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Antinociceptive tolerance revealed by cumulative intracranial microinjections of morphine into the periaqueductal gray in the rat

Michael M. Morgan *, Erin N. Fossum, Carly S. Levine, Susan L. Ingram

Department of Psychology, Washington State University Vancouver, USA

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Abstract

The periaqueductal gray (PAG) appears to play a key role in morphine antinociception and tolerance. The objective of this manuscript is to develop a cumulative dose microinjection procedure so the hypothesized role of the PAG in morphine antinociceptive tolerance can be assessed using dose–response analysis. Rats were implanted with a guide cannula into the ventrolateral PAG. Microinjection of cumulative half log doses of morphine (0.32, 1, 3.2, and 10 μg/0.4 μl) produced antinociception on the hot plate test only at the two highest doses. Microinjection of quarter log doses of morphine into the PAG (1, 1.8, 3.2, 5.6, and 10 μg/0.4 μl) resulted in an ED_{50} for antinociception of 1.8 μg. Systemic administration of the opioid antagonist naloxone increased the morphine ED_{50} to 9.0 μg. Repeated microinjections of saline into the PAG had no effect on nociception. Pretreatment with twice daily injections of morphine, either systemically (5 mg/kg, s.c.) or into the PAG (5 μg/0.4 μl), for 2 days produced a two-fold increase in the ED_{50} for morphine antinociception. These data validate the use of an intracranial cumulative dose procedure to assess morphine potency and demonstrate that microinjection of morphine into the PAG is sufficient to produce tolerance.
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1. Introduction

Opiates such as morphine are the most effective treatment for pain. Unfortunately, the development of tolerance requires escalating doses to produce pain relief. Although opiates act at sites throughout the nervous system, the periaqueductal gray (PAG) appears to be a key structure in tolerance to morphine antinociception. Tolerance to the antinociceptive effect of systemically administered morphine is prevented by selectively blocking opioid receptors in the PAG (Lane et al., 2005), and the antinociception produced by microinjection of morphine into the PAG is reduced with repeated administration (Jacquet and Lajtha, 1976; Morgan et al., 2005a; Siuciak and Advokat, 1987; Tortorici et al., 1999). This reduction in morphine antinociception occurs with as few as four microinjections (Morgan et al., 2005a; Tortorici et al., 1999) and persists for over 1 week (Morgan et al., 2005b; Siuciak and Advokat, 1987).

Although these studies indicate that the PAG contributes to the development of tolerance, the findings are limited because tolerance to PAG morphine administration was assessed using a single morphine dose. Given that a single dose could represent any point along a dose–response curve, it is impossible to determine the magnitude of tolerance. A shift in the morphine dose–response curve is needed to assess the magnitude of morphine tolerance (Kalant et al., 1971). The only dose–response studies of antinociception to PAG microinjections have used a between-subjects design (Fang and Proudfit, 1998; Jensen and Yaksh, 1992; Krzanowska and Bodnar, 1999; Lichtman et al., 1996; Sharpe et al., 1974; Tseng et al., 1980). Given that between-subjects designs require a lot of time and animals, this approach has not been used to assess changes in morphine potency as a result of tolerance. A within-subjects cumulative dose procedure, which is commonly used with systemic administration of drugs (Wenger, 1980), would circumvent this problem and allow the magnitude of tolerance to be quantified. A first step is to determine whether a cumulative dosing procedure can

* Corresponding author. Washington State University Vancouver, 14204 NE Salmon Creek Ave., Vancouver, WA 98686, USA. Tel.: +1 360 546 9726; fax: +1 360 546 9038.
E-mail address: morgan@vancouver.wsu.edu (M.M. Morgan).

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be used to assess morphine antinociceptive potency to intracranial microinjections.

The objective of the present manuscript is to describe a cumulative dose microinjection procedure that can be used to evaluate morphine antinociceptive potency to PAG microinjections. If the PAG contributes to morphine tolerance, then morphine pretreatment should produce a rightward shift in the morphine dose–response curve to PAG microinjection. The proposed studies will validate the use of a cumulative dose procedure for intracranial microinjections to test this hypothesis.

