Up-regulation of μ-opioid receptors in the spinal cord of morphine-tolerant rats

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Though morphine remains the most powerful drug for treating pain, its effectiveness is limited by the development of tolerance and dependence. The mechanism underlying development of tolerance to morphine is still poorly understood. One of the factors could be an alteration in the number of μ-receptors within specific parts of the nervous system. However, reports on changes in the μ-opioid receptor density in the spinal cord after chronic morphine administration are conflicting. Most of the studies have used subcutaneously implanted morphine pellets to produce tolerance. However, it does not simulate clinical conditions, where it is more common to administer morphine at intervals, either by injections or orally. In the present study, rats were made tolerant to morphine by injecting increasing doses of morphine (10–50 mg/kg, subcutaneously) for five days. *In vitro* tissue autoradiography for localization of μ-receptor in the spinal cord was done using [^{3}H]-DAMGO. As compared to the spinal cord of control rats, the spinal cord of tolerant rats showed an 18·8% increase or up-regulation in the density of μ-receptors in the superficial layers of the dorsal horn. This up-regulation of μ-receptors after morphine tolerance suggests that a fraction of the receptors have been rendered desensitized, which in turn could lead to tolerance.

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1. Introduction

Pain is undoubtedly, the most important among the different somatic and visceral sensations. Opioids like morphine form the mainstay for effective treatment of pain. However, opioids also produce serious side effects like tolerance and dependence. Tolerance is indicated by a progressive increase in the dose, needed to produce an equivalent pain relief while dependence is manifested by withdrawal symptoms on discontinuing the drug. The exact underlying cellular mechanism responsible for tolerance and dependence is not known. One of the reasons could be a change in the number or affinity of μ-opioid receptor – the specific opioid receptor – that predominantly binds to morphine (Fang *et al* 1986).

The μ-opioid receptors, acting via G proteins (G_i/G_o subtypes), cause an increase in potassium and a decrease in calcium levels within neurons (North 1993). Consequently, opioids have an inhibitory effect on neurons. Previous studies on changes in μ-receptor density in the central nervous system after morphine-induced tolerance have reported conflicting findings. An up-regulation or a higher density of the receptors was noted in some studies (Pert and Snyder 1976; Lewis *et al* 1984; Brady *et al* 1989; Rothman *et al* 1991; Besse *et al* 1992). However, these studies were mainly radioligand assays and reported an increase in hypothalamus, striatum and amygdala (Brady *et al* 1989) and in the whole brain (Pert and Snyder 1976; Lewis *et al* 1984; Rothman *et al* 1991). In another study, an increased density of μ-receptors or up-regulation (26%) of the receptors was noted in the spinal cord after autoradiography (Basse *et al* 1992). Other studies found no change in the receptor number (Dum *et al* 1979; Gouarderes *et al* 1993). A decrease in receptor density or

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down-regulation has also been reported (cerebral cortex, brainstem and spinal cord, Bhargava and Gulati 1990; brainstem, Bernstein and Welch 1998; spinal cord, Diaz *et al* 2000). Most of the studies have used subcutaneously implanted morphine pellets to produce tolerance. However, it does not simulate clinical conditions, where it is more common to administer morphine at intervals, either by injections or orally.

The present study was undertaken to find out changes, if any, in the μ-receptor expression in the spinal cord of morphine-tolerant rats, using *in vitro* tissue autoradiography. A preliminary report of the present work was earlier presented in abstract form (Gupta *et al* 2002).

2. Materials and methods

2.1 *Induction of tolerance*

Male albino Wistar rats (weight 210 g–235 g) were procured from the Experimental Animal Facility of the All India Institute of Medical Sciences. The animals were housed in well-ventilated cages, and water and food was given *ad libitum*.

For induction of morphine tolerance, the rats ($n = 5$) were subcutaneously injected 3 times a day (15:00, 23:00, 6:00 h) for 5 days with increasing doses of morphine sulphate IP, starting from 10 mg/kg body weight on the first day to 50 mg/kg body weight on the 5th day, at progressive increments of 10 mg/kg, every day. In the control group, rats ($n = 5$) were injected with water for injection IP in the same volume instead of morphine. The development of tolerance was monitored everyday, 40 min after injecting a test dose of morphine (10 mg/kg body weight) near the end of 24 h period (14:00), using the hot plate test (Eddy and Leimbach 1953). Briefly, the rats were placed on the hot plate maintained at a constant temperature of 56°C. The licking of the hindpaw was taken as the end-point. The other reactions that could have been measured are the time required for jumping or the licking of the forepaw. However, opioids affect only the licking behaviour in the hot-plate test and a more consistent response was observed for the hindpaw than the forepaw. A cut off limit of 45 s was kept to prevent tissue injury. The value of 45 s was considered for statistical analysis in such cases as a 100% response. Rats that did not respond were taken away. Statistical analysis was done using Student's *t*-test (paired).

