforms derived from four closely related yet distinct genes designated as α , β , γ , and δ . The predominant isoforms of CaMKII are α and β , which are found in the ratio of 3:1 in the forebrain and 1:4 in the cerebellum, respectively [5]. In the cerebellum, the promoter for the α -isoform of CaMKII uses an alternate transcription initiation site that is weaker compared to the site that is functional in the hippocampus [6]. However, the specific cell type in the cerebellum in which the alternate transcriptional control operates has not been demonstrated. The expression of αCaMKII is confined solely to Purkinje cells in the cerebellum, as observed by immunohistochemical analysis using an antibody specific to αCaMKII [7-9]. The mRNA encoding αCaMKII is also enriched in the Purkinje layer, as seen by in situ hybridisation [10, 11]. αCaMKII plays a role in long-term depression in Purkinje neurons [12]. The CG cell layer is found to be completely devoid of αCaMKII [9]. Instead, the CG cells express βCaMKII , which differs in its biochemical properties compared to αCaMKII , such as increased sensitivity to calcium [13, 14] and the ability to bind to the actin

cytoskeleton [15]. The absence of αCaMKII in CG cells suggests that the calcium signalling pathways in these cells may be unique.

To investigate the mechanisms of calcium signaling in CG cells, we utilized primary cultures of CG cells. Primary CG cells have been employed as model systems for studying neural activity and Ca^{2+} -dependent gene expression [16]. CG cells can be significantly enriched in culture and possess characteristics that resemble those of granule cells in the cerebellum [17]. CG cells are grown in medium containing high KCl to induce depolarization, which mimics stimulation in vivo by the mossy fibers that innervate them. This treatment enhances long-term survival of CG cells in vitro [17-19]. Depolarising conditions facilitate Ca^{2+} entry into these cells via voltage-gated calcium channels [17]. However, some studies have shown that CGs grown in high concentrations of KCl exhibit an aberrant phenotype that differs from that observed in vivo [20].

Our study aimed to develop a primary culture of CG cells for use as a model to study the interaction of transfected αCaMKII with neuronal receptors upon calcium signaling. However, we observed an aberrant expression of αCaMKII in the primary CG culture, despite it being known to be absent in vivo. Previous studies had not reported this anomaly, which intrigued us to investigate the factors behind the induction of αCaMKII expression.

Materials and Methods

Materials

Normal goat serum was from Santacruz, USA. Paraformaldehyde, Triton-X-100, and Tween-20 were from SRL chemicals, India. DAPI, cytosine arabinoside (Ara C), DNase 1, Poly-D-lysine, Laminin, dNTPs, Taq polymerase, RIPA buffer, and monoclonal antibody against β -actin (A-5441) were from Sigma Chemicals, USA. Fluoromount G and optimal cutting temperature (OCT)

compound were from Electron Microscopic Sciences. NBM, Trypsin, Glutamax, and Antibiotic/antimycotic solution were from Invitrogen/GIBCO. Monoclonal antibodies against α (MA1-048) and β subunits (PA1-41150) of CaMKII were from Thermo Scientific, USA. Monoclonal antibody against GluN2B subunit of NMDA receptor (*GluN2B*) (ab93610) was from Abcam, UK. Polyclonal antibody for GABA-A receptor $\alpha 6$ subunit (*$\alpha 6$ GABA*) (AB-5453) was from Millipore, USA. Secondary antibodies with alkaline phosphatase (AP) conjugates, as well as horse radish peroxidase (HRP) conjugates, were from Sigma Chemicals, USA. The secondary antibodies with fluorescence conjugates were from either Sigma or Jackson ImmunoResearch, USA. The kits used included the RNaseasy kit from Qiagen, USA (74104), the DNase digestion kit from Ambion, USA (18068015), and the cDNA synthesis kit, Superscript III from Invitrogen (11304011).