2. Methods

2.1. Subjects

Male Sprague–Dawley rats (200–350 g; Animal Technologies, Livermore, CA) were anesthetized with pentobarbital (60 mg/kg, i.p.) or equithesin (3 ml/kg, i.p.) and implanted with a guide cannula (23 gauge) aimed at the ventrolateral PAG (9 mm long; AP: +1.7 mm, ML: ±0.6 mm, DV: −5.0 mm from lambda) using stereotaxic techniques. The guide cannula was attached to two screws in the skull by dental cement. At the end of the surgery, the rat received a prophylactic injection of the antibiotic cefazolin (15 mg/0.15 ml, i.m.) and a stylet was inserted to plug the guide cannula. The rat was maintained under a heating lamp until awake.

Following surgery, rats were housed individually in a room maintained on a reverse light/dark schedule (lights off at 7:00 AM). Food and water were available at all times except during testing. Rats were handled daily before and after surgery. Testing began at least 7 days after surgery. All procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize the number and potential suffering of experimental subjects.

2.2. Microinjections

Drugs were administered through a 31-gauge injection cannula inserted into and extending 2 mm beyond the tip of the guide cannula. Rats received a sham injection prior to the experiment, in which an injector was inserted into the guide cannula but no drug was administered. This procedure reduces confounds resulting from mechanical stimulation of neurons on the test day. Testing with drug administration began 1 day later. Microinjections were administered in a volume of 0.4 μl and a rate of 0.1 μl/10 s while the rat was gently restrained by hand. The injection cannula remained in place an additional 20 s to minimize backflow of the drug up the cannula track. Following the injection, the stylet was replaced and the rat was returned to its home cage.

2.3. Nociceptive assessment

Nociception was assessed using the hot plate test. The hot plate test consisted of measuring the latency to lick the hind paw when placed on a 52.5 °C plate. The rat was removed from the hot plate if no response occurred within 50 s.

2.4. Experiments

Three experiments were carried out. All experiments used a cumulative dose procedure in which increasing cumulative doses of morphine were injected every 20 min. Nociception was assessed using the hot plate test 15 min after each microinjection. Experiment 1 assessed antinociception evoked from half log cumulative dose microinjections of morphine into the PAG to determine the dose range for morphine antinociception. Experiment 2 examined naloxone reversal of morphine antinociception using quarter log cumulative dose microinjections into the PAG. Experiment 3 used a cumulative dose microinjection procedure to assess the magnitude of tolerance to the antinociception produced by microinjection of morphine into the PAG.

2.5. Histology

Rats were exposed to a lethal dose of Halothane following testing. The microinjection site was marked by injecting Cresyl Violet (0.2 μl) into the PAG. The brain was removed and placed in formalin (10%). At least 2 days later the brain was sectioned coronally (100 μm) and the location of the injection site identified (Paxinos and Watson, 2005).

2.6. Data analysis

The mean (±S.E.M.) baseline hot plate latency for the saline treated rats was 14.0±6.4 s. The cutoff value used to define antinociception was four standard deviations greater than this mean (39.8 s). This procedure was developed so antinociception is defined as a 1 in 31,546 probability of reaching the cutoff latency as opposed to arbitrarily selecting a cutoff value (Morgan et al., 2006). The cutoff value was imposed on the data post-hoc.

Morphine dose–response curves and ED_{50} values were calculated from raw hot plate data using non-linear regression (GraphPad, Prism). The upper and lower limits for calculating ED_{50} values were set at the mean latency following microinjection of the highest morphine dose (10 μg) and the mean baseline latency, respectively. Differences in ED_{50} values were compared using 95% confidence intervals. Only rats with injection cannula in or on the border of the ventrolateral PAG were included in data analysis.

3. Results

3.1. Experiment 1: cumulative half log dose microinjections of morphine into the PAG

The PAG and RVM are part of a descending nociceptive modulatory pathway projecting to the spinal cord. Microinjection of morphine into either site produces antinociception (Jensen and Yaksh, 1986; Morgan and Whitney, 2000; Morgan

et al., 1998). The objective of this experiment was to determine whether a cumulative dose procedure could be used to assess the antinociceptive potency of PAG morphine microinjections. Following a baseline hot plate test, rats were microinjected with increasing cumulative doses of morphine into the PAG ($N=11$). Four microinjections were administered (0.32, 0.68, 2.2, and 6.8 μg/0.4 μl) resulting in half log doses of 0.32, 1, 3.2, and 10 μg/0.4 μl. Half log doses allowed a wide dose range to be covered with a minimal number of injections. Only rats with microinjection sites in or immediately adjacent to the PAG were included in data analysis (Fig. 1, left).