2.2 *Tissue samples*

At the end of fifth day, the rats were sacrificed under ether anesthesia. The spinal cords were dissected out after laminectomy and snap-frozen in liquid nitrogen before being stored in – 20°C freezer. Transverse sections (20 μ) of the cervical region of spinal cord were cut at – 18°C in a cryostat. Sections (5–6) were collected from each of the control and treated rats. As morphine was given systemically, all parts of the spinal cord were exposed to morphine. The cervical region was taken as a reference point to ensure consistency for the present and future studies on spinal cord after morphine tolerance.

2.3 *Autoradiography*

The technique of *in vitro* tissue autoradiography for the localization of μ-receptor has been standardized in this laboratory earlier (Ray and Wadhwa 1999). Briefly, the sections were preincubated in 50 mM Tris-HCl buffer containing 150 mM NaCl and 1 mM EGTA for 30 min at 4°C to remove the endogenous ligands and administered drugs and increase the availability of the ligand to the binding sites. EGTA is a peptidase inhibitor which prevents autolysis of the receptors. After rinsing, the sections were incubated in 50 mM Tris-HCl buffer containing 2 nM of [^{3}H] [D-ala^2, N-methyl-Phe^4, -Gly-ol^5] enkephalin (DAMGO, a highly specific ligand for labelling μ-receptor) for 1 h at room temperature. They were then apposed to [^{3}H] sensitive hyperfilms for 7 weeks at about 4°C. Non-specific binding was determined under the same experimental conditions using 1000-fold excess levorphanol (2 μM) and the cryostat sections exposed to the autoradiographic films.

[^{3}H]-DAMGO was purchased from Amersham, UK. The hyperfi1ms and radioactive standards were from Amersham, UK and levorphanol was from Sigma, USA. Morphine was obtained from a Government agency after obtaining necessary permission from the Narcotics Commissioner.

2.4 *Densitometric analysis*

Densitometric analysis of the autoradiographic images was carried out using Adobe Photoshop version 5·5 software (Adobe Systems Incorporated). The procedure was carried out in the presence of an observer who was blinded to the drugs administered (treated vs control). The software was used to measure the average brightness of pixels within a user defined area. Average brightness of pixels was measured for the superficial laminae of the dorsal horns (DH_{br}), the remaining grey matter (RGM_{br}) and the background (BG_{br}). Density (average darkness per pixel) was calculated from the brightness values (range = 0–255) by subtracting from 255 (DH_d, RGM_d and BG_d). To account for the background density, the functions (DH_d–BG_d) and (RGM_d–BG_d) were calculated for each measurement and analysed. Significance levels were calculated using Student's paired *t*-test.

3. Results

3.1 *Development of tolerance*

Increasing doses of morphine injection produced a corresponding increase in analgesic response as seen from the increase in latency values of the hot plate test, which was significant from day 1 to day 4 ($P < 0{\cdot}05$) as compared to baseline values (figure 1). However, a decrease in the analgesic effect appeared on the 3rd day of injection and the response returned to almost the basal values on the 5th day. On injecting water for injection in a similar schedule in control rats, there was an analgesic response, which was significant on the third day ($P < 0{\cdot}05$) but returned to the baseline on day 5.

3.2 *Autoradiographic findings*

In the spinal cord, μ-receptors were observed over laminae I–II of the dorsal horn (figure 2). This was similar to our previous study, where we had compared Nissl-stained sections with autoradiographs to note the exact site of expression of receptors (Ray 1996). As compared to the spinal cord of control rats (figure 2a), the spinal cord of four of the morphine-tolerant rats showed a highly significant increase in the density of μ-receptor expression in the laminae I–II of dorsal horn (figure 2b, table 1a). When the values of all the treated rats were combined, the density (DH_d–BG_d) was 18·8% higher as compared to the control rats, with $P = 0{\cdot}0006$ (table 1b). No significant change in receptor expression was noted over the remaining parts of gray matter. Non-specific binding was negligible and was at the level of background, which indicated that the autoradiographic labelling was specific for μ-receptors (photomicrograph not given).

