GLUCOCORTICOID MEDIATED SPECIFIC REGULATION OF CALCIUM CHANNEL: AN IN SILICO STUDY

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Abstract

The role played by steroid hormones in managing stress and homeostasis is extremely important from physiological perspective. Glucocorticoid, one of the main steroid hormones bears an important position in this respect. Glucocorticoid confers its activity by precise and specific regulation of certain genes. It adopts a mechanism where it binds to its receptor in the cytosol and translocates to the nucleus and hereby activates or repress particular genes. The voltage gated calcium channels (VGCC) play important roles in neuronal firing. Between the L type and N type VGCC, Glucocorticoid specifically enhance the L type. This is important in regulating the neuronal excitability as enhanced L type current leads the cell to more hyperpolarized state and thus reducing excitability. Here we have studied the factors governing the glucocorticoid mediated control of VGCC. We found that the locus of the glucocorticoid receptor response elements (GRE) and negative GRE (nGRE), nucleosome positioning, the binding affinity of Glucocorticoid receptor (GR) to GRE and nGRE are the factors that lead to specific enhancement of L type over N type VGCC mediated by GR.

Keywords: Glucocorticoid Receptor, Afterhyperpolarization, VGCC, Glucocorticoid Response Elements, Steroid Hormone

I. INTRODUCTION

The myriads different and important physiological roles played by steroid hormones have been an active part of research. Among the steroid hormones Glucocorticoid has been reported to be involved in various processes like metabolic, immune response, cognition, development etc [1-5]. Glucocorticoids are produced by the adrenal cortex and secreted from it when the HPA (hipothalamus-pituitary-adrenal) axis is activated in stress response [6]. After secretion, Glucocorticoids can easily diffuse through the cell membrane and thus its cytosolic presence can easily be discerned. The cytosol has an abundance of Glucocorticoid receptor (GR)s, which are bound by the influxed Glucocorticoids. The GR belongs to nuclear receptor family of transcription factor [7]. Once bound by Glucocorticoids, the GR undergoes some conformational changes that can help it migrate to the nucleus and take part in specific gene regulations. Actually the GR in the cytosol resides as a part of a large multi protein complex. This GR associated protein complex includes hsp90, hsp70 and P23 and immunophilins belonging to the FK 506 family [8, 9, 10]. Until bound by Glucocorticoids the GR remains in a conformation that is transcriptionally inactive and unable to translocate to the nucleus. But as soon as the Glucocorticoid binds to it, the GR undergoes a conformational change and gets dissociated from the chaperone proteins [8]. This lead to the ability of the GR to translocate to the nucleus. The GR has a DNA binding domain(DBD), a ligand binding domain(LBD), a hinge region, two activation functions(AF1 and AF2), two nuclear localization signals,NL1 and NL2. The NL1 and NL2 are responsible for its translocation to the nucleus [11]. Once inside the nucleus the GR can bind to the specific genes to be regulated via its DBD. GR binds to specific genes in response to the presence of consensus sequences called the Glucocorticoid
receptor response elements (GRE) [12, 13]. Moreover, GR has another consensus sequence called negative GRE in some genes. GR can activate or repress specific genes with respect to its binding to GRE and nGRE [14].

In the large profile GR regulated genes, voltage gated calcium channel (VGCC) is of special interest as far as the neuronal excitability is concerned. The VGCC, which remain closed at resting membrane potential get opened up at a threshold membrane voltage as the neuron is excited. This happens at about the peak of an Action Potential (AP) [15]. The VGCC mediated calcium influx contribute in achieving the hyperpolarized stage of the membrane potential mainly through the calcium dependent potassium channels like BK and SK channels. More the calcium influx through the VGCC, more time the cell remain at a hyperpolarized stage and large the amplitude of Afterhyperpolarization (AHP)[16]. The AHP is a significant indicator of intrinsic neuronal excitability. In the process of hippocampal learning tasks the AHP gets lowered and thus the enhanced intrinsic excitability [17]. But the VGCC mediated calcium influx to a greater extent reduces the excitability and can interfere in the process of learning as well. On the other side, this can also be a homeostatic mechanism in maintaining cellular excitability. In their seminal work Chameau et al [18] has reported that out of two VGCC namely the L type and N type, Glucocorticoid specifically enhance L type VGCC over N type. Studies [19, 20] have reported that increase in L type VGCC can lead to more hyperpolarized stage of hippocampal neurons. Chameau et al has shown that the alpha subunit of N type alpha subunit (CaV2.2) of VGCC is down regulated while L type alpha subunit 1C(CaV1.2) remains unaffected and  L type alpha subunit 1D(CaV1.3) is up regulated in response to increased Glucocorticoid. They have also reported that the β4 (beta 4) subunit is drastically up regulated in response to increased Glucocorticoid level.