Microinjection of morphine into the PAG produced a dose-dependent increase in hot plate latency (Fig. 2). The two lowest doses of morphine (0.32 and 1.0 μg) were without effect. Nonetheless, the ED_{50} value (and 95% confidence interval) for morphine microinjection into the PAG was 3.8 μg (1.9–5.6). Although these data show the full range of morphine antinociception, the use of half log doses made the calculation of ED_{50} values imprecise (see ED_{50}s for Experiments 2 and 3).

In order to demonstrate that the antinociception produced by cumulative dose microinjections into the PAG can be applied to other brain structures, a subset of rats ($N=11$) were implanted with a guide cannula aimed at the RVM (12 mm long; AP: −2.3, ML: 0.0 mm, DV: −8.8 mm; Fig. 1, right) and tested with half log doses of morphine. Microinjection of morphine into the RVM produced a dose dependent antinociception. This antinociception was similar to that produced by microinjection of morphine into the PAG except that mean antinociception was lower following microinjection of the highest dose into the RVM (29.3±3.1 s) compared to the PAG (35.5±2.2 s, Fig. 2). This slight difference was caused in part because 5 of the 11 rats had a hot plate latency less than 25 s following microinjection of the highest cumulative dose into the RVM (10 μg). In contrast, only 2 rats injected with 10 μg of morphine into the PAG had a hot plate latency below 25 s, whereas 8 had latencies greater than 34 s.

3.2. Experiment 2: characterization of quarter log dose morphine microinjections into the PAG

Experiment 1 revealed that the effective dose range for morphine microinjections into the PAG is between 1 and 10 ug. Thus, this experiment assessed the potency for antinociception using quarter log doses of morphine into the PAG. Five cumulative injections were administered (1, 0.8, 1.4, 2.4, and 4.4 μg/0.4 μl) resulting in quarter log doses of 1, 1.8, 3.2, 5.6, and 10 μg/0.4 μl. The effect of repeated injections on nociception was assessed by microinjecting saline into the PAG ($N=9$) following the same procedure as the morphine treated group. A second control group ($N=9$) was pretreated with naloxone (1 mg/kg, s.c.) immediately following the first of the five morphine microinjections into the PAG. If cumulative dose