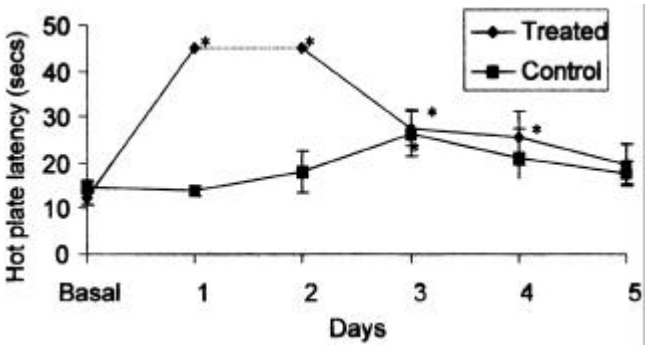

Figure 1. The morphine-treated group showed a hot plate latency period which was significantly higher than the baseline, from day 1 to 4 ($P < 0{\cdot}05$). But the latency period decreased from the 3rd to the 5th day indicating development of tolerance. The control group showed an increase in the latency period, which was statistically significant ($P < 0{\cdot}05$) only on 3rd day.

4. Discussion

In the present study, μ-opioid receptors were noted in the superficial laminae (laminae I–II) of the dorsal horn of spinal cord. Previous studies have reported similar findings. This is because the A*d* and C afferent fibres, which transmit pain from the periphery, terminate mainly on neurons in superficial laminae, where they form synaptic connections. These receptors are mostly presynaptically located on the central processes of neurons of the dorsal root ganglia (Gouarderes *et al* 1991). The remaining receptors are present on a population of small dorsal horn neurons, which are mainly excitatory (Kemp *et al* 1996). The endogenous opioids are also released at these sites,

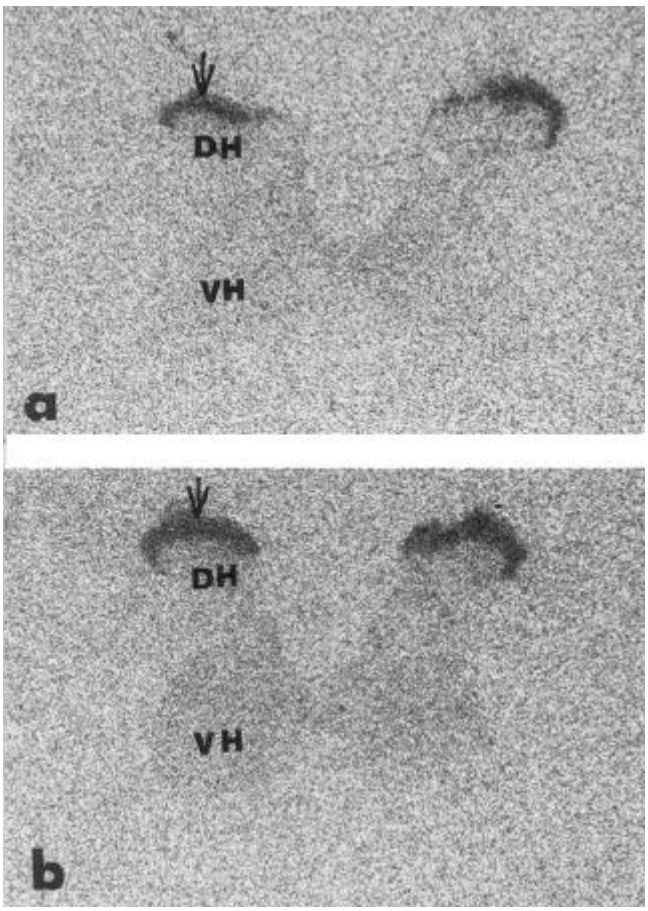

Figure 2. Autoradiographic localization of μ receptors in spinal cord (X26). **(a)** Control: superficial laminae (I–II) (arrow) of the dorsal horn express the receptor. **(b)** Treated: increased expression of receptor over superficial laminae (arrow). VH, Ventral horn; DH, dorsal horn.

which bind on to the μ-receptors and decrease neuronal excitability through mechanisms referred to earlier. The μ-receptor is the predominant opioid receptor in the spinal cord, comprising 70 ± 4% of the total opioid receptors, as compared to *d* (23 ± 2%) and *k* (7 ± 1%) receptors (Besse *et al* 1990).

In morphine tolerant rats, an up-regulation or increase in density of μ-receptors (18·8%, table 1), was noted over the superficial laminae (I–II), as compared to controls. A higher density would imply that receptors have become desensitized, which have been compensated by synthesis of new receptors. Synthesis of new receptors, consequent to morphine tolerance, has been shown recently (Fabian *et al* 2002). It has been suggested that either the structure or conformation of the existing μ-receptors is altered in the morphine-tolerant state (Bernstein and Welch 1998). It has also been shown that the interaction between the receptors and G proteins is altered in tolerance (Tao *et al* 1993). Thus, the increase in receptors could be an attempt by the neurons to preserve homeostasis as a result of which more receptors are synthesized and inserted on the cell membrane. Tolerance would only result if more receptors are rendered desensitized than are synthesized. Moreover, it is possible that desensitized receptors are able to bind to morphine but are unable to transmit the signal to the intracellular machinery.

