Differential expression of MU [μ] Opioid receptors and N-type calcium channels in the amygdala of morphine tolerant rat: An autoradiographic & immunohistochemistry study

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Abstract

Introduction: Recent studies have shown that amygdala plays a pivotal role in the emotional processing of pain. Opioids like morphine relieve pain by suppressing both the sensory and emotional components of pain. On chronic morphine administration, tolerance develops decreasing its analgesic effect. The underlying changes in the amygdala during morphine tolerance are not definitely known. Mu opioid receptors are binding sites for morphine while N-type voltage-gated calcium channels (VGCCs) directly control the release of neurotransmitters from presynaptic nerve endings.

Aims & Objectives: The present study was designed to investigate the status of mu receptors and N-type VGCCs in the amygdala of morphine tolerant rat by autoradiography and immunohistochemistry respectively.

Material & Methods: Wistar rats were made morphine tolerant by repeated administration of morphine for 5 days. Later, the rats were euthanized and cryostat sections of the brain were cut at the region of the amygdala. For autoradiographic localization, the sections were incubated with [³H] labeled DAMGO. For immunohistochemistry, the sections were exposed to primary antibody against N-type VGCCs.

Results: Using image analysis software, a significant down-regulation of mu receptors and an up-regulation of N-VGCCs were observed after tolerance. This differential change in expression would indicate a less effective antinociceptive action of morphine through / though higher neurotransmitter release after tolerance.

Conclusion: In conclusion, the study suggests that morphine tolerance could be associated with altered functional state of the amygdala.

Keywords: Amygdala, Mu-opioid receptor, Voltage-gated calcium channel, Autoradiography, Immunohistochemistry

Introduction

Amygdala's involvement in emotional processing arose from the classic studies of Klüver and Bucy who examined the behavioral effects of medial temporal lobe lesions in monkeys.[1] Amygdala receives nociceptive information from the thalamus and cortical areas and integrates sensory nociceptive information with affective content and contributes to the emotional response to pain [2,3] like anxiety and depression.[4,5]

The various nuclei of amygdala can be divided into three groups: 1) the *deep* or *basolateral group*, which includes the lateral nucleus (Ala), the basal nucleus (Abl), and accessory basal nucleus (Abm); *2)* the *superficial* or *cortical* nuclei (ACo) and *3)* the *centromedial* group composed of the *medial* (Ame) and *central* nuclei (ACe).[6] (Fig. 1) Amygdala has shown to synthesize both endogenous opioids and morphine.[7] It is well known that morphine acts predominantly through the mu-opioid receptors to produce analgesia and euphoria. Amygdala has been reported to express high level of mu-opioid receptors.[8] Considering amygdala's involvement in modulating pain related anxiety, the endogenous morphine could bind to mu opioid receptors. Possibly, these mu opioid receptors could also mediate antinociceptive responses through connections with the periacqueductal gray.[9]

Interestingly, sustained sadness is associated with decreased mu-opioid receptor neurotransmission in amygdala, suggesting that mu-opioid receptors in amygdala regulate affective experiences in humans.[10] Zubieta et al (2001) demonstrated that during sustained pain, there was activation of the endogenous opioid system and significant reduction in mu-opioid receptor availability in the amygdala, as measured with positron emission tomography.[9]

Mu opioid receptors are members of the *G-protein coupled receptor superfamily*.[11] Binding of morphine to mu-opioid receptors **blocks N and P/Q-type Ca²⁺ channels,** [12] decreases adenylyl cyclase activity[13] and opens inwardly rectifying K⁺ channels.[14] Among them, P/Q-type and N-type VGCCs are predominantly localized in cell bodies and presynaptic nerve terminals, and regulate presynaptic activity including neurotransmitter release.[15] An earlier study in our lab have shown that N-type VGCCs are specifically localized on presynaptic terminals in laminae I and II of spinal cord.[16] In contrast, L-type VGCCs were localized in clusters on cell bodies and dendrites, and implicated in specialized somatic functions, but not in neurotransmitter release.[17]

Morphine treatment decreases entry of calcium ions and its uptake by synaptosomes whereas it is increased after chronic morphine administration[19]. Also, chronic morphine treatment, which leads to tolerance, is associated with an increase in vesicular calcium content by allowing increased entry of calcium into neurons[20]. Thus, abnormalities of calcium ion inflow and its storage in the neuron may be related to the development of tolerance following chronic morphine administration. Tolerance is characterized by a decrease in the effectiveness of a drug following chronic administration. Infact, blocking of both L- and N-type VGCCs potentiate morphine induced analgesia and attenuate tolerance.[21,22,23] After chronic administration of morphine, the expression of N-type calcium channels is significantly increased.[16] Therefore, blockade of calcium channels appears to be an important mechanism of action of morphine.