Methods

Immunohistochemistry

All animal experiments were approved by the Institutional Animal Ethics Committee. Rat pups of the required age, postnatal day 7 (P7), were provided by the Animal Research Facility of the [Rajiv Gandhi Centre for Biotechnology \(RGCB\)](#), in accordance with the requirements of the approved experiments. All procedures performed in studies involving animals adhered to the ethical standards of the institution or practice at which the studies were conducted. The cerebella were dissected from P7 pups and immediately immersed in the fixative (4% PFA in 1X PBS, pH 7.4) overnight at 4 °C. They were then cryoprotected in 30% sucrose in 1X PBS overnight at 4 °C for two days. The brains were subsequently embedded in OCT compound. Blocks were created by slowly immersing the brain in liquid nitrogen, and the frozen blocks were stored at -70 °C. Brain samples were sectioned sagittally at a thickness of 14 μ m using a cryostat (Leica 3050S). Sections were processed for immunohistochemical to assess the expression of *α CaMKII*.

Antigen retrieval using citrate buffer was performed to expose any antigen-binding sites that might be inaccessible to the antibody. The sections were subjected to high temperatures of about 90-100 °C in citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0) for about 10-15 minutes. The sections were then washed in 1X PBS three times for about 5 minutes each. The sections were then permeabilized and blocked with 5% normal goat serum (Chemicon) containing 0.2% Triton-X-100

in PBS for 1 h at room temperature, followed by incubation with the primary antibodies in the same solution at 4 °C overnight. The sections were again subjected to washes with 1X PBS three times for 5 minutes each and were incubated with the corresponding secondary antibody conjugated to a fluorophore at room temperature for about 90 minutes. The sections were again washed in 1X PBS three times for 5 minutes each. Between the first and second washes, the sections were stained with the nuclear stain DAPI (1:50,000) by incubating at room temperature. Finally, the sections were mounted with Fluoromount G.

Preparation of primary CG neurons

The primary CG neuronal culture was prepared from P7 Sprague Dawley rat pups. The pups were wiped with 70% alcohol and decapitated. The cerebellum was carefully dissected out. After removing the meninges, the cerebellum was minced into pieces. All dissections were done in sterile ice-cold PBS. The cerebellum was then trypsinized in 0.05% trypsin at 37 °C for about 12 minutes. The tissue was centrifuged at 1000 rpm for about one minute to settle. The trypsin was aspirated out gently, and the tissue was washed with PBS. About 20 μ L of DNase (4000 U) was added to about 2 mL of plain neurobasal medium (NBM) and mixed with the tissue. To disintegrate the tissue and obtain isolated cells, it was subjected to mechanical stress by pipetting approximately 20-50 times. This supernatant was gently harvested, PBS was added, and it was again centrifuged at about 1000 rpm for 5 minutes. The resulting pellet contained the cells. Reconstituted NBM [containing 0.5% B27 supplement, 20 mM added KCl (5 or 10 mM KCl in certain experiments), 1X Glutamax, 33 mM glucose, and 1X Antibiotic/Antimycotic] was added to the cells. Since NBM already contains 5 mM KCl, the total concentration of KCl becomes 25 mM when 20 mM KCl is added. The cells were seeded onto plates pre-coated with poly-D-lysine and laminin. After 24 hours, the medium was removed, and fresh reconstituted NBM was added. In some cases, after 48 hours, the cells were treated with 10 μ M Ara C to prevent the proliferation of glial cells. The cells were maintained in vitro for up to 24 days. The medium was regularly changed, and Ara C treatment was administered intermittently to prevent glial proliferation.

The cells were harvested using 1X PBS on fixed days, specifically DIV 0, 2, 4, 6, 8, 10, 12, 15, 19, and 24. The cells were either lysed using RIPA buffer for Western blot analysis or total RNA was isolated from the cells using RNAeasy kit, in preparation for cDNA synthesis.

Various genes were amplified using suitable primers through reverse transcriptase polymerase chain reaction (RT-PCR). The cells were also subjected to immunocytochemical analysis to assess the expression of proteins of interest.

Transcriptomic analysis

Total RNA was isolated from primary CG cells using the Qiagen RNeasy kit according to the manufacturer's protocol. DNA contamination in RNA preparation mentioned above was removed by DNase digestion using the DNase digestion kit. Reverse transcription was carried out on the RNA preparation to obtain cDNA. The cDNA was subjected to amplification for the gene of interest using the corresponding primers and Taq polymerase.

Western blot analysis

The cells were harvested on different days and were lysed in RIPA buffer. Protein estimation was done using the bicinchonic acid (BCA) method [21]. The lysate was subjected to SDS-PAGE followed by Western blotting to analyze the expression of various proteins of interest. The Western blots were developed using either alkaline phosphatase-conjugated secondary antibodies or by chemiluminescence with HRP-conjugated secondary antibodies.

