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The molecular base of development to tolerance for morphine analgesia, expression of CREB & p-CREB protein in dorsal horn of spinal cordof rats: A New insight to explain the tolerance to opioid analgesia

Satya N. Shukla[1,*], SB Ray[2]

[1]Associate Professor, Index Medical College Hospital & Research Centre, Indore, Madhya Pradesh, [2]Professor, Dept. of Anatomy, AIIMS, New Delhi

***Corresponding Author:**
Email: drsatya2000@gmail.com

Abstract

Introduction: Protein synthesis in cells is regulated by transcriptional activator proteins like *c*AMP response element binding(CREB) protein, which binds to the DNA molecule near the start of the target gene sequence. This results in the activation of promoter region of DNA by RNA polymerase and the beginning of transcription. Phosphorylation of CREB(p-CREB) greatly increases the transcriptional process. In the present work, the expression of CREB and p-CREB were examined in the spinal cord after morphine tolerance. The underlying cellular basis of morphine tolerance is presently unknown. Presumably increased synthesis of CREB and its phosphorylation to p-CREB might be responsible for tolerance as these have been shown to mediate long lasting changes in brain function.

Aims & Objective:

1. To study the alteration in the expression of c-AMP response element-binding (CREB) protein and its phosphorylated form (p-CREB) after chronic morphine administration
2. Immunohistochemical localization of cyclic-AMP response element-binding(CREB) protein expression and it phosphorylated form p-CREBin dorsal horn of thecervical segmentof rat spinal cord
3. Quantitation of expression of CREB and p-CREB with the help of Image Pro Plus 6 Analysis System.

Materials and Method: Adult male Wistar rats were divided to in two groups (n=6/Group) and administered the following: Normal Saline (Group-I), & Morphine (Group-II), for 14 days. Development of Morphine tolerance was determined by the tail-flick test. Expression of CREB and p-CREB was observed by immunohistochemistry using specific antibodies to these proteins. Visualization was done by ABC method. The immuno histochemical staining was quantitatively expressed by measuring the Integrated optical density (IOD) of immunostaining with the help of Image Analysis System driven by Image Pro-plus 6.2 software.

Result and Conclusion: Morphine administration showed increased expression of CREB and p-CREB in dorsal horn of spinal cord. The result of this study demonstrates that CREB and p-CREB may have an important role in the development of morphine tolerance.

Keywords: CREB- *c*AMP response element binding (CREB),p- CREB- Phosphorylation of CREB (p-CREB), Immunohistochemical, IOD- Integrated optical density (IOD), Morphine Tolerance, Tai-Flick Test

Introduction

Pain is most common complain of patients, to force them to consult or make a visit to physicians, and it's the responsibility of physicians or consultants to relieve the pain because pain is the most undesired sensation. While we are treating the patients to provide the relief from pain, by using analgesic drugs like NSAIDs & Opioid analgesics. Most of the patients get relief from Non-opioid analgesics like aspirin, paracetamole but majority of patients those are suffering from pain due to malignancy, the need a opioid analgesic, because of severity of pain and failure to treat the cause behind origin of pain.

So for management of pain in cancer patients, WHO has developed a three-step "LADDER" for management of cancer related pain. Non-opioids, mild-opioids and strong-opioids are prescribed in sequential order till pain is relieved. However, high opioids doses lead to serious side effects like respiratory depression, hallucinations, myoclonus, constipation, somnolence and agitation (Guskin et al., 2001 and Fallon et al., 2005).[(1)]

Opioids like morphine produce side effects ranging from nausea and vomiting, pruritus, over sedation, dizziness and urinary retention to respiratory depression.[(2)] Particularly, on chronic administration, it leads to development of tolerance.[(3)] Combining opioids with certain other drugs (adjuvant analgesics) like ketamine, which is an N-methyl-D-aspartate (NMDA) receptor antagonist, not only increases the analgesia, but also reduces the dose of opioids.[(4)] Previous research done in our laboratory and outside suggests that nimodipine, an L-type calcium channel blocker (L-CCBs), could be one such adjuvant drug.[(5)]