To the best of knowledge, there are no reports on the expression of mu opioid receptors and N-type calcium channels after the development of morphine tolerance. In the present paper, the expression of N-type VGCCs was investigated after morphine tolerance. Moreover, mu opioid receptor expression was also studied after morphine tolerance.

Materials and Methods

A. **Experimental Animals:** Male Wistar rats weighing 150 –200g were procured from the Common Animal Facility of the All India Institute of Medical Sciences. Experimental work was approved by Ethical Committee of the Institute.

All rats were subjected to a screening test regarding baseline pain sensitivity. This was done by tail flick test. The tail flick test was used as a parameter for measuring the analgesic response to morphine. In this test, animal's tail is subjected to a radiant heat stimulus to evoke a tail-flick reaction. Only those rats (n=24) were selected for the study which showed baseline tail flick latency 2-4.5 seconds. The animals (2-3 per cage) were maintained at a 12/12 light/dark cycle with water and food available *ad libitum*.
Selected rats were randomly divided into 2 equal groups for carrying out the following experiments.
1. **Autoradiographic Localization of Mu Opioid Receptors**
2. **Immunohistochemical Localization of N-Type Calcium Channels**
B. **Morphine Treatment:** The treated group was administered escalating doses of morphine respectively from 10, 20, 30, 40 and 50 mg/kg subcutaneously (S.C.) in the hind paw three times a day for 5 days as per earlier established protocol[24]. Similar volume of saline was administered in the control group. Morphine was procured from a Government firm after taking prior permission from Narcotics Control Commissioner of India. Development of morphine tolerance was assessed

by giving a test dose (10 mg/kg-body weight) on days 1, 3 and 5 – 1 hour prior to the start of the second day to both treated and control groups. The amount of test dose was kept constant throughout the experiment. Development of tolerance to morphine was indicated by a decrease in tail-flick latency with continued administration of morphine.

a) **Autoradiography of mu-opiod receptors:** All the animals were sacrificed immediately after the last tail flick test by overdose of inhalation anesthesia. Subsequently, their brains were dissected out and frozen in liquid nitrogen. The tissues were stored in a freezer at -20°C. 20μ thick serial coronal sections containing all the nuclei of amygdala (based upon Paxinos and Watson)[25] were taken with a cryostat and mounted on gelatin-coated slides. The slides were then kept in deep freeze at -20°C until autoradiography was done. The autoradiographic procedure included the following steps:

1. *Preincubation:* The sections were pre-incubated in 50nM Tris HCI buffer containing 150mM NaCI and 1mM EGTA (Ethylene Glycol Tetraacetic Acid) for 30 minutes at room temperature (25°C). It is done to remove the endogenous ligands administered drugs and increase the availability of the binding sites.
2. *Incubation:* The sections were incubated in 50mM Tris HCI buffer containing 2 nM of [3H] [D-ala2, N-methyl-Phe4, -Gly-ol5] enkephalinv (DAMGO, [3H] (Tritium) labeled DAMGO (Amerison plc) for one hour at room temperature. The slides were washed in Tris HCI buffer solution at room temperature to remove excess ligand. Subsequently, slides were dipped in cold distilled water at 4°C and were immediately taken out and dried to inhibit dissociation of bound ligand from mu opioid receptors. Non-specific binding was determined under same experimental conditions, using 1000-fold excess naloxone as compared to DAMGO, during the incubation process. (2 μmol naloxone).
3. Exposure to [3H] sensitive film: Finally, slides were kept in a specially designed box along with a [3H] sensitive autoradiographic films for 8 weeks at room temperature.
4. Development of Film: At the end of 8 weeks, the films were developed in a dark room. Finally, film was dried at room temperature. A clear impression of mu opioid receptor labeled with DAMGO was observed on the film.

[3H]-DAMGO was purchased from Amersham, UK. The hyperfilms and radioactive standards were from Amersham, UK and levorphanol was from Sigma, USA.
5. Quantitative Image Analysis: this was done to quantify the expression of mu opioid receptor in amygdala and its various nuclei. Densitometric analysis of the autoradiographic images was

carried out using Adobe Photoshop version 7.0 software. A total of six sections per animal from both groups were quantified and mean grey value was calculated for whole amygdala and its various nuclei. The identification of nuclei within the amygdaloid complex was made using Nissl stained sections adjacent to autoradiograms of [³H] DAMGO binding.