Quantitation of the Western blots was carried out by densitometric analysis of the images using Quantity One software, version 4.6.3 from Bio-Rad.

Immunocytochemical analysis

Cells seeded on 12 mm diameter coverslips in 24-well plates were fixed with 4% paraformaldehyde after the respective treatments. The cells were washed three times with PBS for 10 min each. Permeabilization and blocking of non-specific binding were carried out by treating the cells with 3% BSA and 0.2% Triton X-100 in PBS. The cells were then incubated in the primary antibody diluted in the same buffer overnight at 4 °C. Subsequently, the cells were washed three times with PBS for 10 min each. The secondary antibody conjugate was then added in the same buffer and incubated for 1 h at room temperature. The cells were washed 3 times with PBS, for 10 min each. Coverslips were mounted using Fluoromount G onto glass slides. DPX mountant was used to seal the coverslips. Images were captured using either an epifluorescence microscope (Leica DMI 4000B inverted microscope with Leica DFC 350 FX model CCD camera and Leica Application Suite software, version 2.8.1)

or a confocal fluorescence microscope (Leica Confocal Microscope, TCS SP2 AOBS, with LCS lite imaging software or Nikon Eclipse Ti A1R microscope with NIS Elements software, version 4.0).

Effect of KCl on primary CG cells

The CG cells were grown in reconstituted NBM containing different concentrations of KCl, specifically 10 mM, 15 mM, and 25 mM (with 5 mM KCl present in NBM and an additional 5, 10, and 20 mM of KCl, respectively), starting from DIV 0. These cells were monitored regularly to ensure they remained viable and healthy at the different KCl concentrations. The cells were either harvested for Western blot analysis or fixed with 4% paraformaldehyde for immunocytochemistry on different days to study the expression of *αCaMKII*.

Results

Immunohistochemical analysis of the rat cerebellum to study the expression of *αCaMKII*

The cerebellum obtained from P7 rat pups was analyzed for the presence of *αCaMKII* in vivo through immunohistochemical analysis. The expression of *αCaMKII* was confined only to the Purkinje layer and was not present in either the external granular layer or the internal granular layer (Figure 1), confirming the previous findings [9] that *αCaMKII* is not expressed in the cerebellar granular layer and is restricted to the Purkinje layer.

Characterization of primary CG neuron preparation

The primary CG neurons prepared from P7 pups were characterised by immunostaining for the presence of various markers. Most of the cells were positive for the neuronal marker microtubule-associated protein (MAP-2), whereas a few cells were stained for glial fibrillary acidic protein (GFAP), showing the presence of astrocytes. The cells were positive for *α6GABA*, the marker for CG cells [22], confirming that most of the cells in the preparation consisted solely of CG cells (Figure 2). The cells were also immunostained for another well-known marker for CG cells, namely Pax6 [23].

RT-PCR analyses for the transcripts of *GluN2B*, *αCaMKII*, *βCaMKII*, *α6GABA*, and *β-actin* were conducted in primary CG cells that were harvested on DIV 2, 4, 6, 8, 10, 12, 15, 19, and 24. Expression of these genes was detected on almost all the days investigated. The marker gene *α6GABA* showed a reduced expression

on DIV 0 and 2 but gradually increased till DIV 10 to 12 as the culture matured. *βCaMKII* was present on all the days, showing a more or less uniform expression pattern. The receptor GluN2B was detected on all the days studied but exhibited reduced expression at early and much later time points, specifically DIV 0, 2, and DIV 19, 24, respectively. This is consistent with earlier studies indicating that in CG cells, the GluN2B subunit is completely downregulated and replaced by GluN2A and GluN2C by DIV 21 [24, 25]. Surprisingly, *αCaMKII* was also found to be present at low levels on all the days, with the expression further decreasing at later time points, similar to the pattern observed for *GluN2B* (Figure 3).