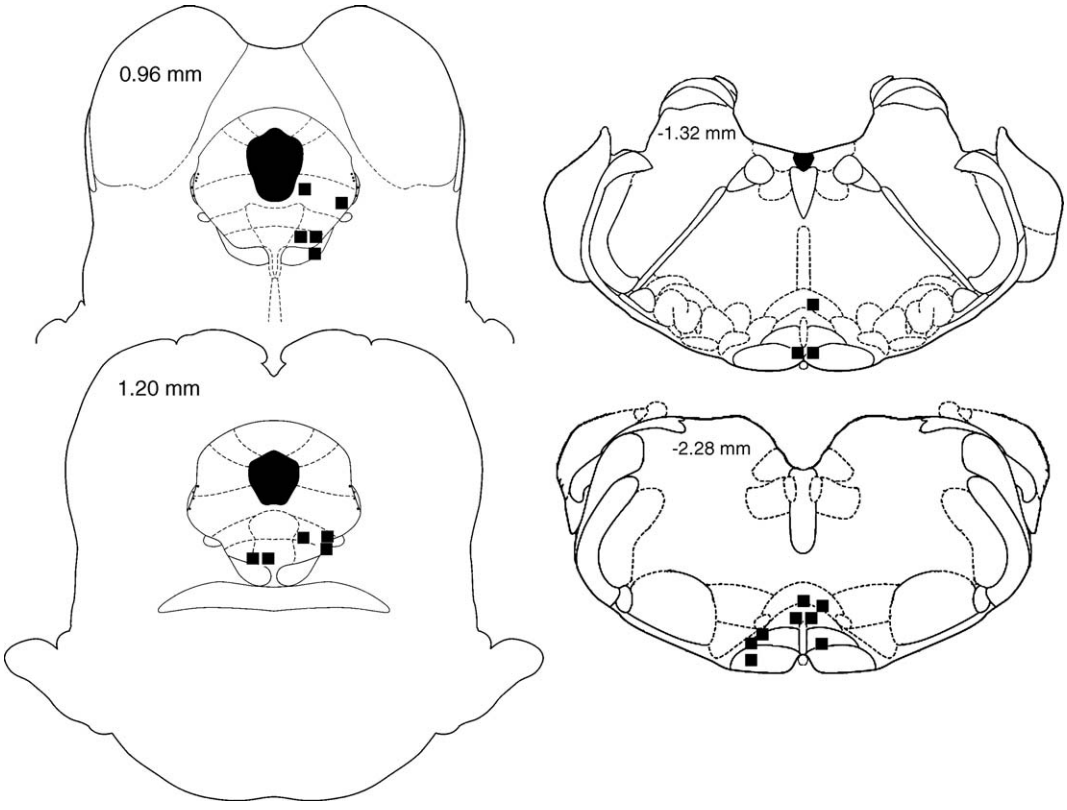

Fig. 1. Location of PAG and RVM injection sites. Only rats with injection sites in or immediately adjacent to the PAG or RVM were included in data analysis (Paxinos and Watson, 2005). All rats were injected with cumulative doses of morphine. There were no obvious differences in morphine antinociception across the different PAG or RVM injection sites. The PAG injection sites shown here are similar in location to those in Experiments 2 and 3.

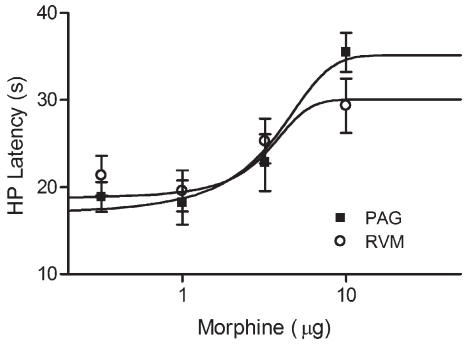

Fig. 2. Half log cumulative dose microinjections into the PAG and RVM. A dose-dependent increase in hot plate latency occurred with increasing cumulative doses of morphine. Half log dose intervals were used to provide a full dose range of antinociceptive effects at PAG and RVM sites. Antinociception was evident only after administration of the two highest doses. Based on these data, subsequent experiments focused on microinjection of quarter log doses into the PAG.

microinjections of morphine produce antinociception via opioid receptor binding, then naloxone administration should produce a rightward shift in the morphine dose–response curve.

An incremental dose-related increase in mean hot plate latency was produced by morphine administration (Fig. 3). Baseline hot plate latency rose from 14.5±0.5 s to 36.3±1.7 s following administration of a cumulative morphine dose of 10 μg/0.4 μl. The ED_{50} (and 95% confidence interval) for the morphine treated rats was 1.8 μg (1.4–2.2 μg). Systemic administration of the opioid antagonist naloxone (1 mg/kg) caused a large rightward shift in the dose–response curve to morphine microinjection (Fig. 3). The ED_{50} for antinociception in naloxone treated rats was 9.0 μg (6.8–11.3 μg).

Repeated microinjections of saline into the PAG had no effect on nociception. Mean hot plate latency never exceeded 19 s following any of the five saline injections. Although a slight increase in hot plate latency from a baseline of 14.0 s to 18.7 and 18.9 s occurred following the fourth and fifth injections, this increase was driven by 2 of 9 rats with hot plate latencies greater than 20 s.

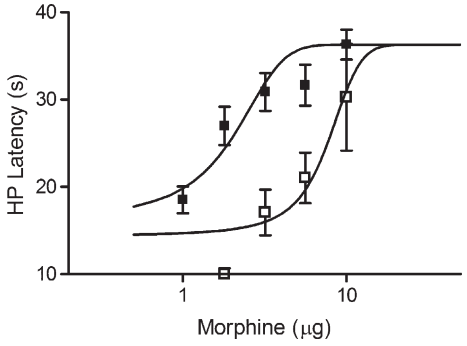

Fig. 3. Quarter log cumulative dose microinjections of morphine into the PAG produces graded antinociception. A dose dependent increase in antinociception occurred with five quarter log cumulative doses of morphine. Systemic administration of naloxone (open squares) caused a reduction in morphine potency from an ED_{50} of 1.8 μg to 9.0 μg. No antinociception was evident with five repeated injections of saline into the PAG (see text).