The software was used to measure the average brightness of the pixels within each nuclei of amygdala was measured which was manually outlined. The same procedure was done separately for the whole amygdala. The mean gray values for whole amygdala were evaluated by calculating mean and standard error and then applying the *unpaired t-test* to test significance. Similarly, mean and standard error was calculated for individual nuclei, Then *one-way ANOVA* was applied followed by *Bornferroni's Multiple Comparison Test* to test significance (*p<0.05* was taken to be significant*).*

b) **Immunohistochemical localization of N-type calcium channels:** The animals were sacrificed at the end of the fifth day by anesthetizing with Sodium Pentobarbitol (100mg/kg i.p.). Then perfusion fixation was done using 4% Paraformaldehyde solution. The brain was dissected out, trimmed to obtain region of amygdala in 4% Paraformaldehyde solution at 4˚C for 24 hours. For cryopreservation, the brain was placed in 10%, 20% and 30% sucrose solution respectively.

Serial tissue sections (20µ thick) were cut in a cryostat (-20˚C) and stored in a cryopreservative antifreeze buffer at -20˚C. The selected sections were processed for single labeling, free floating immunohistochemistry.

The sections were washed with 0.1M PBS + 0.25% Triton-X-100 (3 changes for 10 min.)

Quenching was done with endogenous peroxidase with 80% methanol + 0.5% v/v H_2O_2 for 30 minutes. *Non-specific binding* sites were blocked with 10% *Normal Goat Serum* (NGS) in 0.1M PBS + 0.25% Triton-X-100.

The sections were then incubated in *primary antibody Anti-Ca$_V$ 2.2* (1:100) (SIGMA, USA) for 60 h at 4˚C. Subsequently, the sections were incubated in goat anti-rabbit biotin conjugated IgG (*secondary antibody).*

Subsequent to buffer wash, *Avidin Biotin Complex* – dilutions were made according to Vectastain Elite ABC kit in PBS-Tx (Vector Laboratories Inc., Burlingame, CA). *3, 3´ diaminobenzidine complex -* 0.025% W/v v (DAB, Sigma) was used as a chromogen to visualize antigen-antibody complex.

Following buffer wash, sections were mounted on gelatin coated slides.

**Quantification of immunoreactivity:** The immunostained sections were visualized using a Leica microscope and difference in the intensity of immunoreactivity between the controls and treated groups was assessed by image analysis using the Leica Q WIN. The sections were viewed under 2.5-x magnification and 'acquired' on the computer monitor. Using a graphic 'pen & tablet', the amygdalar nuclei were outlined in a measuring frame and the intensity was 'detected'. For each nucleus, the grey values of both, the left and the right sides from six cranio-caudal levels were pooled to obtain a mean grey value for each sample. The identification of nuclei within the amygdaloid complex was made using Nissl stained sections adjacent to immunostained slides. To compare the control with treated groups, statistical analysis of data from immunostained sections was done by unpaired test (*p<0.05* was taken to be significant*).*

Results

Development of tolerance: Chronic administration of morphine lead to a significant decrease in the tail flick latency. (Table 1, Fig. 2) The animals for autoradiographic study and those for the immunohistochemical localization showed similar pattern in the development of morphine tolerance.

Table 1: Tail flick latency values for animals used in autoradiographic & immunohistochemical studies