The primary CG cells were subjected to cell lysis, followed by Western blotting on DIV 2, 4, 6, 8, 10, 12, 15, 19, and 24 (Figure 4A). *βCaMKII* showed a gradual increase in expression starting from DIV 2 to DIV 24. *GluN2B* was present on all the days analyzed, but its expression decreased towards DIV 19 and DIV 24. Consistent with the transcriptomic data, the presence of *αCaMKII* was also detected on all the days studied, except for DIV 0, where there was no detectable expression (Figure 4B).

KCl induces the expression of *αCaMKII* in CG cells

The expression of *αCaMKII* in primary CG cells was contrary to expectations. The colocalization of *αCaMKII* with *α6GABA* shows that the expression of *αCaMKII* is indeed present in CG cells (Figure 5).

We investigated the factor that could be responsible for inducing *αCaMKII* expression in primary CG cells. One of the prominent components added to the culture medium is KCl; hence, we tested whether KCl is responsible for inducing *αCaMKII* expression. The primary CG cells were grown in different concentrations of KCl to study its effect on the expression of *αCaMKII*. It was found that the cells grown in 25 mM KCl exhibited *αCaMKII* expression, as detected by Western blotting (Figure 6) and immunocytochemistry (Figure 7). There was no detectable expression or very faint expression of *αCaMKII* in cells grown in 10 mM KCl and 15 mM KCl on DIV 4 (Figures 6 and 7), and on DIV 8 (Figures 6 and 7). However, the effect was pronounced when cells were treated with 25 mM KCl on both DIV 4 and 8, with increased expression observed on DIV 8. This indicates that KCl is responsible for inducing the expression of *αCaMKII* in cultured CG cells.

We also tested the effect of KCl by altering its concentration in the medium. When cells grown in 10 mM KCl

for 2 days were switched to a medium containing 25 mM KCl, there was a clear increase in *αCaMKII* expression compared to cells maintained in 10 mM KCl, further demonstrating that KCl induces *αCaMKII* expression in a concentration-dependent manner.

Discussion

αCaMKII is an essential molecule involved in calcium signal transduction in excitatory synapses involved in learning and memory. However, CG cells, which are primarily excitatory, are found to be devoid of *αCaMKII* in vivo [9]. The promoter for *αCaMKII* in the cerebellum is weak, resulting in negligible transcription [6]. Although some of the functions supported by *αCaMKII* could be carried out by *βCaMKII*, which is expressed in CG cells, it is still intriguing how CG cells compensate for the absence of *αCaMKII*, given that there are key differences in the biochemical properties between *βCaMKII* and *αCaMKII*, particularly regarding affinity for calmodulin, actin binding, etc. [13]. To understand the molecular mechanisms associated with calcium signaling in CG cells, we established a primary culture of CG cells using standard procedures adapted from previous reports [26, 27]. The preparation mostly consisted of CG cells, as they were immunostained positively for *α6GABA* and *Pax6* [22]. In the cultured CG cells, the expression of the *GluN2B* subunit (the receptor to which *CaMKII* is known to bind) was consistent with in vivo studies [24, 25].

Surprisingly, we found that the primary culture of CG cells showed the presence of *αCaMKII*, as demonstrated by mRNA expression analysis, Western blotting, and immunocytochemical analysis. Although mRNA of *αCaMKII* was present in CG cells at very low levels on DIV 0 (Figure 3), Western blot analysis showed that *αCaMKII* protein level could not always be detected (Figures 4B and 6), which is consistent with data from immunohistochemical analysis of rat cerebellum (Figure 1). This suggests that the expression is induced by some factor, which could possibly be a component of the medium used to grow and maintain the cells. Previous research shows that KCl, a major component of the medium used for the maintenance of CG cells, can induce the expression of certain genes in CG cells [20]. According to this study, various genes are upregulated and downregulated due to the chronic depolarization occurring in CGs maintained in 25 mM KCl compared to those in 5 mM KCl, as shown by gene expression analysis. Some of the upregulated mRNAs include those for channels, like *Itpr1*, neurotensin receptor 2, voltage-gated K⁺ channels, ligands, like BDNF, and enzymes, like