Here, in this study we have looked for various factors that lead to the specific enhancement of L type VGCC over N type. We have adopted an insilico approach in this study. Firstly, we analysed the promoter region of the N type alpha subunit (CaV2.2), L type alpha subunit 1C (CaV1.2) and L type alpha subunit 1D (CaV1.3). As it has been reported that GRE and nGRE present at an upstream promoter region is an important factor for the binding of GR to the DNA [8], we looked for the location of GRE and nGRE with respect to the promoters. We also analysed the nucleosome positioning in the sequences of CaV2.2, CaV1.2, and CaV1.3. Nucleosome positioning can significantly affect transcription factor binding, recruitment and assembly of various proteins needed for transcription initiation. Oppositely nucleosome positioning can affect gene repression as well. Perlmann et al showed that the GR binds to the GRE when it is exposed to the outside of the major groove and not bound into nucleosome [21]. Then we have done docking analysis of GR binding to the GRE and nGRE of the respective genes to look for binding affinity through energy minimization process. We have also done the docking of β 4 subunit SH3 and GK domain with the AID of L type and N type Alpha subunits. We have not got any significant differences in the binding affinities among them. This has been done keeping in view the reports in many studies which say that the Beta subunit interact via SH3 & GK domain with the AID of alpha subunit which foment the migration of the channel towards plasma membrane [22]. But the exact mechanism of alpha and beta subunit interaction and channel migration remains an open question till date [23].

II. METHODS

2.1. Sequence retrieval

In our study we have used the following sequences retrieved from NCBI Gene Bank database [24]:

CaV1.2 Accession No. NG_008801.2

CaV1.3 Accession No. NG_032999.1

CaV2.2 Accession No. NG_042271.1 and

Beta-4 Subunit Accession No. NG_012641.1
2.2. Analysing promoter region

We performed promoter prediction analysis using Neural Network Promoter Prediction (NNP) (Berkeley Drosophila Genome Project) [25]. Results obtained are shown below in result section.

2.3. Response Element Position Analysis

Using Bioedit [26] software package we performed a manual search for the position of Negative Glucocorticoid Response Element (nGRE) in the respective sequences mentioned above for all the nGRE combinations as in the Table 1. For Glucocorticoid Response Element (GRE) we have used NUBIScan server [27].

Table 1: (Showing Possible nGRE Combinations)

| Sl. No. | nGRE Combinations       | Sl. No. | nGRE Combinations       |
|---------|-------------------------|---------|-------------------------|
| 1       | CTCCGGAGA                | 10      | CTCCCTAGGAGA            |
| 2       | CTCCAGGAGA               | 11      | CTCCCAAGGAGA            |
| 3       | CTCCCTGGAGA              | 12      | CTCCCGAGGAGA            |
| 4       | CTCCGGAGGA               | 13      | CTCCCTCGGAGA            |
| 5       | CTCCCGGAGA               | 14      | CTCCCCGGGAGA            |
| 6       | CTCCAAGGAGA              | 15      | CTCCCTGGGAGA            |
| 7       | CTCCATGGGAGA             | 16      | CTCCGTTGGGAGA           |
| 8       | CTCCACGGAGA              | 17      | CTCCCGGGGAGA            |
| 9       | CTCCAGGGGAGA             | 18      | CTCCCGGGGAGA            |

2.4. Nucleosome Position Prediction

To determine the nucleosome position we have used NuMap server [28]. All of the sequences are subjected to prediction. Results obtained are included in result section.