Tail Flick Latency Values	TFL-Baseline (seconds)	TFL-Day 1 (seconds)	TFL-Day 2 (seconds)	TFL-Day 3 (seconds)
Autoradiographic study Control group	2.79+0.55	10.00	10.00	10.00
Treated group	2.87+0.34	10.00	7.7+0.48	4.07+0.29
Immunohistochemical study Control group	3.01+0.28	10.00	10.00	10.00
Treated group	3.35+0.44	9.79+0.37	7.17+0.62	3.76+0.28
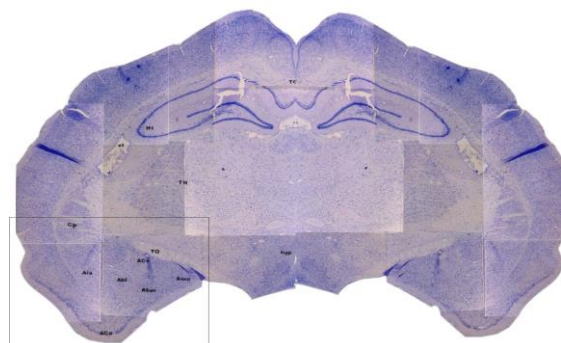
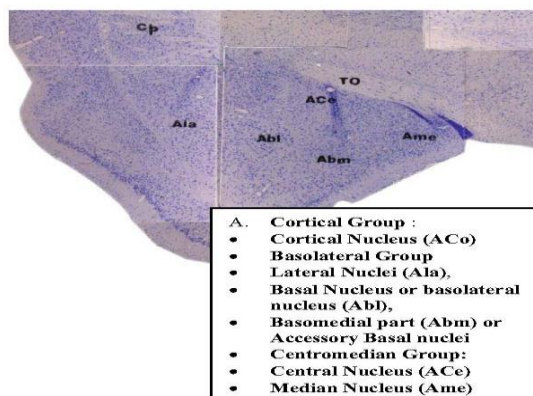**Fig. 1: Amygdala and related structures in Nissl stained section of rat brain**

Fig. 2: Various amygdaloid nuclei are seen, other structures identified are:

TO: Optic Tract; Cp: Caudate Putamen; TH: Thalamus; HI: Hippocampus

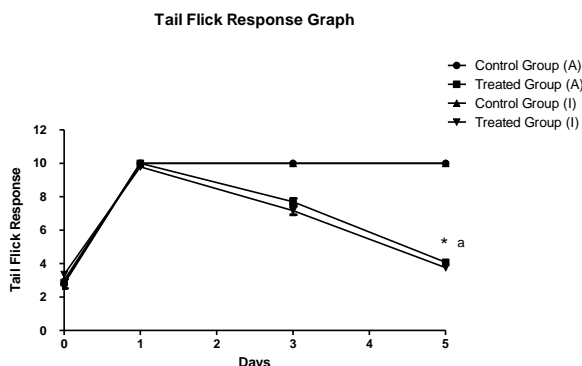**Fig. 3: Tail flick response shown on day 0, day 1, day 3 and day 5. The tail flick response time decreased significantly (p<0.05) on day 5 in treated as compared to control groups (*), tail flick response also decreased significantly (p<0.05) on day 5 compared to day 1 in the treated group (a) showing significant development of tolerance. (A) Signifies autoradiography group (I) Signifies immunohistochemistry group**

Autoradiographic Labelling: Mean gray value for the entire amygdala was 53.75±8.53 and 39.8±3.96 respectively. The treated group showed significant decrease (p<0.05) in the expression of mu opioid receptors. In control group, high levels of expression were noted in the medial and lateral nuclei (mean gray value of 120.25±7.27 and 100.26±9.02 respectively) and moderate level of expression was seen in central and basal nuclei (mean gray value of 87.75±10.81 and 84.2±5.31 respectively). (Table 2, Fig. 2) Similarly, in the treated group, higher levels of expression were noted in the medial and lateral nuclei. The expression was significantly increased in the cortical nuclei (p<0.05) of amygdala whereas it was significantly decreased in all other nuclei. (Fig. 4)

Table 2: Comparison of mu opioid receptor density in nuclei of amygdala in control and teated rats. The expression was significantly decreased in all the nuclei (*) except cortical nuclei in which it was significantly increased (a)

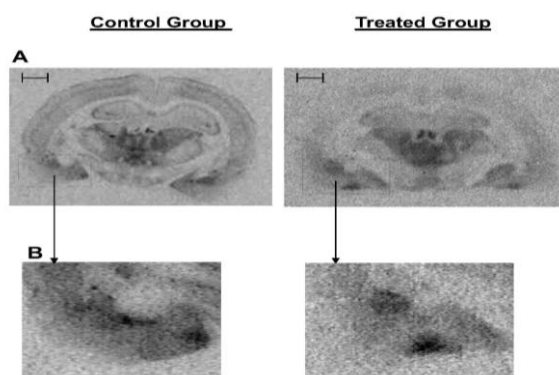| Nuclei Group	Central	Medial	Cortical	Lateral	Basal
Control Group	87.75±10.81	120.25±7.27	71.25±5.61	135±8.67	84.2±5.31
Treated Group	62.6±2.16*	74.66±5.5*	101.16±2.22*	98.1±9.17*	66.83±3.97*

Fig. 4:

(A) Autoradiographic labeling of mu opioid receptor in a coronal section of control and morphine tolerant rat. The region of amygdala has been outlined by a rectangular box; Scale Bar = 646 μm