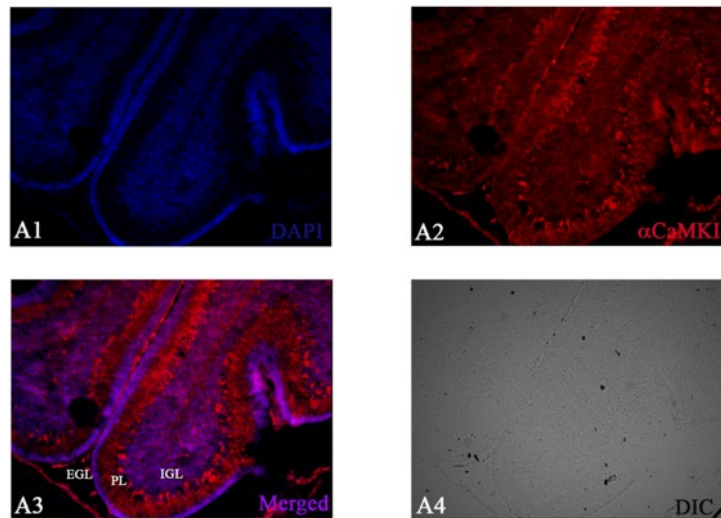


Figure 1. Immunohistochemical analysis of P7 rat cerebellum for the expression of *αCaMKII*

A1) DAPI staining, A2) Staining with anti- α CaMKII mouse primary antibody and Cy3-conjugated anti-mouse secondary antibody, A3) Merged view of A1 and A2 images, A4) Phase contrast image of cerebellar lobes.

Abbreviations: EGL: External granular layer; IGL: Internal granular layer; PL: Purkinje layer.

Cyt c oxidase. It is also known that different durations of KCl-induced depolarization lead to the activation of different signaling pathways based on the concentration of Ca^{2+} entry, resulting in differential transcriptional activity and gene expression [28]. Our study also showed a similar effect, namely the induction and expression of *αCaMKII*, which is not typically expressed in vivo.

In our experiments, *αCaMKII* is expressed when the cells are maintained in elevated concentrations of KCl, specifically 25 mM. Lower concentrations of KCl, such as 10 mM and 15 mM, did not induce significant expression of *αCaMKII*. However, these conditions do not support long-term survival of CG cells [17-19]. Depolarization induces the opening of VGCCs, facilitating the entry of calcium, which promotes cell survival [17]. Although depolarization induced by KCl and subsequent Ca^{2+} influx is mediated through different channels, such as NMDAR and AMPAR, certain studies have clearly shown that the major mediators of calcium influx are L-type VGCCs [29, 30]. This influx also induces specific activity-dependent changes in neurons in response to downstream signaling of Ca^{2+} influx. It is also possible that in our study, KCl could mediate membrane depolarization, which subsequently activated VGCCs, resulting in the induction of *αCaMKII*.

The study suggests that the mechanisms by which *αCaMKII* expression is prevented in CG cells in vivo are likely reversed when CG cells are in culture due to the depolarization caused by KCl. This observation is supported by studies showing that retinoic acid stimulates *αCaMKII* gene expression in PC12 cells, which have a distinct transcription initiation site and are similar to that present in cerebellar tissue. This is different from the *αCaMKII* transcription initiation site in the hippocampus and accounts for the difference in the level of expression of *αCaMKII* between these tissues [6]. It is possible that the KCl-induced expression of *αCaMKII* in CG cells depends on this difference in the transcription apparatus. Understanding the mechanisms of *αCaMKII* induction in the CG primary culture system may help elucidate the mechanisms by which expression is suppressed in vivo. Recent studies have investigated the action of scorpion toxin Bmk NT1, which induces neurotoxicity via PKC/CaMKII-dependent ERK1/2 signaling in primary CG cells [31]. Although these studies have not addressed whether the pathway is mediated specifically by *αCaMKII* or *βCaMKII* signaling, it would be interesting to determine which of the kinases are involved.

Conclusion

The study shows the expression of *αCaMKII* in the CG primary culture cells, although the same is not present

