BEHAVIORAL AND ELECTROPHYSIOLOGICAL EVIDENCE FOR TOLERANCE TO CONTINUOUS MORPHINE ADMINISTRATION INTO THE VENTROLATERAL PERIAQUEDUCTAL GRAY

D. A. LANE, a V. TORTORICI b AND M. M. MORGAN a*

aWashington State University Vancouver, 14204 Northeast Salmon Creek Avenue, Vancouver, WA 98686, USA
bInstituto Venezolano de Investigaciones Cientificas, Caracas 1020-A, Venezuela

Abstract—Repeated microinjections of morphine into the ventrolateral periaqueductal gray (vPAG) produce tolerance to the antinociceptive effect of morphine [Behav Neurosci 113 (1999) 833]. These results may be a direct effect of morphine on cells within the vPAG or be caused by cues linked to the microinjection procedure (i.e. associative tolerance). The objective of this paper was to determine whether continuous administration of morphine within the vPAG will produce tolerance. Tolerance was assessed by measuring changes in behavior and changes in the activity of neurons in the rostral ventromedial medulla (RVM), the primary output target of the PAG. Rats were implanted with an osmotic minipump that released morphine (2.5 or 5 μg/h) or saline into the vPAG continuously. Continuous administration of morphine produced an increase in hotplate latency when measured 6 h after initiation of treatment. Tolerance to this antinociception was evident within 24 h. After 3 days, rats were anesthetized and the activity of RVM neurons was assessed. Although acute morphine administration into the RVM inhibits the activity of RVM on-cells and enhances the activity of off-cells, these neurons appeared normal following 3 days of continuous morphine administration. Systemic naloxone administration produced hyperalgesia that was associated with a marked increase in on-cell activity and a complete cessation of off-cell activity. The loss of morphine inhibition of nociception, measured behaviorally and electrophysiologically, demonstrates that tolerance is caused by a direct action of morphine on vPAG neurons.

Key words: antinociception, opiate tolerance, PAG, rostral ventromedial medulla, withdrawal.

Systemic administration of opiates produces antinociception, at least in part, by activation of the periaqueductal gray (PAG). Microinjections of morphine into the PAG produce antinociception (Jensen and Yaksh, 1989; Yeung et al., 1977). Repeated microinjections of morphine into the ventrolateral PAG (vPAG) produce tolerance as witnessed by a decrease in antinociception across trials (Siuciak and Advokat, 1987; Tortorici et al., 1999).

The PAG produces antinociception via a descending system that includes the rostral ventromedial medulla (RVM). The RVM is of particular interest because two classes of neurons within the RVM, on- and off-cells, have been shown to modulate nociception. On-cells respond to nociceptive stimuli with a sharp increase in activity just prior to a nociceptive reflex, whereas off-cell activity decreases prior to a nociceptive reflex. Acute morphine administration, whether systemic, into the PAG, or into the RVM, results in decreased on-cell activity and continuous off-cell activity (Cheng et al., 1986; Fields et al., 1983; Morgan et al., 1992; Tortorici et al., 2001). These findings indicate that on-cells facilitate and off-cells inhibit nociception. Repeated microinjections of morphine into the vPAG result in a loss of inhibition of RVM on-cells and a loss of activation of off-cells (Tortorici et al., 2001).

Tolerance to morphine may be caused by direct effects of morphine on cells within the vPAG or because of contextual cues associated with drug administration (i.e. associative tolerance). Associative tolerance follows a classical conditioning paradigm in which cues become linked to the drug administration procedure and elicit a conditioned response in the opposite direction to the unconditioned response (Siegel, 1975, 1976). In other words, cues linked to morphine administration activate processes that counteract morphine antinociception. The repeated microinjections used in previous studies examining morphine tolerance included cues that could have contributed to the development of tolerance (Jacquet and Lajtha, 1975; Lewis and Gebhart, 1977; Siuciak and Advokat, 1987; Tortorici et al., 2001, 1999). By administering morphine without cues, the degree to which tolerance develops because of morphine’s action on cells within the vPAG can be assessed.

The objective of this manuscript is to determine whether continuous morphine administration into the vPAG produces tolerance. Tolerance will be assessed using both behavioral and electrophysiological measures so as to determine the location of the changes underlying tolerance. If tolerance is caused subsequent to the RVM, then RVM neurons should show normal responses to PAG morphine. However, it is hypothesized that tolerance is caused by a change in the vPAG and thus, neurons in the RVM should be tolerance to PAG morphine.

