Nimodipine down-regulates CGRP expression in the rat trigeminal nucleus caudalis

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L-type calcium channel blockers like verapamil are used in the prophylaxis of migraine. However, their effect on the expression of CGRP in the trigeminal nucleus caudalis (TNC) is unknown. It is important because an earlier study had shown that olcegepant, a CGRP receptor antagonist, acts at the level of the trigeminal spinal nucleus rather than the trigeminal ganglia. Nimodipine was used in the present study as it crosses the blood-brain barrier. The objective of the study was to determine the pattern of expression of calcitonin gene-related peptide (CGRP) in the TNC after administration of nimodipine and/or morphine. Wistar rats were injected with saline, morphine, nimodipine or morphine + nimodipine for 14 days. Subsequently, the lowest part of the medulla oblongata containing the spinal nucleus was removed and processed for immunohistochemical localization of CGRP. The density of expression was quantified using Image J software. The results were statistically analyzed. CGRP expression was noted over the superficial part of the TNC, which decreased significantly after nimodipine administration. Conversely, morphine produced an up-regulation. The expression was unchanged with reference to saline in the morphine + nimodipine treated group. Decreased expression of CGRP in the trigeminal nucleus caudalis after nimodipine is being reported for the first time. Also, whether CGRP expression can be used as a marker for predicting the therapeutic efficacy of an anti-migraine drug is currently being investigated.

Keywords: Calcium channel blocker, cAMP response-element binding protein, Image J, Immunohistochemistry, Migraine, Trigeminal ganglia

The caudal part of the spinal nucleus of the trigeminal nerve (trigeminal nucleus caudalis or TNC) receives unmyelinated (C) and thinly myelinated (Aδ) nerve fibers from the trigeminal ganglia. These carry the sensations of pain and temperature from the head and face. The trigeminal ganglia in humans contain the highest density of neurons (approximately 50%) expressing the calcitonin gene-related peptide (CGRP)2. Release of CGRP from the peripheral and central terminals of neurons of the trigeminal ganglia produces vasodilatation of the meningeal blood vessels and facilitation of glutamatergic neurotransmission like that for α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors respectively2. Migraine is a periodic unilateral headache which is associated with excess release of CGRP and this can be detected in the peripheral blood2. Opioids like morphine produce analgesia by preventing the release of neurotransmitters and neuromodulators from presynaptic nerve terminals by closing N- and P/Q-type voltage-dependent calcium channels (VDCCs)4. Mu-opioid receptors are expressed in the TNC which are further up-regulated following noxious stimulation5. An electrophysiological study has shown that acute morphine (1–10 mg/kg) administration attenuates the activity of TNC neurons following noxious stimulation of the duramater6. In fact, resistant cases of migraine associated with pregnancy or coronary heart disease are sometimes treated with morphine, meperidine or other opioid analgesics7. However, the status of CGRP expression in the TNC after chronic morphine administration is not known. In the spinal cord of morphine tolerant rats, a higher expression of CGRP was noted8,9. Interestingly, the increase was noted in the superficial laminae of the spinal cord, which is associated with the transmission of pain and temperature.

Calcium channel blockers like verapamil are also used in the prophylaxis of migraine10. However, there is no report, to the best of knowledge, on their effect on the expression of CGRP. Nimodipine was chosen for the present study as it crosses the blood-brain barrier. Nimodipine is an L-type voltage-sensitive calcium channel (L-VSCC) antagonist, commonly

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used for treating subarachnoid haemorrhage. It is a short-acting 1,4 dihydropyridine derivative. Also, both morphine and nimodipine were co-administered and their effect on CGRP expression noted in the TNC.

**Materials and Methods**

Wistar rats (adult male, weight 175-220 g) were divided randomly into following 4 groups of 6 animals each: Group A animals were injected with physiological saline twice daily, subcutaneously (sc), animals in Group B received morphine sulphate ip (20 mg/kg twice daily for 7 days followed by 30 mg/kg twice daily for next 7 days sc), Group C rats received nimodipine through ip route once daily while Group D animals received both morphine and nimodipine as for Groups B-C (nimodipine was administered 20 min before morphine). The doses of the drugs were the same as has been reported earlier. The total time period of drug administration was 14 days. The animals were maintained under a 12:12h L:D cycle and provided food and water ad libitum. Prior permission for animal experimentation was obtained from the Institutional Animal Ethics Committee.

At the end of day 14, the rats were anaesthetized with pentobarbital injection (100 mg/kg) and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer saline (PBS). The lowest part of the medulla oblongata bordering the spinal cord was dissected out after craniotomy and preserved in PBS for a further period of 3 days. Subsequently, these were immersed sequentially for a day each in 15% and 30% sucrose solution. Cryostat sections (20 µ thick) were cut in the transverse plane at -20°C and collected as free-floating sections in 0.1M phosphate buffer saline (PBS). An average of 11-12 sections per rat was collected. The caudal part of the trigeminal nucleus caudalis (TNC) was localized using 1% Cresyl Violet stain. Later, the sections were processed for immunohistochemical localization of CGRP. Initially, these were washed in 0.1M PBS containing 0.25% Triton X (PBS-Tx). Endogenous peroxidase was quenched by incubating the sections in 80% methanol containing 0.3% hydrogen peroxide for 30 min. Later, sections were exposed to 10% Normal goat serum in PBS-Tx for 2 h. Following this, sections were directly exposed (without washing) to primary antibody against CGRP (1:400; Calbiochem, USA) for 48 h. CGRP expression was visualized by the ABC method (Vector laboratories, USA.) using 3,3-diaminobenzidine tetrachloride (DAB) as the chromogen. Finally, the sections were taken on slides and dehydrated followed by clearing. These sections were mounted with cover slips using DPX. Positive control was also processed simultaneously on cryostat sections of the spinal cord removed from the saline treated experimental animals. Negative controls were also run without the primary antibody in sections of the medulla oblongata.