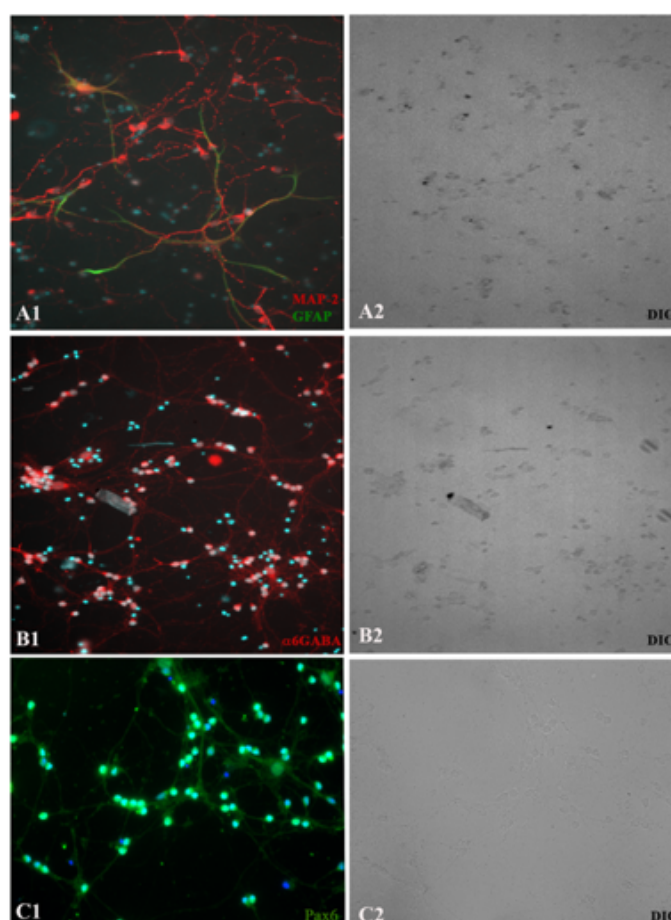


Figure 2. Characterization of primary CG neurons using markers

A1) MAP2 in red + GFAP in green, A2) DIC of A1, B1) $\alpha 6$ GABA, B2) DIC of B1, C1) Pax6, and C2) DIC

Note: The nuclear staining in blue in all the images is DAPI.

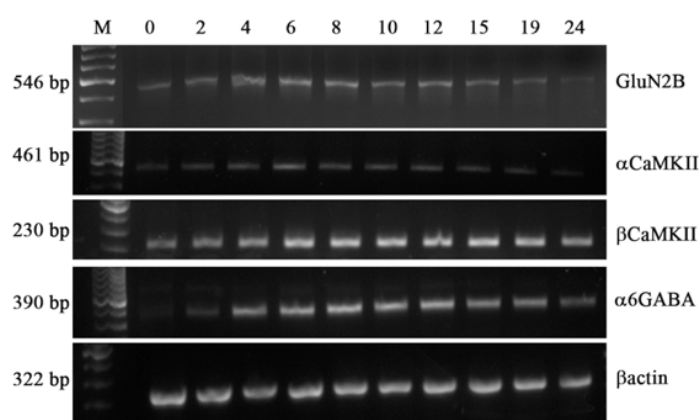
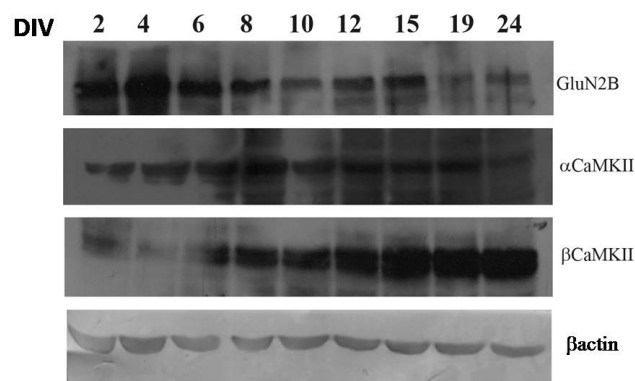
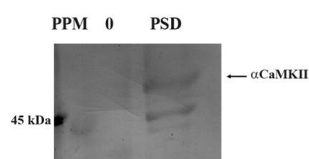


Figure 3. Analysis of the expression of different genes in primary CG cells by reverse transcriptase PCR

Note: Different panels show the expression of *GluN2B* (546 bp), *αCaMKII* (461 bp), *βCaMKII* (230 bp), *α6GABA* (390 bp), and *β-actin* (322 bp), respectively. Lanes marked M show a 100 bp DNA ladder as a molecular size marker. Lanes marked 0 to 24 indicate the various DIV on which the samples were selected for analysis. *β-actin* serves as the loading control.



