Expression of opioid receptor-like 1 (ORL1) & mu opioid receptors in the spinal cord of morphine tolerant mice

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Received May 31, 2004

**Background & objectives:** The mechanism underlying the development of tolerance to morphine is not clearly understood though a number of factors have been implicated. One of the likely factors may be increased activity of anti-opioid peptides like nociceptin (also known as orphanin FQ or N/OFQ). N/OFQ and morphine bind to opioid receptor-like 1 (ORL1) receptor and mu-opioid receptor respectively. The present work was undertaken to investigate the density of ORL1 and mu (µ) receptor expression in the spinal cord of mice after inducing morphine tolerance.

**Methods:** Swiss albino mice were injected with either morphine (experimental group, n=15) or saline (control, n=15), twice a day for 9 days. The development of tolerance was noted by the hot-plate test. Cryostat sections of the cervical region of spinal cord were labeled with specific ligands to localize ORL1 and mu receptors. The density of receptor expression over laminae I-II of spinal cord was evaluated using image analysis system.

**Results:** The morphine treated mice developed tolerance by day 9 as evident by the hot plate test. Both receptors were selectively expressed at a higher concentration over the superficial laminae (I-II) of the dorsal horn, indicating a role in pain processing. An increased expression of ORL1 receptors was also noted over the gray matter around the central canal. Quantitative analysis showed an increased expression of ORL1 and mu receptors though the increase was not statistically significant.

**Interpretation & conclusion:** The present study showed that both, ORL1 and mu-opioid receptors were expressed in areas of the spinal cord, concerned with transmission of pain signals. The density of these receptors increased in the superficial laminae (I-II) though not significantly from control after morphine tolerance. The increase in ORL1 receptors could oppose the analgesic action of morphine, contributing to tolerance. Further studies need to be done to elucidate the mechanism of morphine tolerance.

**Key words** Autoradiography - morphine - N/OFQ - opioid receptors - spinal cord - tolerance

Though morphine remains the gold standard among analgesic drugs, its effectiveness is limited by rapid development of tolerance and dependence. The µ-opioid receptor, through which morphine, remifentanil or tramadol act, is widely distributed in the nervous system. The mechanism underlying development of tolerance to morphine is not clearly understood though a number of factors have been implicated. One such factor is the presence of anti-opioid peptides in the nervous system, which counteract the analgesic action.
of morphine\textsuperscript{1}. One of the important anti-opioid peptides is nociceptin (also known as orphanin FQ or N/OFQ), which binds to Opioid receptor-like 1 (ORL1) receptor\textsuperscript{4}.

N/OFQ is a heptadecapeptide, similar to endogenous opioid peptides; particularly dynorphin A and it opposes the analgesic effect of morphine after intracerebroventricular (icv) injection\textsuperscript{5}. The anti-opioid status of nociceptin is confirmed by the fact that icv administration of antibody against nociceptin reverses chronic morphine tolerance by about 50 per cent in rat\textsuperscript{6}. In mice with a deletion of the gene for ORL1 receptor (receptor knock out), tolerance to morphine is attenuated\textsuperscript{7}. The ORL1 receptor, composed of 370 amino acids, belongs to the superfamily of G protein coupled receptors and shows considerable structural homology to the kappa opioid receptor\textsuperscript{8}.

An increased synthesis of N/OFQ has been convincingly demonstrated in the brain of morphine tolerant rats\textsuperscript{9}. The overall increase of N/OFQ content in periaqueductal gray, amygdala and cerebrospinal fluid was 81, 55 and 52 per cent respectively, after morphine tolerance\textsuperscript{9}.