This is not in synchrony with the evidences presented in the earlier paragraph, where related research has clearly shown that L-VGCC are present on the post synaptic neurons and affect the transcription of certain genes, based upon the activation of CREB protein. The present research work investigated the status of CREB), based on the hypothesis that the

chronic administration of morphine, leads to development of tolerance to morphine induced analgesia, is because of enhanced expression of CREB and p-CREB in Dorsal horn of spinal cord of experimental animals like in this study, rats. Now, why on dorsal horn of spinal cord, because whole gray matter of spinal cord is divided in to 10 laminae by Rexed, those are known as Rexed`s laminaes. This concept of laminae is suefull in experimental works only & provides information about the localization of terminal degenerating fibres after section of posterior nerve roots. Rexed`s laninae I, which is also well known as substantia gelatinosa is responsible for relay of the sensations like pain and temperature, & modification of transmission of sensory input. That's why in present work we emphasize over alerted expression of CREB & p-CREB in dorsal horn of spinal cord of control & morphine treated group. Tolerance is defined as decreased analgesic response to opioids after long term administration of drug to relieve the pain from patients. Short term used of drugs developed tolerance because of desensitization of opioids receptors, while long term administration of opoids, leads to development of tolerance- whereas sustained administration leads to the development of classical or chronic tolerance. Short-term receptor desensitization, which may underlie the development of tolerance, probably involves phosphorylation of the mu and delta receptors by PKC. Long-term tolerance may be associated with increases in adenylyl cyclase activity—a counter-regulation to the decreased cyclic AMP levels seen after acute opioid administration. Chronic treatment with mu-receptor opioids causes super activation of adenylyl cyclase. This effect is prevented by pretreatment with pertussis toxin, demonstrating involvement of Gi/Goproteins, and also by cotransfection with scavengers of Gprotein–βγdimers, indicating a role for this complex in super activation (Goodman & Gillman, 2008).[6]

Further, an early study had noted that chronic morphine administration produces an increase in CREB, particularly phosphorylated CREB (Li and Clark, 1999). However, there is no any study on the effect on CREB, particularly phosphorylated CREB, after morphine -administration. We had investigated the spinal cord only, inspite of systemic administration of morphine because a recent study has clearly demonstrated that spinal mu-opioid receptors mediate the analgesic action of systemic opioids, by inhibiting the ascending nociceptive pathway, rather than activating the descending pain inhibitory pathway from periaqueductal gray and rostoventral medulla (Chen et al., 2006).[7]

Aims and Objective

1. To study the alteration in the expression of c-AMP response element-binding (CREB) protein and its phosphorylated form (p-CREB) after chronic morphine administration
2. Immunohistochemical localization of cyclic-AMP response element-binding(CREB) protein expression and it phosphorylated form p-CREB in dorsal horn of the cervical segment of rat spinal cord
3. Quantitation of expression of CREB and p-CREB with the help of Image Analysis System driven by Image Pro-plus 6.2 software Analysis System

Materials and Method

Male Albino Wistar rats (n=12) were used in the present study. These rats were obtained from Experimental Animal Facility of AIIMS after prior approval of the project by Institutional Animal Ethics Committee. The animals were kept in cages, with no more than 3 animals in one cage. They were maintained at a 12 hours: 12 hours light/dark cycle with water and food available ad libitum. Rats were randomly divided in 2 groups of 6 rats/group for the present study: (i) Group I: Control group- treated with physiological saline; (ii) Group II: Morphine group-treated with morphine (10 mg/kg) subcutaneously twice daily for 14 days.

The study of morphine analgesia & development of tolerance to morphine induced analgesia, previously studied at our laboratory with the help of Tail-Flick test and data regarding from day 1 to day 14, both morning & evening were collected and statically analyzed and published in our own paper.(Shukla S. N. et al 2014).[8]

Group I- Control group- treated with physiological saline

Group II- Morphine group-treated with morphine (10mg/kg) subcutaneously twice daily for 14 days.

Drugs:

1. **Normal saline-** Normal saline is 0.9% NaCl (sodium chloride or salt), and it was purchased from market.
2. **Morphine-** Ampoules of morphine sulfate (15 mg/ml/ampoule) were obtained from a Government Licensed dealer after getting requisite permission from Office of The Commissioner Of Excise, L&N Block; Vikas Bhawan; New Delhi. It was procured in small batches because of restriction in its availability due to its abuse potential (Shukla et, al 20141).[1]

Experimental Design: The experimental work was divided into 2 parts

Part I- Immunohistochemical localization of cyclic-AMP response element- binding (CREB) protein expression and its phosphorylated form (p-CREB) in dorsal horn of the cervical segment of rat spinal cord

Part II- Quantitation of expression of CREB and p-CREB with the help of Image Analysis System.

Group I (n= 6): Saline Group: This was the control group, which was injected normal saline, subcutaneously two times a day at 12 hours interval for

14 days. The injection was given at the lateral aspect of thigh. The volume of normal saline was equivalent to the dose of morphine, in volume. The injections were given with help of sterile tuberculin syringe. Tail-flick response was taken after 40 minutes of injection.