3.3. Experiment 3: dose–response analysis of tolerance to PAG morphine

The objective of this experiment was to test the hypothesis that cumulative dose microinjections of morphine into the PAG can be used to assess changes in morphine potency. Rats were injected with morphine or saline twice a day for 2 consecutive days to induce tolerance. Some rats received repeated systemic injections of morphine (5 mg/kg, s.c., $N=7$) or saline ($N=7$) and other rats received repeated microinjections of morphine (5 μg/0.4 μl; $N=18$) or saline ($N=14$) into the PAG. All rats were injected with cumulative quarter log doses of morphine into the PAG on Day 3 as described in Experiment 2. It is hypothesized that morphine pretreatment would cause a rightward shift in the morphine dose–response curve as expected with the development of tolerance.

Pretreatment with systemic morphine for 2 days diminished the antinociceptive potency of morphine microinjected into the PAG compared to saline pretreated rats (Fig. 4A). The ED_{50} for antinociception to PAG morphine administration in saline-pretreated rats was 1.7 μg (1.2–2.3). In morphine-pretreated rats the ED_{50} value was 3.5 μg (2.0–4.9 μg). This two-fold shift in the dose–response curve indicates that cross-tolerance developed from systemic to PAG morphine administration.

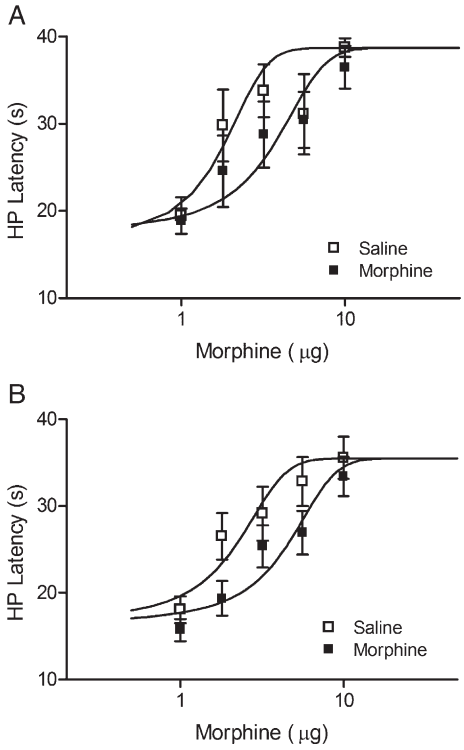

Fig. 4. Tolerance induces a rightward shift in the PAG morphine dose–response curve. A) Rats pretreated with systemic morphine are cross-tolerant to PAG microinjection of morphine. Repeated systemic administration of morphine caused a rightward shift in the dose–response curve to PAG microinjection of morphine compared to saline pretreated controls. B) Repeated PAG morphine microinjections induces tolerance. Rats pretreated with PAG morphine showed a rightward shift in the dose–response curve to cumulative dose microinjections of morphine into the PAG compared to saline-pretreated rats.

A similar two-fold shift in the ED_{50} for PAG morphine microinjection occurred in rats made tolerant to repeated morphine microinjections into the PAG (Fig. 4B). The ED_{50} for rats pretreated with four morphine microinjections during the 2 days preceding testing was 4.4 μg (3.4–5.4). This value was significantly greater than the ED_{50} value for PAG morphine antinociception in rats pretreated with saline microinjections into the PAG (ED_{50}=2.1 μg; 1.5–2.7).

The shape of the morphine dose–response curves did not differ between naïve rats tested in Experiment 2 and the saline and morphine pretreated rats tested with morphine in Experiment 3. In fact, the ED_{50} for morphine antinociception following cumulative microinjections into the PAG was remarkably similar between the morphine naïve groups in Experiments 2 (1.8 μg) and 3 (1.7 and 2.1 μg).