EXPERIMENTAL PROCEDURES

Subjects

Male Sprague–Dawley rats (weighing 260–430 g) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and implanted with
a guide cannula (23 gauge) aimed at the vPAG using stereotaxic coordinates (Paxinos and Watson, 1986). The guide cannula was affixed to the skull with two screws and cranioplasti cement. The rats were housed individually following surgery with food and water available ad libitum. Lights were maintained on a reverse 12-h light/dark cycle (off at 7:00 a.m.). Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of Washington State Universiti es IACUC. Efforts were made to minimize the number and potential suffering of experimental subjects.

The rats were handled daily to decrease stress associated with handling. Three days following guide implantation, each animal received a “fake injection” in which an 11 mm injector was inserted into the guide cannula but no drug was administered. This procedure habituated the animals to the testing procedure and reduced effects resulting from mechanical damage to neurons on test day. The day after the fake injection, all rats received a microinjection of morphine (5 \( \mu \)g/0.4 \( \mu \)l) to determine whether antinociception could be evoked from the injection site. Antinociception was assessed using the hotplate test. The hotplate test measures the latency to lick a hind paw when a rat is placed on a 52 °C surface. Rats were removed from the hotplate if they did not respond within 40 s. Animals that did not demonstrate antinociception (hotplate latency <12 s) from the acute morphine microinjection were not included in the study.

Experiment 1: behavioral effects of continuous morphine

The day following the morphine pretest, animals underwent a second brief surgery to implant an Alzet osmotic mini-pump (Du-rect Corp., Cupertino, CA, USA). Rats were anesthetized with halothane and the caudal end of the previous scalp incision (made for cannula placement) was reopened via blunt dissection and the osmotic pump was pushed s.c. until it was positioned between the rat’s scapulae. The pump was connected to 3A vinyl tubing, which exited through the scalp incision. The tubing was attached to a 31-gauge injector. The injector was bent at a 90° angle with an 11 mm injector portion and a 2 mm portion attached to the tubing. Once the injector was inserted into the guide cannula, the injector was covered in cranioplasti cement to keep the injector perma-
nently in place. Following pump implantation, rats were placed back in their home cages. Nociception was assessed using the hotplate test 2, 6, 24, and 48 h following osmotic pump implantation.

Morphine or saline was administered continuously throughout the experiment via the osmotic mini-pump. Rats received pumps filled with either 5 or 10 \( \mu \)g/\( \mu \)l of morphine sulfate (gift from National Institute on Drug Abuse) or saline. The osmotic pumps infused solution at a rate of 0.5 \( \mu \)l/h. Thus, rats received either 2.5 or 5 \( \mu \)g of morphine per hour into the vPAG.

Experiment 2: physiological effects of continuous morphine

A subset of the rats used in the behavioral study, were anesthe-
tized with sodium pentobarbital (55 mg/kg, i.p.) (Nembutal; Abbott Laboratories, Abbott Park, IL, USA) 48–96 h post osmotic pump implantation for single unit recording of RVM neurons. A PE-20 catheter was inserted into the jugular vein for continuous admin-
istration of the anesthetic methohexital sodium (Breval; Monarch Pharmaceuticals, Bristol, TN, USA). Rats were placed in a ste-
reotaxic frame and a small craniectomy (2×2 mm) was performed caudal to the PAG injection site to allow recording of neurons in the RVM.

The rats were allowed to recover partially from pentobarbital so that it was possible to elicit a tail flick (TF) reflex to immersion of the caudal end of the tail (5 cm) into 52 °C water. Once this anesthetic plane was reached, methohexital sodium was infused at a constant rate (15–30 mg/kg/h) through the jugular catheter using an infusion pump. This maintained animals in a lightly anesthetized state with a baseline TF latency of approximately 5–6 s and prevented any signs of discomfort or spontaneous movement.

A stainless steel microelectrode (FHC, Bowdoinham, ME, USA) was stereotaxically lowered into the RVM to record the activity of RVM neurons. Single unit activity was isolated from background noise and neurons were characterized as on- or off-cells based on a burst or pause in activity immediately preceding the TF reflex (Fields et al., 1983). Only on- and off-cells were studied. TF latencies were assessed every 5 min until three con-
secutive stable baseline latencies were obtained. After the third baseline test, rats were injected with naloxone (1 mg/kg, s.c.) to induce withdrawal. At least three additional TF latencies were obtained at 5 min intervals. The osmotic pumps continued to deliver morphine into the vPAG throughout testing.