Group II (n= 6): Morphine Group: The animals in this group were treated with morphine sulphate (10mg/kg of body weight) twice a day at 12 hours interval for 14 days**.** Injections of morphine were given with tuberculin syringe, subcutaneously over the lateral aspect of thigh. Successive injections were given in alternate limbs. Tail-flick response was taken after 40 minutes of injection. Decrease in values of tail-flick near the latter-half of the experiment indicated the development of tolerance to morphine.

Morphine analgesia was studied on Groups I and II, and assessment of analgesia was done by tail-flick test. The values of tail-flick latency were almost equal to baseline values for group I, throughout the experiment, while for group II, values of tail-flick latency were almost equal to the cut off time (9.15±1.762), at day 1, but gradually the values decreases over the time period of experiment and at the end of experiment, tail-flick values reaches to base line value. This pattern of gradual decreases of latency is interpreted as the development of tolerance to analgesic effect of morphine.(*Satya Shukla and Subrata Ray* 2015) [9

Part I

CREB and p-CREB protein in dorsal horn of the cervical segment of rat spinal cord: At the end of this period of 14 days, the rats were sacrificed on 15th day under deep anaesthesia by Pentobarbitol sodium (100mg/kg i.p.). Fixation was done by perfusion of 4% paraformaldehyde in 0.1 M phosphate buffered saline, through transcardiac perfusion. For transcardiac perfusion, anaesthetized rat was put in a tray, and thoracic cavity was opened at the level of diaphragm. After the opening of thoracic cavity, a cannula was inserted though apex of left ventricle and it was placed in the ascending aorta. The cannula was fixed in position with help of an artery forceps. The cannula was connected with perfusion pump, through which initially 100 ml of 0.1M phosphate buffered saline was infused to washout the blood. This was followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline, which was slowly infused over the time period of 1 hour.

To dissect out the spinal cord, a longitudinal incision was given in midline over the dorsal aspect, over the vertebral column. After the incision, skin was retracted laterally; spinal muscles were cut on both sides of vertebral column, and retracted laterally to expose the vertebral column. After that the cervical region of spinal cord was extracted by laminectomy. It was kept in 4% paraformaldehyde solution at 4^{0}C for 5-7 days for fixation. For cryopreservation, the spinal cord was placed in 20% and 30% sucrose solution respectively, till the spinal cord sank to the bottom in the solution. Cervical spinal cord was cut on a cryostat at 20µm thickness at -18^{0}C. For cryosectioning, the part of cervical spinal cord was put on the tissue holder, with cervical region facing upwards and was fixed with the help of OCT. Sections were processed for single labeling, free floating immunohistochemistry (IHC) to visualize different level of expression of CREB and p-CREB protein. For free floating IHC, sections were taken to a multivial culture plate containing 0.1M PBS-Tx. Anti-CREB and anti p-CREB antibodies were purchased from Sigma (USA). The procedure of IHC was standardized for both antibodies at different dilutions.

Protocol of Immunohistochemistry procedure: The detail protocol of Immunohistochemical localization of various molecules like different types of calcium channels, opioid receptors like µ, Ƙ and delta, then CREB & p-CREB was earlier standardized in our laboratory, Research Lab. For Neuroscinces, 1027-28 at Department of anatomy by various research scholars' like Ray S B, Wadhwa S, Verma D, Shukla S N, et, al.[(9)]

Part II - Immunohistochemical localization, for the expression of CREB and p-CREB protein in horn of the cervical segment of rat spinal cord