2.5. Protein and DNA Modelling & Molecular Docking

For molecular docking study we developed a model for DNA Binding Domain (DBD) of the GR with the help of I-TASSER [29-31] protein Modelling Server. We opted for the protein modelling due to unavailability of complete structure in PDB database. For DNA modelling we have used Abalone software package.

We performed molecular docking study with GR DBD & CaV1.2 GRE region, GR DBD & CaV1.3 GRE region and GR DBD & CaV2.2 GRE region and Beta-4 GRE & GR DBD region. For all the molecular docking we have used ClusPro Server ver2.0 [32-36] with default parameter. Visualization was done using PyMol [37].

III. RESULTS

3.1. Promoter Analysis

3.1.1. For CaV1.2 (L-Type)

NNP gives a putative promoter at position 87114 – 87164 with confidence score of 0.88 for CaV1.2.

3.1.2. For CaV1.3 (L-Type)

NNP gives a putative promoter at position 4724-4774 with confidence score of 0.92 for CaV1.3

3.1.3. For CaV2.2 (N-Type)

Promoter prediction using NNP predicts different sites for possible promoter site however we chose position 4954-5004 with score 0.97 for CaV2.2.

3.1.4. For β4

For promoter analysis of beta-4 subunit gene we employed the whole nucleotide sequence to NNP which gives presence of promoter at position 4891-4941 with score 0.98.
3.2. Response Element Analysis

3.2.1. Negative Glucocorticoid Response Element (nGRE)

In our analysis we didn’t find presence of any negative Glucocorticoid Response Element at crucial site in L-Type calcium channel (both CaV1.2 & CaV1.3). There was total 18 possible combination of nGRE most of them lay outside Promoter region. However, nGRE presence was found in a crucial position N-Type calcium channel (CaV2.2) at position 6163.

3.2.2. For β 4

We searched presence of nGRE in beta-4 subunit gene sequence but couldn’t find presence of any.

3.2.3. Glucocorticoid Response Element (GRE)

3.2.3.1. CaV1.2 Sequence analysis using NUBIScan server shows presence of probable GRE TGGACAggaTGTCCC at the position 67411.

3.2.3.2. CaV1.3 NUBIScan shows presence of AGCACAgaTGTTCA at 265.

3.2.3.3. CaV2.2 The ACCACAatcTGTGCG motif found in CaV2.2 gene sequence at position 2776 near transcription start site.

3.2.3.4. β4 In case of beta-4 subunit we submit the sequence to NUBIScan server shows presence of GRE at position 4372.

3.3. Nucleosome Position Prediction

3.3.1. CaV1.2

Nucleosome position prediction using NuMap server shows presence of low nucleosome occupancy at position 67,411 with score 26 (Figure 1A)

3.3.2. CaV1.3

In CaV1.3 nucleosome prediction showed score 22 at nucleotide position 265 (Figure 1B)

3.3.3. CaV2.2

As we searched for nucleosome occupancy around negative GRE site (at position 6163) downstream of promoter we found score 42.0 (Figure 1C)

3.3.4. β4

NuMap server predicts nucleosome occupancy score around position 3878 is 32.55 which is relatively low as compared to overall nucleosome occupancy of the DNA sequence (Figure 1D)

![Fig. 1 Nucleosome prediction graph, (A) Nucleosome Position Prediction for CaV1.2, (B) Nucleosome Position Prediction for CaV1.3, (C) Nucleosome Position Prediction for CaV2.2, (D) Nucleosome Position Prediction for β4](image-url)
3.4. Docking Analysis

3.4.1. L type

*CaV1.2 Promoter DNA+ GR DBD:*

The Cluspro score is -1456. The docking pattern is as depicted in figure 1(A) in Appendix 1.

*CaV1.3 Promoter DNA+ GR DBD:*

The Cluspro Score is -1459. The docking pattern is as depicted in figure 1(B) in Appendix 1.

3.4.2. N-TYPE

*CaV2.2 Promoter DNA+ GR DBD:*

The Cluspro Score is -1459. The docking pattern is as depicted in figure 1(C) in Appendix 1.