Statistical analysis

Hotplate data were analyzed using a two factor (dose×time) repeated measures ANOVA with an \( \alpha \) level of 0.05. Post hoc comparisons were completed using Tukey test. All other data were analyzed using Student’s \( t \) test with an \( \alpha \) level of 0.05.

RESULTS

Experiment 1: behavioral effects of continuous morphine

Behavioral data were collected from 37 rats. All injections were in the vPAG or within 0.3 mm of the vPAG (Fig. 1). Ten rats received 2.5 \( \mu \)g/h of morphine into the vPAG, 15 received 5 \( \mu \)g/h of morphine into the vPAG. Saline was infused into the vPAG in 10 rats. Morphine infusion at more dorsal sites produced continuous explosive running. Mor-
phine infusion was immediately stopped in these rats to prevent injury or death. Thus, data from these rats were not included in the analysis. Two rats received 5 \( \mu \)g/h of morphine approximately 1 mm below the vPAG as place-
ment controls. Antinociception was not produced form the placement control rats at any of the test times (2, 6, 24, or 48 h) following osmotic pump implantation.

Infusion of morphine into the vPAG initially produced a significant increase in hotplate latency compared with the saline-treated animals (\( F(2,32)=24.59, P<0.05 \)). Post hoc comparisons indicate significantly higher hotplate latencies for the 2.5 \( \mu \)g/0.5 \( \mu \)l/h dose of morphine compared with saline controls 2 h after drug infusion began and at two and 6 h post drug infusion for the 5 \( \mu \)g/0.5 \( \mu \)l/h dose of mor-
phine (Fig. 2). As drug infusion continued, hotplate laten-
cies for rats receiving morphine significantly decreased across trials (\( F(3,60)=4.47, P<0.05 \)). This return to control levels 24 h after drug infusion began indicates that toler-
ance developed to vPAG morphine infusion (Fig. 2).
Experiment 2: physiological effects of continuous morphine

A subset of the rats used in experiment 1 (5 μg/h dose of morphine or saline), was used for electrophysiological recording of RVM cells in experiment 2. Data were collected from 23 RVM neurons (12 on-cells and 11 off-cells) in 19 rats. Osmotic pump administration into the vPAG was maintained throughout the recording period.

The rats were tested on the TF test while neurons within the RVM were simultaneously recorded. The mean TF latency for the morphine (6.44 ± 0.80 s) and saline (5.65 ± 1.25 s) groups did not differ (t(14) = 1.42, P > 0.05), even though morphine was still being administered to the morphine group. This finding confirms the hotplate data showing that tolerance has developed.

Continuous morphine administration into the vPAG had no effect on mean spontaneous on-cell activity (morphine group = 3.67 ± 1.74 Hz; saline group = 1.67 ± 0.80 Hz; t(10) = 1.041, P > 0.05) or spontaneous off-cell activity (morphine group = 23.00 ± 7.50; saline group = 17.50 ± 4.75; t(9) = 0.642, P > 0.05). Acute morphine administration into the vPAG causes off-cells to become continuously active and on-cells to become inactive (Cheng et al., 1986; Morgan et al., 1992; Tortorici and Morgan, 2002). The present data show that these effects are lost with continuous morphine administration into the vPAG.

Noxious heat caused a burst of activity in on-cells that preceded the TF reflex (Fig. 3). This burst of activity was evident in both morphine- and saline-treated rats indicating that the inhibitory effect of PAG morphine administration had been lost (i.e. rats were tolerant). There were no significant changes in heat-evoked on-cell activity (t(10) = 1.680, P > 0.05) or heat-evoked off-cell activity (t(9) = 0.044, P > 0.05).

Naloxone administration had a dramatic effect on RVM cells in rats receiving morphine, whereas it had virtually no effect on RVM cells in rats receiving saline. Fig. 4 shows an increase in spontaneous on-cell activity (t(10) = 3.73, P < 0.05; top left of Fig. 4) and a complete inhibition of

![Fig. 1. Location of injection sites in the PAG. Triangles indicate 2.5 μg/h morphine, squares indicate 5 μg/h morphine administration, circles indicate saline administration, and X's indicate 5 μg/h morphine placement controls. Distance anterior to the interaural line is indicated at the bottom of each section (Paxinos and Watson, 1986).](image)