To our knowledge, there exists one study, which reported up-regulation of μ-receptor in the spinal cord using autoradiography (Besse *et al* 1992). However, our work was different in that morphine was administered subcutaneously for 5 days in an escalating dose, which is similar to the treatment for cases of acute and severe pain like burns. Increase of μ-receptors in specific areas of the brain like the hypothalamus and striatum in morphine tolerance has also been reported (Brady *et al* 1989). Up-regulation was also noted in hepatic encephalopathy (Bergasa *et al* 2002).

Many studies have also reported a decrease of receptors or down-regulation (Tao *et al* 1987, 1990; Bhargava and Gulati 1990; Bernstein and Welch 1998; Diaz *et al* 2000). This is also thought to be a mechanism for development of tolerance as less receptors are available for morphine to bind to and produce analgesia. Rapid phosphorylation of μ-receptors due to activity of G protein coupled receptor kinases (GRKs) and *b*-arrestins could be responsible. The role of *b*-arrestins in μ-receptor desensitization has been recently shown (Bohn *et al* 2000). Some studies have even shown no change in the receptor

Table 1. Comparison of the average density of **(a)** the dorsal horn in the autoradiographic images of control with each of the treated rats, **(b)** the dorsal horn and the remaining grey matter of control rats, and treated rats combined.

(a)

Comparison of the dorsal horn density in control rats with each of the treated rats						
		Treated rats				
	Control rats	1	2	3	4	5
Average DH_d–BG_d	96·31	120·30	119·76	98·33	120·14	116·20
Standard deviation	5·15	7·22	12·81	6·57	6·55	5·09
t-test sig. with control (2-tailed, homoscedastic)	–	< 0·0001	0·0025	0·556	< 0·0001	< 0·0001
Increase (%)	–	24·9	24·3	–	24·7	20·6

(b)

Average background density (BG_d): control = 68·538; treated = 64·757

Dorsal horn			Remaining grey matter		
	Control	Treated		Control	Treated
Average density (DH_d)	164·852	179·185	Average density (RGM_d)	89·788	87·336
Average DH_d–BG_d	96·313	114·427	Average RGM_d–BG_d	21·250	22·656
Standard deviation	5·146	11·525	Standard deviation	9·036	8·656
t-test sig. (2-tailed,homoscedastic)* = 0·0006			*t*-test sig. (2-tailed, homoscedastic)** = 0·721		
Percentage increase = 18·8%			–		

DH_d, Dorsal horn density; RGM_d, remaining grey matter density; BG_d, background density.
*Levene's test for equality of variance = 0·06.
**Levene's test for equality of variance = 0·80.

density (Dum *et al* 1979; Gouarderes *et al* 1993). It is possible that the amount of morphine injected and the route of administration could play a role in the varying levels of μ-receptors, observed under different experimental conditions. The receptor density did not alter when morphine was administered through continuous intrathecal route (Gouarderes *et al* 1993). A decrease in receptor density was reported when morphine pellets were implanted subcutaneously (Bhargava and Gulati 1990). The development of tolerance is also related with the route of administration. A recent study has shown that tolerance to morphine develops significantly less frequently on direct intrathecal administration into the spinal canal than on systemic administration through subcutaneous morphine pellet implantation (Riba *et al* 2002). In the present study, an escalating dose was used (10–50 mg/kg) which produced appreciable degree of tolerance within a short period of time. This was thought to resemble clinical situations where morphine is administered through injections or orally, thrice daily for prolonged periods of time.

The increase seen in the latency values in control rats, which was significant on day 3, might be related with the secretion of endogenous opioids, in response to stress (Kelly 1982). This would not be an important factor in the treated group due to sedation produced by morphine.

We believe that the present experiment could also serve as a model by which the extent of tolerance can be adjudged after co-administration of other drugs to modify the development of tolerance.

5. Conclusion

The present study was undertaken to find out changes in the density of μ-opioid receptor in the spinal cord of rats after morphine tolerance. As compared to control rats, the spinal cord of morphine-tolerant rats showed an 18·8% increase or up-regulation in the density of μ-receptors in laminae I–II of the dorsal horn. This was significant because nerve fibres which transmit pain from the periphery, terminate on neurons in laminae I–II. This up-regulation could be an adaptive response to desensitized μ-receptors caused by morphine tolerance.

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