4A

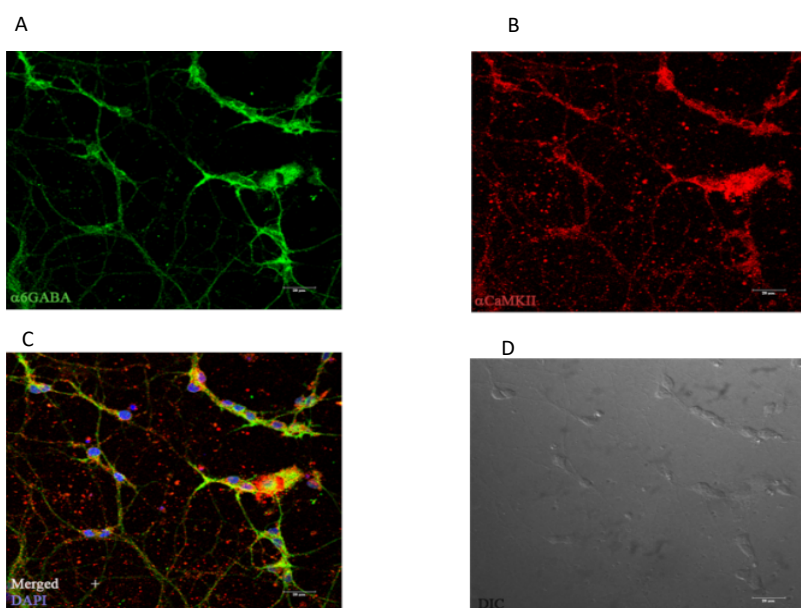


4B

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Figure 4. Analysis of expression of various proteins in primary CG cells by Western blotting – a temporal study

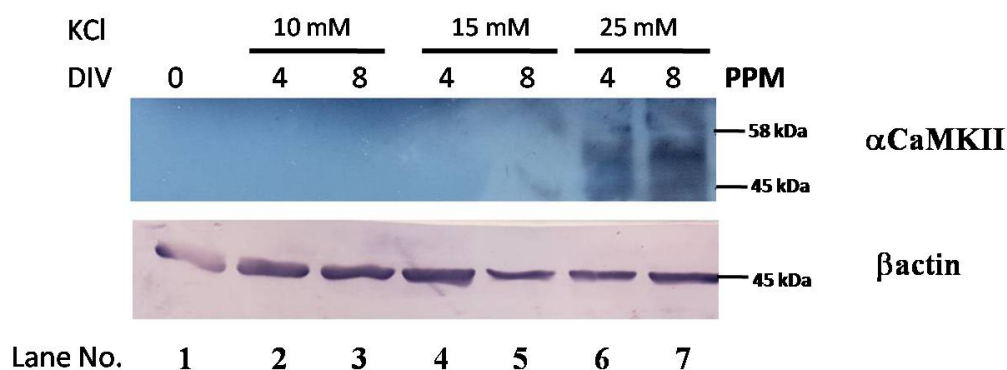
Note: 4A shows the expression of GluN2B (180 kDa), α CaMKII (50 kDa), β CaMKII (60 kDa), and β -actin (45 kDa). Lanes marked as 2 to 24 show the various DIV on which the samples were analyzed for the particular protein. Each lane contained 100 μ g of protein, and β -actin served as the loading control. 4B shows α CaMKII blot. PPM indicates marker lane, 0 indicates the CG cell preparation on DIV 0, i.e. before seeding, and PSD indicates the lane with the rat forebrain brain postsynaptic density sample used as a positive control for α CaMKII expression. The upper band shows the α CaMKII band. The lanes contained 100 μ g of protein from the cell lysate and 20 μ g of protein from the PSD.