![Fig. 2. Change in hotplate latency during continuous morphine administration into the vPAG. There was a significant increase in hotplate latencies in both morphine groups following osmotic pump implantation. Tolerance to this antinociceptive effect was evident in both groups by 24 h post-implantation.](image)

![Fig. 3. Representative traces of on- and off-cell heat-evoked activity following 72 h of morphine infusion into the vPAG. Placing the tail in 52 °C water (H) cause a burst of activity immediately prior to the TF in the on-cell. This occurred whether morphine or saline was administered into the vPAG, suggesting the development of tolerance. The activity of off-cells, in contrast, pauses immediately prior to the TF. This effect occurred whether morphine or saline was administered into the PAG.](image)
off-cell activity ($t(10)=3.065, P<0.05$; bottom left of Fig. 4) within minutes of naloxone administration. Examples of the drastic changes in ongoing on- and off-cell activity are shown in Fig. 5. Naloxone administration in rats receiving saline into the vPAG did not alter spontaneous on-cell ($t(10)=0.126, P>0.05$) or off-cell activity ($t(10)=0.246, P>0.05$).

Heat evoked cellular activity was also altered by naloxone administration. Naloxone administration in rats that received morphine produced a significant increase in heat-evoked on-cell activity ($t(10)=2.361, P<0.05$; top right of Fig. 4), and a decrease in heat-evoked off-cell activity in rats that received morphine ($t(9)=4.247, P<0.05$; bottom right of Fig. 4). These changes in cellular activity correlated with changes in TF latencies. Administration of naloxone caused a significant decrease in TF latencies for rats receiving morphine as compared with saline controls ($t(14)=3.99, P<0.05$), indicating the development of hyperalgesia (Fig. 6). Naloxone administration had no effect on heat-evoked on-cell ($t(8)=0.730, P>0.05$) or off-cell activity ($t(8)=0.04, P>0.05$) in rats receiving saline into the vPAG.

These findings are consistent with numerous other reports of tolerance to morphine administration into the PAG (Jacquet and Lajtha, 1976; Lewis and Gebhart, 1977; Siuciak and Advokat, 1987; Tortorici et al., 1999, 2001, 2003). However, this finding is original in showing tolerance to continuous morphine administration into the vPAG. All previous reports of tolerance to PAG morphine administration used repeated microinjections to produce tolerance. Repeated systemic administration of morphine has been shown to produce associative tolerance, that is, a higher degree of tolerance when drug administration is linked to a specific environment (Schnur et al., 1992; Siegel, 1975, 1976). Subsequent work has shown that almost any stimulus can potentially be a cue to evoke associative tolerance including the drug or drug administration procedure itself (Cepeda-Benito and Tiffany, 1993; Grisel et al., 1996; Walker et al., 1981). Although repeated microinjections of morphine into the vPAG have been shown to produce tolerance, the degree to which this tolerance is caused by morphine (non-associative tolerance) or by cues predicting morphine administration (associative tolerance) was not known. In the present study, tolerance developed when morphine was administered without handling the animals (no cues for drug administration) indicating that associative cues are not necessary for tolerance to develop.

Associative and non-associative tolerance have been shown to have physiologically distinct mechanisms in the amygdala (Mitchell et al., 2000) and spinal cord (Grisel et al., 1996). The vPAG may be part of a system that produces non-associative tolerance; however, whether the vPAG also contributes to associative tolerance has not been determined.

**DISCUSSION**

Continuous morphine administration into the vPAG resulted in a rapid onset of tolerance. This tolerance was evident both behaviorally and electrophysiologically. Morphine administration caused an increase in hotplate latency that disappeared within hours of continuous administration. This tolerance was also evident in the normal response of RVM on- and off-cells after 3 days of morphine administration into the PAG.

![Fig. 4. Spontaneous- and heat-evoked on- and off-cell activity before and after naloxone administration. These graphs show tolerance to morphine in that cells respond the same regardless of morphine or saline administration (pre-naloxone data). Naloxone precipitated withdrawal is associated with increased on-cell activity and a decrease in off-cell activity. Naloxone administration caused a significant increase in spontaneous (top left) and reflex relate (top right) on-cell activity in rats receiving morphine. Naloxone administration produced a significant decrease in spontaneous (bottom left) and reflex related (bottom right) activity in rats receiving morphine. Naloxone administration had no effect on the activity of saline-treated rats.](image-url)
Acute morphine administration into the vPAG results in continuous off-cell activity and inhibition of on-cell activity (Cheng et al., 1986; Morgan et al., 1992; Tortorici and Morgan, 2002). This cellular activity is associated with inhibition of nociceptive behavior (increase in TF latencies). However, with continuous morphine administration, tolerance was demonstrated by “normal” on- and off-cell activity. That is, off-cells paused and on-cells had a burst of activity immediately preceding the TF response despite continuous infusion of morphine into the vPAG. Moreover, there were no significant differences in TF latencies between rats receiving morphine or saline when tested 48–96 h after initiation of morphine administration. A similar tolerance was demonstrated by repeated microinjections of morphine into the vPAG (Tortorici et al., 1999).