3.4.3. β4

β4 GRE DNA + GR DBD

The Cluspro Score is -1412.9. The docking pattern is as depicted in figure 1(D) in Appendix 1.

The docking analysis of Beta 4 subunit SH3 and GK domain with the AID of L type and N type Alpha subunits did not show significant variation of binding pattern and binding energy (Result not shown).

All the results are summarised in table 2.

| Subunit | Promoter region (BDGP Promoter Prediction) | GRE position (NUBIScan) | nGRE position | Nucleosome Position (NuMap) | GR DBD and DNA binding energy (ClusPro 2.0) |
|---------|---------------------------------------------|-------------------------|----------------|----------------------------|---------------------------------------------|
| CaV1.2  | Region: 87114 - 87164 Score: 0.88          | Position: 67411          | -              | at 67411 Score: 26          | Lowest Energy: -1456.1                     |
|         | Region: 4724-4774 Score: 0.92              | Position: 265            | -              | at 265 Score: 22            | Lowest Energy: -1459.3                     |
|         | Region: 4954-5004 Score: 0.97             | Position: 2776           | 6163           | at 6163 Score: 42           | Lowest Energy: -1391.8                     |
| β4      | Region: 4891-4941 Score: 0.98             | Position: 4372           | Not Found      | at 4372 Score: 24           | Lowest Energy: -1412.9                     |

Table 2: Results Summary

IV. DISCUSSION

In this study we tried to decipher the differential regulation pattern of L type and N type VGCC by Glucocorticoid as reported by Chameau et al [18]. This kind of regulation of VGCC bears much importance in many physiological processes especially in neuronal excitability as an increase in the L type VGCC will enhance AHP which keeps the cell in more hyperpolarized state and thus decrease excitability [19, 20]. This aspect is also very important in memory formation [17]. We have looked for the various possible factors that can lead to this variable regulation of VGCC by glucocorticoid. Glucocorticoid mediates its regulation through GR in the genetic level. Firstly, we
have analysed the promoter region in all the sequences retrieved from NCBI with the help of insilico promoter prediction tool, then the analysis of the putative positions of GRE and nGRE in the sequences of L type, N type alpha subunits and the β4 subunit and the Nucleosome positioning near the GRE in the gene sequence.

4.1. Non significant change in transcription of CaV 1.2 & CaV 1.3 by Glucocorticoid

Promoter for CaV 1.2 positioned in 87114 to 87164 is well in compliance with the NCBI reported transcription start site (TSS) of 87778. So the promoter position just 351 bp upstream is a well characterised result. The GRE positioned at 67411 (TGGACAggaTGTCCC) is the only putative one found upstream of the promoter. This is almost 20 kbps upstream of the TSS. The Nucleosome position score near the GRE position is 26 which is comparatively low. This suggests that probability of the GRE region being outside nucleosome is high and GR can bind there at ease. Despite the availability of the GRE the CaV 1.2 shows no significant regulation by Glucocorticoid[18]. This can be pertained to the far off GRE from the promoter. Although studies have showed that GRE can be positioned several sequences upstream of the promoter [8], 20kbps upstream is too far a position for conferring regulatory effect. Moreover, the GR binding and unbinding is a dynamic process where GR shifts between bound and un-bound state to its response elements [38]. So, binding of GR at a very distant position from the TSS although with great ease can fail to confer its regulatory activity due to its grand distance from the promoter. Although there might exist other reasons for the absence of regulation. Lucas et al [39] showed that for an effective regulation by GR, along with GRE there should present sites for other transcription factor binding in the vicinity of the GRE which forms hormone response domains.

In the case of CaV 1.3 the promoter is predicted at 4724 - 4774 and the TSS lies at 5119. So the predicted promoter lies 395 bp upstream. The GRE is positioned at 265 (AGCACAgcaTGTTCA) is the putative one. It is 4429 bp upstream from the promoter. The nucleosome positioning score is 22 which is low here too. Despite these, the almost insignificant regulation of the CaV 1.3 can be attributed to the factors described by Lucas et al [39] as mentioned above.