Localization of CREB

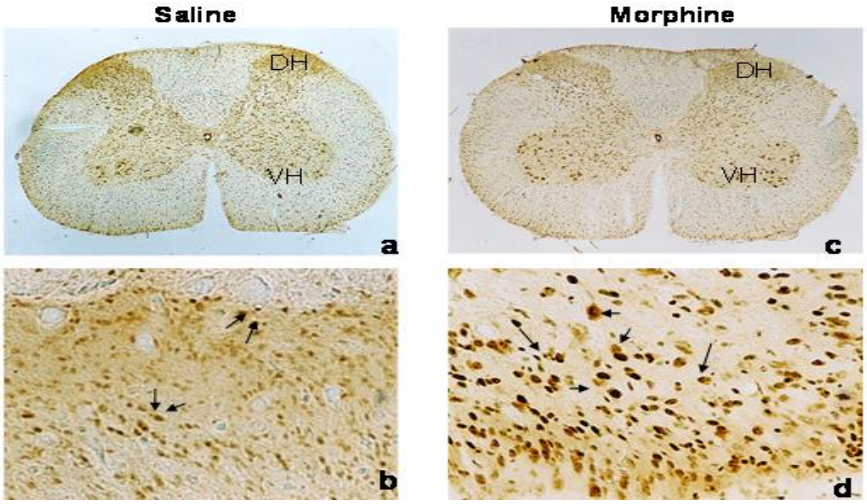

Fig. 1a, b: Showing immunohistochemical staining of the section of cervical spinal cord of rat with Anti-CREB antibody in saline treated group, c, d: Showing increased immunohistochemical staining of the section of cervical spinal cord of rat with Anti-CREB antibody in morphine treated group. Arrows are pointed on immunoreactive positive neurons in laminae I & II of dorsal horn

Localization of p-CREB

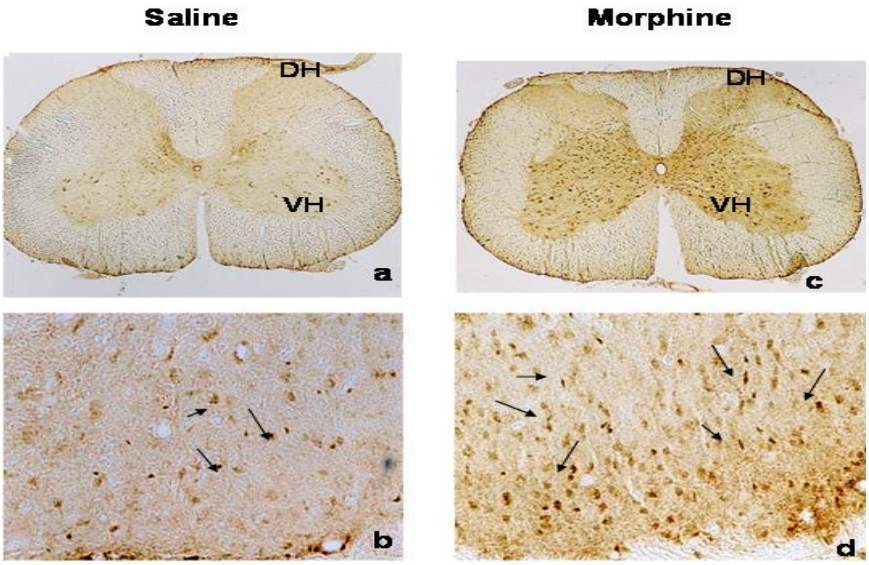

Fig. 2a, b: Showing immunohistochemical staining of the section of cervical spinal cord of rat with Anti-p-CREB antibody in saline treated group c, d: Showing increased immunohistochemical staining of the section of cervical spinal cord of rat with Anti-p-CREB antibody in morphine treated group. Arrows are pointed on immunoreative positive neurons in laminae I & II of dorsal horn

Part III: Quantitation of expression of immunohistochemical staining with the help of Image Analysis System-

For the quantitation of immunoreactivity, 6 sections per animal were selected. These were visualized under a Microscope at 20X magnification for quantitation of CREB and p-CREB expression. The microscope was attached with a digital camera and a computer system. The immunohistochemical staining was quantitatively expressed by measuring the Integrated Optical Density (IOD) of immunostaining with the help of Image-Pro-Plus 6.2 software.

Detailed procedure of measuring the Integrated Optical Density (IOD)

1. Viewing of the immunohistochemical stained sections under 4X.
2. Random selection of 6 sections per animal.
3. Now the selected sections were focused under 20X magnification.
4. The region of lamina I & II of dorsal horn was focused and the image was captured.
5. A rectangular frame was imposed over the region of laminae I & II in photograph.
6. The photograph was converted into Gray scale-8.
7. Now the Integrated Optical Density was measured of selected region of measuring frame.
8. The value of IOD was taken in a Microsoft Excel sheet.
9. The same procedure was applied for all 6 sections of each animal, and for each group.
10. The average value of IOD of all 6 sections was calculated and taken in a Microsoft excel sheet.

The same procedure was applied for every group. So the average values of IOD for each group were measured for both Anti-CREB and Anti p-CREB immunostaining

Statistical analysis: To compare the control with treated groups, statistical analysis of the values of Integrated optical density was done by Kruskal Wallis one way ANOVA, followed by "Tukey's Multiple Comparison Test" (multiple range 't' test) (*p<0.05* was taken to be significant*).*

1. Kruskal-Wallis one way ANOVA
2. Tukey's Multiple Comparison Test

Result

Morphine analgesia was studied on Groups I & II, and assessment of analgesia was done by tail-flick test. The values of tail-flick latency were almost equal to baseline values for group I, throughout the experiment, while for group II, values of tail-flick latency were almost equal to the cut off time (9.15±1.762), at day 1, but gradually the values decreases over the time period of experiment and at the end of experiment, tail-flick values reaches to base line value. This pattern of gradual decreases of latency is interpreted as the development of tolerance to analgesic effect of morphine. As shown in Fig. 1 & 2. Increased expression of CREB & p-CREB, in morphine treated group shows that, CREB & p-CREB have a molecular base for development of tolerance to morphine induced analgesia.

