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Differential responses to morphine-induced analgesia in the tail-flick test

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Abstract

We compared acute and chronic antinociceptive effects of morphine in animals with high reactivity (HR) versus low reactivity (LR) to novelty. Antinociception was assessed by tail-flick test. Rats were i.p. injected with either saline or morphine (1.5mg/kg or 3mg/kg) every 12 hours for 7 days according to the treatment group. On day one of the experiment, LR animals in the 1.5mg/kg morphine group showed significantly higher tail-flick latency than HR. Moreover, significant tolerance to the antinociceptive effects of morphine at the used doses was observed in LR but not HR animals. However, effects of chronic morphine treatment on tail-flick latency in rat groups with similar morphine-induced acute anti-nociception were undistinguishable. The difference in tail-flick latency between HR and LR rats observed after acute 1.5mg/kg morphine injection was eliminated if β-funaltrexamine (3mg/kg, i.p.) was administered 24 hours before the test, an indication that μ opioid receptors are responsible for the difference observed. Studies to anatomically characterize the difference in the acute analgesic effect of morphine in HR vs. LR animals did not however yield any significant difference in μ opioid receptor mRNA levels in locus coeruleus (LC), ventral periaqueductal gray (vPAG), nucleus raphe magnus (NRM) and nucleus reticularis paragigantocellularis (NRPG) between these two groups of animals.

In conclusion, our results show that differences in novelty-seeking behavior can predict inter-individual variability in morphine-induced antinociception in rats. Such variability is dependent upon activation of μ opioid receptors, but does not correlate with μ opioid receptor expression in LC, vPAG or ventral medulla.

Introduction

Studies of between-subject variability in animals have been proven useful in uncovering the biological mechanisms underlying behavioral and physiological responses in humans. For example, genetic studies comparing Wistar-Kyoto (WKY) and Wistar-Kyoto hyperactive (WKHA) rats have provided insights on the biological factors related to the syndrome of hyperactivity in humans [19], while recently comparison of animals with different running abilities has helped establishing a link between aerobic capacity, mitochondrial function and cardiovascular and metabolic disease [26].

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In a model of individual differences in rodents, novelty-seeking behavior is a predictor of responses to drug of abuse, and animals that exhibit a high locomotor response to novelty (HR) show higher propensity to self-administer drugs than their counterpart (LR) [21]. The potentially homologous dimension in humans, sensation-seeking behavior, has been widely studied and related to inter-individual differences in vulnerability to drug of abuse [3,29].

Differences in opiate addiction between HR and LR animals have been widely investigated. When administered morphine, HR animals show higher morphine-induced psychomotor activity [7] and greater levels of morphine self-administration than LR [1]. Moreover, studies on opioid-induced withdrawal have shown differences in the behavioral and motivational aspects of withdrawal between HR and LR animals, and identified a difference in β_1 adrenergic receptors expression in the dorsal part of the bed nucleus of stria terminalis as an important component in the biological mechanisms underlying these differences [2].

Even though clinical studies showing a negative correlation between sensation seeking and endorphin levels in the cerebrospinal fluid of chronic pain patients [11] suggest a possible link between novelty seeking, endogenous opioid peptides and nociception, morphine-induced antinociception has not been thoroughly compared in HR vs. LR rats. White and collaborators, using a paradigm where rats were administered cumulative doses of morphine, have shown that HR animals are less responsive than LR to low doses of morphine in the tail-flick test [25]. The biological correlates responsible for such difference in morphine-induced analgesia are however unknown. Moreover, the possibility that a difference in the analgesic effects of morphine between HR and LR animals could extend to differences in tolerance has not been investigated to date.

In experiment 1, we compared tail-flick latency in HR vs. LR animals during a 7-day period where rats received either a saline or morphine i.p. injection every 12 hours.

The analgesic properties of morphine have been traditionally ascribed to its agonistic properties on μ opioid receptors [15]. In experiment 2, we used a selective μ opioid receptor antagonist to investigate if the difference in tail-flick latency between HR and LR animals observed in experiment 1 is indeed dependent upon μ opioid receptors activation.

Locus coeruleus (LC), ventral periaqueductal grey (vPAG), nucleus raphe magnus (NRM) and nucleus reticularis paragigantocellularis (NRPG) play an important role in morphine-induced antinociception as measured by tail-flick test [12,13,27]. Since inter-individual differences in tail-flick latency were eliminated in experiment 2 by the μ opioid antagonis β-funaltrexamine, in experiment 3 we compared μ opioid receptor mRNA levels in HR vs. LR animals by *in situ* hybridization in the brain regions mentioned above, looking to uncover the biological differences that underlie the behavior observed.

Materials and methods

Animals

For all experiments, a total of 189 outbred Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA), were used. Animals were adult males weighing approximately 225–250 g upon arrival; they were housed three per cage in a room adjacent to the testing room, and maintained on a 12/12 h light/dark cycle (lights on at 0700 hours). Rats were acclimated to the animal quarters for 1 week before any experimental procedure. Experiments were conducted between 1300 and 1700 hours, during the light portion of the cycle. Food and water were available *ad libitum*. Animals were treated in accordance with National Institutes of Health guidelines on laboratory animal use and care.

Experiment 1

In this experiment, we measured the effects of acute and chronic morphine administration on tail-flick latency in HR vs. LR animals.