(B) Comparative analysis of mu opioid receptor density in audiographs in both control and treated groups

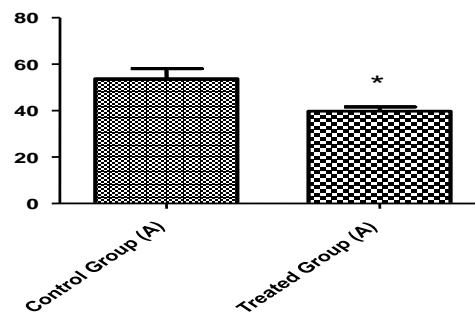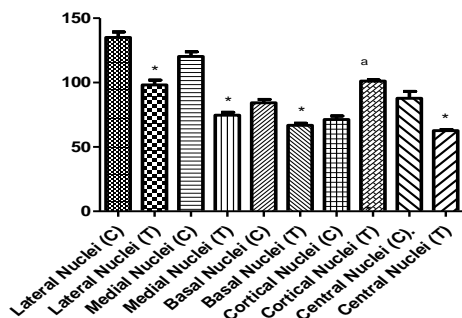**Fig. 5: Bar diagram of mu opioid receptor density in the amygdala. The density was found to be significantly decreased (p<0.05) in the treated group after the development of morphine tolerance as compared to the control group (*)**

Immunohistochemical Study: Expression of N-type calcium channel was noted in the amygdala as well as its component nuclei (Fig. 5). The different nuclei of amygdala did not show significant difference in the expression of N-type calcium channels in either control

or treated groups. However, the N-type calcium channel expression was significantly greater (p<0.05) in treated group (108.4±3.81) as compared to control group (74.57±2.02) (Fig. 6).

Mu Opioid Receptor Density in Nuclei Of Amygdala

Fig. 6: Bar diagram showing mu opioid receptor density in various nuclei of amygdala. The expression was significantly decreased in all the nuclei (*) except cortical nuclei in which it was significantly increased (ᵃ)
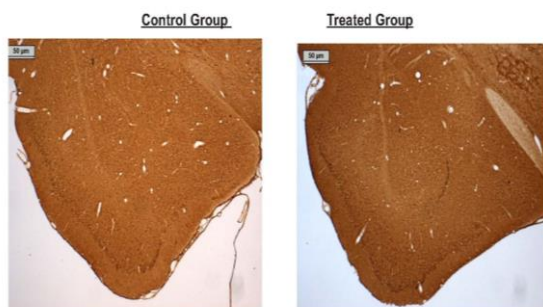
Fig. 7: Comparative analysis of N-type calcium channel expression in immunostained sections of control and treated groups
An increased expression of anti-Cav2.2 is noted in the morphine treated group over the amygdala

N-TYPE CALCIUM CHANNEL INTENSITY IN AMYGDALA
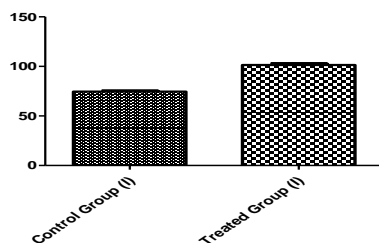
Fig. 8: Bar diagram showing N-type calcium channel intensity in amygdala of immuno-stained sections of control and treated groups. N-type calcium channel intensity is significantly increased (p<0.05) in the immunostained sections of treated group as compared to control group

Discussion

In the present study, induction of morphine tolerance led to alteration in the expression of Mu opioid receptors and N-type calcium channels. Regarding Mu opioid receptors, significantly decreased expression was observed in the amygdala. In contrast, expression of N-type calcium channels was significantly increased after the development of morphine tolerance. An earlier study demonstrated that opioid tolerance is accompanied by up-regulation of L-type calcium channels and down-regulation of mu opioid receptors in both cerebral cortex and spinal cord of rats.[26] Other related studies have shown that chronic exposure to opioid drugs increases the number of L-type calcium channels in rat brain.[22,27]