However, the status of ORL1 receptors during morphine tolerance remains to be completely elucidated. Increase in ORL1 receptor density along with increased synthesis of N/OFQ would indicate an important role of anti-opioids in the development of tolerance. A previous study has reported increased ORL1 receptor expression in the spinal cord of rat after morphine tolerance\textsuperscript{10}. In this study, relatively high dose (7.5 µg/ml/h) of morphine was administered through osmotic minipump for 7 days. However, this does not completely simulate the use of morphine for therapeutic purposes, where comparatively lower doses are given through intraspinal or intramuscular routes. In the present study, expression of µ and ORL1 receptors was studied in the spinal cord of mice after injecting morphine intraperitoneally. The dose of morphine (10 mg/kg intraperitoneally twice daily for 9 days) used in the present study was based on an earlier report on morphine tolerance\textsuperscript{11}. The µ-opioid receptor was studied since downregulation of µ receptors is thought to underlie the phenomenon of opioid tolerance\textsuperscript{12}.

**Material & Methods**

Male albino mice (Swiss strain), weighing between 20-25 g, obtained from the Experimental Animal Facility of the All India Institute of Medical Sciences (AIIMS), New Delhi, were used in the study. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC). In the experimental group, mice (n=15) were injected with morphine at a dose of 10 mg/kg intraperitoneally (ip), twice daily for nine days at 0800 and 1700 h. Development of tolerance to morphine was determined by the decreased antinociceptive response in the hot plate (UGO basile) test, which was done 40 min after administration of morphine at the end of days 1, 5 and 9. In brief, mice were kept on a hot plate maintained at 51-52°C. The end point was taken when the mice either licked or raised the hindpaw or tried to jump off. The hot plate latency was also done for control group mice (n=15). These mice were injected with normal saline ip, in an equal volume to that of morphine, twice daily for nine days. At the end of 9th day, the mice were sacrificed by decapitation under ether anaesthesia. The cervical region of the spinal cord was dissected out after laminectomy. The tissue was snap-frozen in liquid nitrogen before being stored at -20°C. Cryostat sections (20 µ thick) were cut at -18°C and collected on gelatin-coated slides. Adjacent sections were stained with 0.5 per cent cresyl violet (Nissl staining) to localize the gray and white matter. The rest of the sections were stored at -20°C.

On the day of experiment, the sections were preincubated in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA) and 1mg/ml bovine serum albumin (BSA) for 30 min at room temperature (25°C) for ORL1 receptors and at 4°C for µ receptors. This was done to remove endogenous opioids bound to the receptors. A stock solution of 50 mM Tris-HCl buffer (pH 7.4) containing 1mM EGTA was made and used throughout the experiment.

**Autoradiography for ORL1 receptors:** The procedure was in accordance to that standardised earlier\textsuperscript{10}. Briefly, the sections were washed in Tris-HCl buffer twice for 5 min each. The sections were then incubated with tritium labeled nociceptin, (leucyl-3H) Nociceptin.
1 nM in Tris-HCl buffer containing 1mg/ml of BSA for 2 h. At the end of incubation, the sections were washed in cold Tris-HCl buffer at 4°C, four times for three minutes each. Final rinsing was done rapidly in cold distilled water. The sections were dried under a stream of hot air. Nonspecific binding was determined on some of the tissue sections using 1 µM (1000 fold excess of tritiated ligand) of plain nociceptin as competitor for radiolabelled nociceptin in the incubation buffer. A 1000 fold excess concentration of unlabelled nociceptin would displace the bound radioactive nociceptin from the receptors. This would not produce an image during autoradiography. The remaining experimental procedures were the same as for specific binding.

μ receptors: The procedure has been standardised earlier in our laboratory13. In brief, the sections were rinsed in Tris-HCl buffer at 4°C, twice for 1 min each. The sections were then incubated in Tris-HCl buffer, containing 2nM tritium labeled (D-ala²,N-methyl-phe⁴,-gly-ol⁵) (tyrosyl-3,5-²H) enkephalin [²H(DAMGO)] (specific ligand for µ receptor) and 1 mg/ml of BSA for one hour at room temperature. The sections were rinsed initially in cold Tris-HCl buffer, twice for 1 min each before being rinsed in cold distilled water. Finally, the sections were dried under hot air. Nonspecific binding was determined using 2 µM naloxone under the same conditions.