Both on and off-cells project to the spinal cord (Fields et al., 1995; Vanegas et al., 1984), allowing the RVM to inhibit or facilitate pain. TF latencies are shorter when on-cells are spontaneously active (Heinricher et al., 1989), and continuous on-cell activity has been linked to hyperalgesia (Bederson et al., 1990). These findings suggest that on-cells facilitate nociception. The pronounced increase in on-cell activity and facilitation of the TF reflex following naloxone administration is consistent with this interpretation.

The enhanced activation of on-cells following naloxone administration in rats treated with vPAG morphine for 3 days appears to be caused by morphine withdrawal. Morgan et al. (1992) found that acute morphine administration into the PAG resulted in a decrease in on-cell activity. This on-cell activity returned to baseline levels following a systemic injection of naloxone. Thus, the increase in on-cell activity in the present study must be a product of continuous morphine administration in the vPAG. In contrast, Bederson et al. (1990) found a significant increase in on-cell activity in rats that received acute systemic morphine followed by naloxone.

**Fig. 5.** Top: Rate meter record of an on-cell with continuous morphine administration into the vPAG before and after naloxone administration. TF indicates when the rat flicked its tail following placement into 52 °C water. Each TF is associated with a burst of activity despite morphine administration into the vPAG. Administration of naloxone produced a large increase in on-cell activity (spontaneous and heat evoked). Bottom: Rate meter record of an off-cell with continuous morphine administration into the vPAG before and after naloxone administration. Each TF is associated with a pause in off-cell activity despite morphine administration into the vPAG. Administration of naloxone produced an almost complete cessation of off-cell activity.
Therefore, the conflicting findings may be due to the different sites of action of morphine.

Regardless, systemic naloxone administration following chronic morphine administration produced withdrawal symptoms such as shorter TF latencies and drastic changes in the activity of RVM on- and off-cells. This finding is important because it indicates that tolerance to repeated administration of morphine is not due to the loss of morphine’s action on the cell, but a change in how the descending system functions. Many theories of tolerance suggest that a continuous presence of morphine results in desensitization and/or downregulation of µ-opioid receptors on the cellular membrane (Harrison et al., 1998). If these theories are accurate and morphine loses its ability to affect the cell, application of an opiate antagonist, should produce a significant change in cellular activity or nociceptive behavior. In contrast, naloxone administration resulted in a dramatic increase in on-cell activity and shorter TF latencies. Thus, morphine does not lose its effect on the cell, the cell’s response to morphine changes.

An important question is which neurons are changing as tolerance develops to morphine administration into the vPAG? Tolerance could be caused by a change in opioid sensitive neurons in the vPAG or anywhere along the descending system. Our finding that RVM neurons appear normal despite morphine administration into the vPAG suggests that the change precedes the RVM. This is consistent with the finding that activation of PAG output neurons does not produce tolerance (Morgan et al., 2003).

In the present study, morphine was only administered to the vPAG supporting the idea that changes seen in the RVM are driven by changes in the vPAG. Although morphine has been shown to directly activate some PAG neurons (Osborne et al., 1996), morphine antinociception is generated by disinhibition of GABAergic interneurons which leads to activation of PAG output neurons (Behbehani et al., 1990; Depaulis et al., 1987; Moreau and Fields, 1986; Stiller et al., 1996). Tolerance does not occur with repeated activation of PAG output neurons (Morgan et al., 2003), thus, the mechanism for tolerance must be located in the opioid-sensitive GABAergic neurons that inhibit PAG output neurons. In vitro single cell recordings showing changes in the intracellular signaling in opioid-sensitive GABAergic neurons in the PAG in morphine tolerant rats are consistent with these behavioral findings (Chieng and Christie, 1996; Ingram et al., 1998).

In conclusion, this study demonstrates that tolerance to morphine administration into the vPAG can occur independent of associative cues. It appears that tolerance is caused by a direct action of morphine on neurons in the vPAG.

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