In the control group, higher expression of mu opioid receptors was noted in the medial and lateral nuclei of amygdala and high expression was seen in the central and basal nuclei. Similar findings were noted earlier by immunohistochemical localization by Ding et al., (1996)[28] who observed high levels of mu opioid receptor expression in the medial, lateral and basal nucleus, whereas lower levels have been observed in the central nucleus. On the contrary, Atweh (1977) showed high density in cortical nuclei, moderate density in medial, basal and central nuclei and low density in lateral nuclei determined by the autoradiographic localization of stereospecific [³H] diprenorphine binding sites.[29] Though Mansour (1987) had reported very high expression of mu opioid receptors in cortical and lateral nuclei, moderately high expression in medial nucleus and lower expression was seen in central nucleus.[30] Daunais et al. (2001) noted high concentration of [³H] DAMGO binding sites within the basal and medial nuclei and low concentration was noted in the central nuclei. However, overall expression of mu opioid receptor does not determine the efficacy of the receptor.[31] Whereas the concentration of [³H] DAMGO binding site in the central nucleus was very low, the concentration of DAMGO-stimulated G-protein activation in this nucleus, as measured with ([³⁵S] GTPγS) binding, was relatively high compared to other portions of amygdala containing much higher concentrations of [³H] DAMGO binding sites. The converse was true in the medial nucleus where high concentration of [³H] DAMGO binding sites were associated with lower levels of DAMGO stimulated G-protein activation. This apparent mismatch suggests that central nucleus may be an area where there is a high efficiency of agonist-stimulated G-protein activation such that one receptor could activate a large number of G proteins.[31] This goes down well with the established role of the central nucleus in pain modulation which has earned it the name "*nociceptive amygdala* (Neugabeur, 2004). Considering the role of mu opioid receptor in analgesia, it is noteworthy that the nucleus involved in processing of nociceptive information will show a higher density in

the control animals. Nociceptive information from thalamus and cortex reaches the amygdala as polymodal inputs to the lateral nucleus, from where it is transmitted to the central nucleus, the output nucleus. The central nucleus has substantial projections to the hypothalamus, bed nucleus of the stria terminalis (De Olmos, 1985) and several nuclei in the midbrain, pons, and medulla (Veening, 1984) Projections to the brain stem are to three main areas: the *periaqueductal gray* (Rizvi, 1991), which leads to vocalization, startle, analgesia and cardiovascular changes, the *parabrachial nucleus*, which is involved in pain pathways (Rizvi, 1991); and the *nucleus of the solitary tract* (NTS), which is connected with the vagal system (Van der Kooy et al.,1984).

In the present study, there was significant down-regulation of mu-opioid binding sites in the various nuclei of the amygdala except the cortical nuclei where up regulation was seen after morphine tolerance. Overall, expression was also decreased. Decrease in receptor expression may be linked to morphine tolerance. This down regulation on prolonged morphine administration may result in less binding sites for morphine thereby blocking the action of endogenous as well as exogenous opiates leading to a state known as opioid induced hyperalgesia[38] which contributes to 'analgesic tolerance'. It can be compared to persistent painful conditions where there is increased endogenous opioid release is seen accompanied by a down-regulation of opioid receptors.[9]

However, the reason for selective increase in the cortical nucleus requires further study.

Down regulation of mu opioid receptors after morphine tolerance was observed by *Tao et al. (1987) in* striatum, midbrain and cortex.[39] *and by Bhargava and Gulati (1990)*[40] in spinal cord, pons, medulla, and cerebral cortex.

Various nuclei of amygdala did not show significantly different N-type calcium channel expression in either control or treated groups. However, statistically significant upregulation was noted after chronic morphine administration. This could signify the increased activity of amygdale because N-type calcium channels are necessary for the release of neurotransmitter. Saegusa et al., (2001) demonstrated that mice lacking N-type VDCCs show suppressed responses to inflammatory and neuropathic pain.[43] They also demonstrated that mice lacking the N-type Ca^{2+} channel displayed reduced anxiety-like behaviors. Presumably, N-type calcium channels in amygdala could also lead to increased level of anxiety in morphine tolerance. Finn et al., (2003) reported that intra-amygdaloid injection of N-type calcium channel blocker ω-conotoxin resulted in significantly reduced conditioned freezing behavior to electric shocks with no effect on ultrasound-induced unconditioned aversive behavior. Since amygdala determines pain related anxiety, its functional status

during chronic morphine administration may contribute to increased pain perception.

Ikemoto et al (2002) reported up-regulation of addicsin expression during morphine tolerance which affects glutamate uptake in the amygdala.[26] Addicsin is 98% identical with rat glutamate-transporter-associated protein 3-18 (GTRAP3-18) The increased uptake of excitatory transmitter glutamate could be associated with upregulation of N-type calcium channels which would determine the release of glutamate from synaptic terminals.

In conclusion, the present study shows definitive changes in certain nuclei of amygdala which could determine its altered function at the time of morphine tolerance.

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