The slides bearing the dried sections were exposed to tritium sensitive hyperfilms for 8 wk at about 4°C in specially designed cassettes. Autoradiographic standards containing calibrated amounts of radioactivity were simultaneously exposed to the films. The films were developed in Kodak D19 developer for 5 min before being fixed in 30 per cent Hypo for 4 min. Films were then washed in running tap water. The autoradiographic images were photographed under the light microscope.

Quantitative analysis of autoradiographs: For quantitative analysis, both the autoradiographic standards and the autoradiographs were initially scanned. Optical density values from autoradiographic standards with relation to specified amount of radioactivity present in them were used to draw a standard curve. The laminae I-II were outlined together and the optical density in lamina I-II were determined in control (3-4 sections per animal) and experimental (3-4 sections per animal) groups. The background density was deducted to obtain the actual values. These values were then fitted to the standard curve to derive the receptor density. However, the values in nCi/mg indicate relative receptor density for comparison rather than actual receptor density. The software used was quantity 1 software from Biorad (USA).

Morphine sulphate was procured from Hospital Dispensary, AIIMS, (Leucyl-3,4,5-²H) Nociceptin (specific activity 133 Ci/mmol) was purchased from NEN, USA, plain nociceptin from Tocris Cookson, UK (²H) DAMGO (specific activity 63 Ci/mmol), hyperfilms and autoradiographic standards were procured from Amersham, UK. Naloxone hydrochloride was from Samarth Pharma Pvt. Ltd., Mumbai.

Statistical analysis: Student’s "t" test (unpaired) was applied to analyse the data, and P<0.05 was considered significant.

Results

Hot plate test: A significant increase (P<0.01) in the latency period required for nociceptive reponse was observed in experimental mice on day 1 as compared to days 5 and 9 of morphine treatment (Fig.1), followed by a sharp decrease in the latency values between days 1 to 5 and a smaller decrease between days 5 and 9. Thus, the treated mice developed tolerance to morphine as shown by the decrease in antinociceptive response between days 1 to 9.

Cresyl violet staining: Gray and white matter along with the laminar distribution of the various neuronal groups was observed in cryostat sections (Fig.2A).

Autoradiographic localization of ORL1 receptors: The ORL1 receptors were expressed selectively in the gray matter (Figs.2 B & C). An increased expression was seen in the superficial part of the dorsal horn which correspond to laminae I-II of the spinal cord and in lamina X. In the morphine tolerant group, a similar expression of ORL1 receptors as compared to controls was observed.
Fig.1. Hot plate test to show development of morphine tolerance. Values are mean±SEM for 15 mice in each group. *P<0.01 compared to days 5 and 9 in the same group.

Fig.2. (A) Cresyl violet stained section of cervical region of the mice spinal cord showing gray and white matter. The superficial laminae (I-II) (arrow head) contain predominantly smaller neurons as compared to remaining gray matter. Lamina X (enclosed by brackets) is situated around central canal. (B) Autoradiographic localization of ORL1 receptor in spinal cord in control animal. Higher expression of the receptor is noted in the superficial laminae (arrow head) and around the central canal (arrow). No expression of receptor is observed over white matter. (C) Autoradiographic localization of ORL1 receptor in morphine tolerant animal. An increased expression is noted in the superficial laminae (arrow head) and lamina X (arrow). The bar represents 250 µm.
Nonspecific image was at the level of background density (photomicrograph not given). This indicated that the autoradiographic image was due to specific binding of the ligand $[^3]H$ nociceptin to ORL1 receptors.

**Quantitation of receptor density:** The density of ORL1 receptor in control and treated animals were $0.187\pm0.09$ and $0.230\pm0.06$ nCi/mg respectively. For $\mu$ receptors, it was $0.632\pm0.14$ in control and $0.717\pm0.13$ nCi/mg in morphine treated animals. Though both ORL1 and $\mu$ receptors showed an increase in density in the treated group as compared to controls, it was not statistically significant ($P>0.05$).