4.2. Significant upregulation of β4 subunit by Glucocorticoid

The promoter for β4 subunit positioned at 4891 – 4941 which is situated well at 178 bps upstream from the TSS of 5069. The GRE (AGGACAtacTGGTCT) is positioned at 4372 which is just 519 bps upstream of the promoter. Again the Nucleosome score in the region is 24 which is also very low and hence the chance of the GRE exposure to be bound by Glucocorticoids increases. So the proximity of the GRE to the promoter, the reduced occupancy of nucleosome in the GRE region and a well positioned promoter will enhance the regulation of the β4 subunit by Glucocorticoids.

4.3. Downregulation of CaV 2.2 by Glucocorticoids

It has been reported that the genes repressed by Glucocorticoid has a consensus nGRE in its sequence [14]. The presence of nGRE is very crucial for the repression of a gene by Glucocorticoid. Since, Chameau et al [18] reported the down regulation of the CaV 2.2 in response to Glucocorticoid we looked for the presence of nGRE along with GRE in its sequence.

The promoter for CaV 2.2 is positioned at 4954 which is very well situated at 192 bps upstream from the TSS of 5146. The GRE (ACCACAatcTGTGCC) upstream of the promoter has been found to be positioned at 2776, just 2178 bps upstream of the promoter, which is quite good a position for conferring the regulation by GR when it binds here. The nGRE(CTCCGGAGA) was found at 6163 which is 1017 bps downstream the promoter. Out of total 27 combination possible nGREs as mentioned in the method section, CTCCGGAGA has been the only nGRE found to be present in the entire sequence of CaV 2.2. Now the question arises that if the nGRE is present at a position downstream of the promoter how the GR binding here can confer its repressing regulation. Studies show that in many cases the gene regulatory elements can be positioned at locations downstream of the promoter which is nicely reviewed by Matson et al [40]. Keeping in view of all these reports we here propose that although the transcription starts in absence of any nGRE near promoter, when the RNA polymerase in the process of transcribing the gene encounters the nGRE
and GR bound to it gets disassembled from the DNA and thus transcription stops. Hudson et al [41] reported that the nGRE opposes the homodimerization of the GR upon binding. This is due to the orientation of the D box present in the GR. In this process the GR binds as monomers to the nGRE in different positions. Also it has been shown that binding of GR to nGRE as monomers help recruiting proteins and other factors that disassemble the RNA polymerase from the DNA stopping transcription. Likewise in this case of CaV2.2 the GR binds as monomers to the nGRE and recruit those factors that can disassemble RNA polymerase and the transcription gets stopped. Thus the down regulation of CaV 2.2 occurs.

As mentioned earlier, we have not got any significant differences in the binding affinities β4 subunit SH3 and GK domain with the AID of L type and N type Alpha subunits(results not shown). This has been done keeping in view the reports in many studies which say that the Beta subunit interact via SH3 & GK domain with the AID of alpha subunit which foment the migration of the channel towards plasma membrane[22, 23].

So, the increased current by L type VGCC over N type VGCC observed by Chameau et al [18] is for the differential regulation of L type and N type subunits and β4 subunit by GR. The gene of N type subunit has been downregulated by GR while that of the L type subunits (CaV 1.2 & CaV 1.3) remain almost unchanged. This in time will reduce the occupancy of N type VGCC in the cell. At the same time the gene of β4 subunit has been upregulated and as a result its cellular occupancy will be increased. So the increased β4 subunit will interact with L type subunits more than that of N type subunits and thus will migrate the L type subunits to the membrane more than the N type. This results in the increased current through L type VGCC over the N type.

V. CONCLUSION

The differential regulation pattern of the genes corresponding to L type and N type VGCCs and that of β4 subunit by Glucocorticoids lead to the increased current by L type over N type VGCC.

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Figure 2 Molecular Docking Study (a) CaV1.2 GRE (Green) and GR DBD (in Red), (b) CaV1.3 GRE (Green) and GR DBD (Red), (c) CaV2.2 GRE (Green) and GR DBD (Red), (d) Beta-4 (in Green) and GR DBD (in Red).