Table 1: Values of IOD for immunoreactivity (Mean±SEM)

Groups	IOD of CREB	IOD of p-CREB
Group I	12.88926 ± 1.1163	12.60581 ± 3.5275
Group II	25.12314 ± 2.9556	27.44218 ± 1.2703

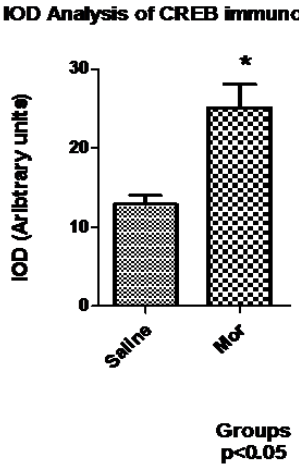

Fig. 3: Bar diagram showing the IOD values for various group

There is significant increase in the expression of CREB in morphine treated group with comparison to saline treated group

(*indication statistically significant difference between group)

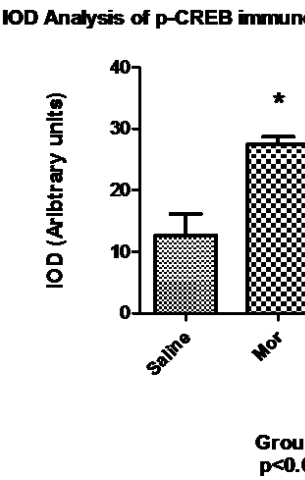

Fig. 4: Bar diagram showing the IOD value for various group

There is significant increase in the expression of p-CREB in morphine treated group with comparison to saline.
(* indicating statistically significant difference between groups)

Discussion

Calcium–calmodulin-dependent protein kinase IV (CaMKIV) activates the cAMP response-element binding protein (CREB) by phosphorylating it at Ser 133 (Deisseroth et al., 1996 and Deisseroth et al., 1998).[10] Phosphorylated CREB recruits the CREB-binding protein, which leads to the activation of CRE (cAMP response element) - containing promoters and ultimately to gene expression (Matthews et al., 1994; Ginty et al., 1997 and Soderling et al., 1999).[11] CaMKIV is a calcium-dependent protein kinase that is detected in both the nuclei and cytoplasm of neurons, and is the only CREB-phosphorylating protein kinase that is detected predominately in the nuclei of neurons (Jensen et al., 1991; Nakamura et al., 1995 and Kang et al., 2001).[12]

Interestingly, a large number of p-CREB-positive neurons were also found in the dorsal part of central canal (lamina X), where dorsal commissures interconnect the two sides of the dorsal horn. Lamina X neurons receive afferent inputs similar to that of laminae I–II (Brown et al., 1981).[13]

Summary and Conclusions

Morphine is the most effective analgesic drug used for management of chronic pain, but development of tolerance to its analgesic effect is a major limiting factor. . It was hypothesized that tolerance could be due to increased Ca^{2+} entry into neurons, which then leads to Phosphorylation of CREB and transcription of pronociceptive gene like c-fos.[14]

A study by Ru-Rong Ji et al., (1997) has shown that majority of neurons containing p-CREB-positive nuclei in the spinal cord after formalin stimulation was observed in laminae I–II and V–VI. These are spinal cord regions in which a majority of noxious primary afferents terminate and in which the cell bodies of dorsal horn nociceptive neurons are localized (Sugiura et al., 1986 and Besson et al., 1987).[15]

Molecular basis of the tolerance to morphine analgesia, is shown by the results of the present study, there is increased activation of CREB and p-CREB in morphine tolerance. So we have observed the increased expression of CREB & p- CREB in dorsal horn of spinal cord of Morphine Tolerant rats, which may be quite valuable finding of present study, which will be going on to opens a lots of doors/ approaches to understand molecular basis for development of tolerance to morphine (Opioid) induced analgesia. And this study would be very helpful for inventions of new drugs, betterment of management of pain in terminally ill or those are in most advance stage of cancer, at which point, physicians eagerly want to provide a good palliative care to their patients.

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