**Discussion**

The present study showed that both ORL1 and $\mu$ receptors were expressed over the gray matter of the spinal cord in control and morphine tolerant mice. In control animals, higher expression of receptors was noted over the dorsal laminae (laminae I & II) of the spinal cord. Increased ORL1 expression was also observed over the central gray (lamina X) of the spinal cord.

_Nonspecific binding using 10³ fold excess naloxone, an antagonist of $\mu$ receptor, was at the level of background._

**Fig.3.** (A) Autoradiographic localization of $\mu$ receptor in control animal. Higher expression of receptors is present in laminae I-II (arrow head). (B) $\mu$ receptor localization in morphine tolerant animal. An increased expression was noted particularly in laminae I-II (arrow head). The bar represents 250 $\mu$m.
cord. Both these areas (dorsal laminae and central gray) also receive AO and C afferent fibres conveying nociceptive information from the periphery. Thus, these receptors play important role in modifying the transmission of pain signals. Earlier studies using radioligand binding, immunohistochemistry and in situ hybridization have also reported high density of ORL1 receptors over the dorsal laminae of the spinal cord.

Non significant increase seen in density of ORL1 receptors after morphine tolerance could be dose related. In a previous study, an up-regulation of

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ORL1 receptors in the spinal cord was observed after morphine tolerance produced by continuous morphine infusion into the intrathecal space by an osmotic minipump for seven days. The dose of morphine administered was much higher (7.5 µg/ml/h for 7 days) as compared to the present study. This appears so because we have observed a robust analgesic response lasting for about 5 h after intrathecal administration of 2.5 µg of morphine. A dose of 10 mg/kg morphine injected ip or sc in mice appears to be equivalent to 2.5 µg of morphine by intrathecal route; a ratio of 1:100. In another report, a specific antagonist of nociceptin (SB-612111) could reverse morphine tolerance. In this study, morphine tolerance was produced by administration of 50 mg/kg of morphine sc, once daily, for 4 days, which would have produced a high degree of tolerance. Expression of ORL1 receptors may also be determined by the degree of tolerance produced by mode of drug administration. For example, the degree of tolerance produced after morphine administration through intermittent boluses (15 mg/kg sc injected twice daily) for 5 days was less than that produced after continuous morphine administration (75 mg pellets implanted sc) for the same duration.

The expression of µ receptor after morphine tolerance increased but did not differ significantly from control. Earlier studies on density of µ receptor in spinal cord after morphine tolerance noted up-regulation, unchanged status and down-regulation (Table). In one study, ingestion of morphine in drinking water for 21 days led to almost 76 per cent increase in expression of µ receptor in the spinal cord of rat with only a slight increase in the brain. The authors suggested that factors regulating expression of µ receptor in the spinal cord could be different from that in the brain. In another study, a significant downregulation of µ receptors in spinal cord of morphine tolerant rat was reported and it was hypothesized that decrease in receptors is directly related to tolerance, as there would be lesser number of receptors for morphine to bind and produce analgesia (classical hypothesis of morphine tolerance). The variability in the findings may also be due to differences in the route or chronicity of administration of morphine.

It appears that tolerance to morphine develops despite variable alterations in the number of µ receptors on the cell surface. The primary reason may be due to altered interaction between the receptor and G proteins or adaptations within brain circuits. It is possible that many of the receptors may be desensitized and as such become unresponsive to morphine. It is also possible that the desensitized receptors are labeled autoradiographically by [³H]DAMGO. Enhanced internalization of µ receptors leads to delay in development of tolerance. The slight increase could well be due to increased synthesis of new µ receptors in response to desensitization of existing receptors. Recently, an early upregulation of µ receptors was noted in the microsomal fraction of forebrain after morphine tolerance. This was correlated with de novo synthesis of new receptors as well as activation of latent opioid receptors. Despite higher expression of receptors, tolerance occurs probably due to greater fraction of receptors that are desensitized than that synthesized/activated. More studies are required to elucidate the mechanism of morphine tolerance.

Acknowledgment

Authors acknowledge the Department of Biotechnology, Government of India, New Delhi and All India Institute of Medical Sciences (AIIMS), New Delhi for financial support.

References


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