4. Discussion

The present data show that pretreatment with systemic or PAG administration of morphine causes a rightward shift in the morphine dose–response curve. This finding confirms and extends previous results indicating that PAG neurons contribute to morphine tolerance. These data also indicate that a cumulative dose procedure for intracranial microinjections is a valid and useful tool to assess morphine potency.

A number of studies have reported tolerance to the antinociceptive effect of morphine microinjection into the PAG with repeated administration (Jacquet and Lajtha, 1976; Lewis and Gebhart, 1977; Morgan et al., 2005a; Morgan et al., 2005b; Siuciak and Advokat, 1987; Tortorici et al., 2001; Tortorici et al., 1999). Tolerance also has been reported with continuous administration of morphine into the PAG (Lane et al., 2004). Although these studies are consistent, none assessed tolerance using a full dose–response analysis. Given that drug tolerance is characterized by a parallel rightward shift in the dose–response curve (Fernandes et al., 1977), these previous studies examining PAG-mediated tolerance are inconclusive. Microinjection data is particularly difficult to interpret because a loss of antinociceptive potency can occur because of damage to neurons with repeated microinjections. However, cell damage cannot account for the rightward shift in the PAG dose–response curve reported here because rats pretreated with systemic morphine were subjected to PAG microinjections on the test day only.

The rightward shift in the PAG morphine dose–response curve measured behaviorally is consistent with changes in PAG neurons in rats pretreated with morphine. These changes include a reduction in conductance at voltage-gated potassium channels and upregulation of cAMP (Chieng and Christie, 1996; Ingram et al., 1998). Although it is not clear how these cellular changes relate to antinociceptive tolerance, the use of dose–response curves provides a method to assess the behavioral effects of manipulating intracellular signaling cascades. Studies examining the effect of disrupting these signaling pathways are currently underway in our laboratory.

Morphine potency to PAG microinjections was surprisingly stable across studies. ED_{50} values were 1.7, 1.8, and 2.1 μg in the three control groups tested with cumulative doses of morphine. The one exception was an ED_{50} value of 3.8 μg in rats tested with half log doses of morphine instead of quarter log doses as in the other experiments. This difference highlights the importance of using doses that fall along the slope of the dose–response curve. Other factors also can influence morphine potency. We have shown that the nociceptive test, the type of dosing procedure (i.e., cumulative vs. between-subjects), and the method of calculating ED_{50} influence the potency of systemically administered morphine (Morgan et al., 2006). These influences make comparison of morphine potency between studies difficult.

The obvious advantages of cumulative dose microinjections are a reduction in the number of animals and time needed to complete a study. These factors are especially important with microinjection studies in which a significant amount of time is required to surgically implant injection cannulae. Another advantage specific to the microinjection procedure is the consistent injection site for all doses. Microinjections of morphine into the lateral and ventrolateral PAG produce drastically different behavioral reactions (Morgan et al., 1998) including differences in susceptibility to morphine tolerance (Morgan and Liebeskind, 1987; Tortorici et al., 1999). A cumulative dose procedure overcomes this problem by injecting each dose into the same place.

Finally, cumulative dose microinjections can be used at sites throughout the brain, not just the PAG. A dose dependent increase in antinociception occurred with cumulative injections of morphine into the RVM. Although morphine potency with RVM microinjections (ED_{50}=1.6 μg) was similar to that produced with PAG morphine microinjections, morphine efficacy appeared lower with RVM microinjections. Unfortunately, assessment of efficacy is hindered by morphine concentration because injection of doses greater than 10 μg/0.4 μl are difficult to get into solution. Although higher doses can be injected using a cumulative dose approach because low concentrations are administered in an additive manner, the finding that high doses of morphine can have anesthetic effects on RVM neurons is a limitation (Heinricher et al., 1994). Nonetheless, the present experiments demonstrate that cumulative dose microinjections are a useful means of assessing morphine potency, especially in studies of drug tolerance.

Cross-tolerance from systemic to PAG morphine administration demonstrates that PAG neurons play an important role in the development of tolerance to morphine. Our recent data showing that tolerance to systemic morphine is attenuated by selectively blocking opioid receptors in the PAG (Lane et al., 2005) indicates that the PAG is necessary for the development of tolerance. The present finding that tolerance develops with repeated microinjections shows that PAG neurons also are sufficient to produce tolerance.

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