Images of the stained sections were captured under the low power of the microscope (2X objective) using the ProgRes CT5 camera and the images were saved in ProgRes CapturePro 2.7 Image analysis software (Jenoptik, Germany). The area of the CGRP expression in these sections, which corresponded to the superficial part of the TNC, was selected bilaterally and the average brightness of pixels assessed by Image J software (version 1.40; NIH, Bethesda, USA). Background staining intensity was determined from the remaining part of gray matter and deducted from that noted over TNC to obtain the specific value. This was deducted from 255 to obtain the darkness value representing the density of expression of CGRP.

Statistical analysis was done using one way analysis of variance (ANOVA) followed by post hoc comparison using the Bonferroni multiple comparison test (GraphPad Prism version 5, San Diego, USA). \( P<0.05 \) was considered statistically significant.

**Results**

Cresyl Violet stained cross-sections of the lower level of the medulla showed the spinal tract and the adjacent TNC (Fig. 1). Immunohistochemical localization of CGRP was noted in the superficial part of the TNC in all the groups. However, the density of expression of CGRP was different in these groups. The remaining areas of the TNC as well as the spinal tract of the trigeminal nerve did not show expression of CGRP. Dense CGRP expression was noted over laminae I-II of the spinal cord in the control group. However, no immunostaining was observed in the negative control group.

Quantitative analysis of the CGRP expression showed that it increased significantly \((P<0.01)\) after morphine administration in comparison to saline (Fig. 2). Nimodipine treatment decreased the expression in relation to the morphine treated group \((P<0.001)\) and the saline treated group \((P<0.05)\). Conversely, morphine + nimodipine showed a significant increase in comparison to the nimodipine group \((P < 0.001)\)
though not from the morphine treated group. Also, it was not significantly different from the saline treated group.

Discussion

The present study shows for the first time that chronic nimodipine administration significantly decreases CGRP expression in the TNC. Interestingly, the area of expression of CGRP was the same in all the treated groups, i.e., over the superficial part of the TNC. This is identical to its expression over the superficial laminae (I-II) of the spinal cord. Since, CGRP plays a key role in migraine, nimodipine could be useful in preventing or even treating this disorder. In fact, L-VSCCs have been reported to mediate nociceptive neurotransmission in the TNC along with P/Q and N-type-VSCCs. Moreover, certain L-VSCC blockers (amlodipine and verapamil) are being used in the prophylaxis of migraine. Despite this, clinical trials involving nimodipine have not shown a definitive advantage when compared to other drugs like flunarizine. The reason could be due to difference in the route of administration of nimodipine (oral dose in the trials vs. intraperitoneal administration in the current study). The dose of 2 mg/kg ip once daily used in the present study was extrapolated from the oral dose of 120 mg/day in humans (since the metabolic rate of rats is about 10 times higher than in humans, the oral dose in rats would be 20 mg/kg, which in turn becomes 2 mg/kg/day ip; toxicity studies in our laboratory showed that 2 mg/kg ip would be a safe dose). Vehicle
alone treated group was not included in the present report because preliminary studies did not show any change in CGRP expression. Nimodipine could have inhibited CGRP expression by reducing Ca\(^{2+}\) influx. Ca\(^{2+}\) influx can activate Ca\(^{2+}\) calmodulin protein kinase, which then phosphorylates cAMP response element binding protein (CREB). CREB is a transcriptional activator which enhances CGRP expression\(^{16}\). CGRP is almost exclusively expressed in neurons and controlled solely at the transcriptional level\(^{17}\).

Morphine alone treated group received repeated doses of morphine over a period of 14 days. It was earlier reported by us that this leads to significantly higher level of morphine tolerance\(^ {18} \). The present work correlates morphine tolerance with highly significant increase in the expression of CGRP. Sustained morphine treatment has been shown to increase CGRP release from cultured neonatal dorsal root ganglion neurons\(^ {19} \). A number of studies have observed similar increase of CGRP level in the spinal cords of morphine tolerant rats\(^ {8,9} \). It has been reported that antagonists of CGRP prevented the development of tolerance\(^ {20} \). Increased CGRP activates p38 MAPK (Mitogen activated protein kinase) in microglia and Extracellular signal regulated kinase (ERK) in astrocytes and increases the synthesis of Interleukin-1\( \beta \) (IL-1\( \beta \)), Interleukin-6 (IL-6), Tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and microsomal prostaglandin synthase \(^ {1} \). Conversely, blockade of ERK and p38 signaling pathway suppressed the tolerance. In the morphine + nimodipine group, there was higher expression of CGRP. Possibly, chronically administered morphine reversed the inhibition of CGRP expression, produced by nimodipine. The beneficial effect of morphine in migraine could be due to other mechanisms, particularly after acute administration. Currently, CGRP expression in the TNC is being determined as a marker of efficacy of various drugs used in the treatment of migraine. An earlier study has shown that inhibition of CGRP-mediated neurotransmission occurs in the spinal trigeminal nucleus rather than in the neurons of trigeminal ganglion by olcegepant, a CGRP receptor antagonist\(^ {21} \). Fluorometric calcium ion recording from the neurons of the TNC would have added proof of concept data. However, a recent study has shown by enzyme immunoassay that CGRP release is in fact controlled by voltage-gated calcium channels\(^ {22} \).

In conclusion, the present study shows the beneficial effect of L-VSCC blockers could be related to decreased expression of CGRP in the TNC.

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References