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Figure 5. Immunocytochemical analysis of primary CG cells grown for 8 DIV in 25 mM KCl to study the expression of α CaMKII

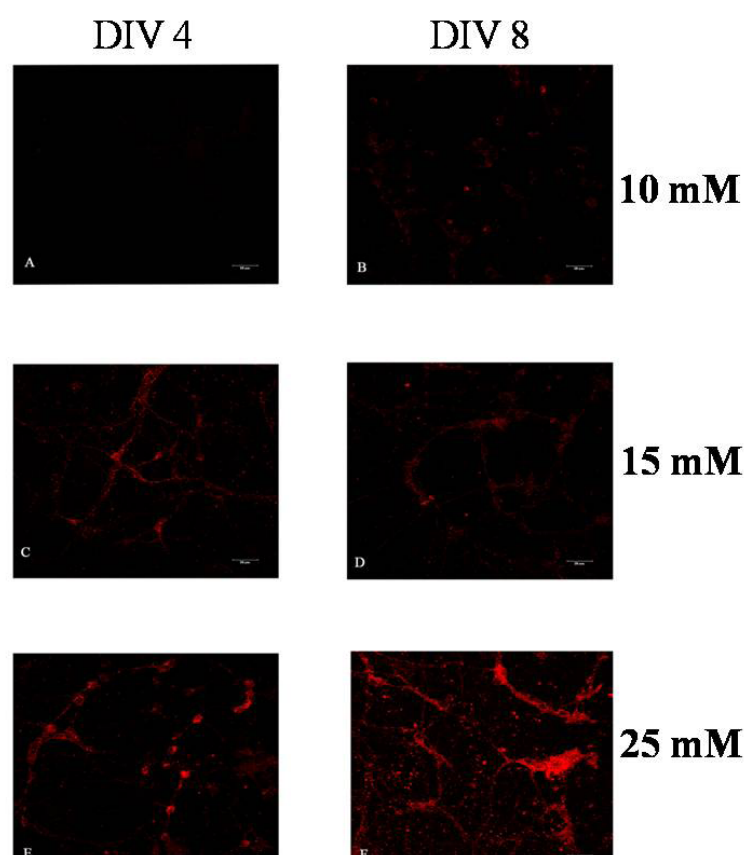
Note: A shows staining for the CG cell marker, α 6GABA, B shows staining for α CaMKII, C shows an overlay of A and B along with DAPI staining for nuclei, demonstrating the localization of α CaMKII and α 6GABA in the same neurons, and D shows the DIC image of the same.



CJNS

Figure 6. Western blot analysis of the expression of *αCaMKII* in CG cells grown in different concentrations of KCl

Note: The upper panel shows the blots of *αCaMKII*, whereas the lower panel shows the blots of *β-actin*, which serves as the loading control. The lanes represent: 1) DIV 0, 2) DIV 4, 10 mM KCl, 3) DIV 8, 10 mM KCl, 4) DIV 4, 15 mM KCl, 5) DIV 8, 15 mM KCl, 6) DIV 4, 25 mM KCl, and 7) DIV 8, 25 mM KCl. Each lane contained 50 μ g of protein.



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Figure 7. Immunocytochemical analysis of primary CG cells grown in various concentrations of KCl to study the expression of *αCaMKII*

Note: Staining was performed with Cy3-conjugated anti-mouse secondary antibody. The images in the left panel show *αCaMKII* expression on DIV 4, while those in the right panel show expression on DIV 8. The upper, middle, and lower panels display samples treated with 10 mM, 15 mM, and 25 mM of KCl, respectively.

in vivo in the animal. The induction and expression of α CaMKII are attributed to the higher concentrations of KCl used to maintain the cells in vitro. Previous studies have shown that KCl can lead to aberrant expression of genes in primary cells cultured in vitro. This study reports for the first time the concentration-dependent induction of α CaMKII expression in CG primary cells, despite its absence in vivo. Although primary cultures of CG cells have been used as an in vitro model, culture conditions that induce alterations in the expression of key proteins, such as α CaMKII, could lead to significant changes in the signaling mechanisms within these cells. Therefore, primary cultures of CG cells could serve as a model to delineate the mechanisms involved in inducing the expression of α CaMKII, which would help in understanding the tight regulation that suppresses the expression of this enzyme in vivo. While this emphasizes the need for caution in extrapolating data from CG cell cultures to in vivo conditions, such alterations may also reveal mechanisms that are otherwise difficult to detect using in vivo systems.

Ethical Considerations

Compliance with ethical guidelines

All study procedures were done in compliance with the ethical guidelines of the Declaration of Helsinki 2013. This study was approved by the [Rajiv Gandhi Centre for Biotechnology \(RGCB\)](#), Thiruvananthapuram, India, (Code: IAEC/100/OMK/2010).

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Conflict of interest

The author declared no conflict of interest.

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