Locomotor activity test—After 7 days of habituation to the housing conditions, 75 rats were tested for locomotor activity during a 60 min exposure to the mild stress of a novel environment. Locomotor activity was measured between 1300 and 1700 hours. Each rat was placed in a 43L 21.5 W 24.5 H (in cm) clear acrylic activity monitor and locomotor activity was monitored by means of two banks of photocells connected to a microprocessor. Rats that exhibited locomotor counts in the highest third of the sample were classified as HR (*n*=25), whereas rats that exhibited locomotor counts in the lowest third of the sample were classified as LR (*n*=25) [2].

Tail-flick test—One week after animals were sorted according to their locomotor activity, antinociception was assessed in the tail-flick test by an experimenter blind to the rats' locomotor counts and treatment. On two consecutive days prior to testing, rats were rapidly but gently restrained with a soft towel and placed on the tail-flick apparatus to habituate them to the procedure. On the first day of testing basal tail-flick latency was compared in HR vs. LR animals (n=25 per group). Radiant heat from a 90 W bulb was focused on 4–7 cm from the tail distal end, and the time from the onset of the heat stimulus to withdrawal of the tail from the heat source recorded. Tail flick latency for each rat was calculated as the average of three consecutive measurements. In order to prevent tissue damage, cut-off time for each measurement was set to 15 seconds. Two days after baseline measurements, HR and LR rats were tested for anti-nociceptive effects of morphine in the tail-flick test. HR and LR animals were randomly assigned to one of the following treatment groups: saline, 1.5mg/kg morphine (morphine sulphate, Mayne Pharma, Paramus, NJ) or 3mg/kg morphine (*n*=6-10 per group). Rats were i.p. injected with either saline or morphine every 12 hours for seven days according to the treatment group and tested on the tail-flick apparatus every other day 30 minutes after treatment. Since most of the rats in the 3mg/kg morphine group reached the 15 seconds cut-off time set for the test, higher morphine doses were not used. During morphine administration, animals' weight was also daily recorded as an indirect measure of morphine-induced feeding.

Experiment 2

Since morphine at a dose of 1.5mg/kg induced a significantly higher acute analgesic effect in LR than in HR animals, in experiment 2 we investigated if such difference was due to morphine agonistic action on μ opioid receptors.

Ninety rats were sorted according to their locomotor activity in a novel environment between 1300 and 1700 hours, and HR and LR animals were habituated to the tail-flick procedure as above. HR and LR rats were then assigned to two different treatment groups: saline or 3mg/kg β-funaltrexamine (β-funaltrexamine hydrochloride, Tocris, Ellisville, MO). Rats were i.p. injected with either saline or the μ opioid antagonist β-funaltrexamine [10] 24 hours before testing, according to the treatment group, and all rats received 1.5mg/kg of morphine 30 minutes before tail-flick test (*n*=6-10 per group). Tail-flick latency was calculated as above.

Experiment 3

In this experiment we compared μ opioid receptor mRNA levels in LC, vPAG, NRM and NRPG in HR vs. LR animals.

Locomotor activity was measured as above. One week following the locomotor activity test, eight HR and eight LR animals were euthanized by rapid decapitation, their brain immediately removed, frozen in isopentane cooled to -30 to -40°C, and stored at -80°C.

In situ *hybridization*

The *in situ* hybridization method used in this study is described in detail by Isgor *et al* [9]. Briefly, tissue was sectioned at -20°C at a thickness of 12 μm, mounted onto poly(L-lysine)-coated slides, and stored at -80°C until use. Before probe hybridization, tissue was fixed in 4% paraformaldehyde at room temperature, rinsed with aqueous buffers, and dehydrated with graded alcohols. The riboprobe was synthesized with incorporation of 35 S-UTP and 35 S-CTP and hybridized to tissue overnight at 55°C. Sections were then washed with increasing stringency, dehydrated with graded alcohols, air-dried, and exposed to film. Exposure time was chosen to maximize signal. Sections were then dipped in liquid film emulsion and stored in light-tight boxes. Exposure time was chosen to maximize the detection of radioactive grains, and after sufficient time was elapsed, sections were developed, dehydrated, and coverslipped in a xylene-based mounting medium (Permount, Fisher Scientific, Fair Lawn, NJ).

A riboprobe was synthesized from a cDNA fragment for the μ opioid receptor generated in our lab. The signal detected was consistent with previously published data [23].

Image analysis

Six tissue sections per brain region (bregma −9.7 to −10.3 mm, bregma −8.0 to −8.72 mm, bregma -10.5 to −11.3 mm and bregma −10.3 to −11.0 mm for LC, vPAG, NRM and NRPG respectively) were selected from each animal. Digital images of the brain sections were captured from X-ray films in the linear range of the gray levels using a CCD camera (TM-745, Pulnix, USA). Integrated optical density for μ opioid receptor mRNA was determined for each section using the Micro Computer Imaging Device (Ontario, Canada) image analysis system. For each animal, data from multiple sections were averaged to obtain a representative value (mean density).

Statistical analysis

Data for basal tail-flick latency in experiment 1 were analyzed using a Student's unpaired *t* test. Data for weight changes during chronic morphine administration in the same experiment were analyzed using a two-way analysis of variance (ANOVA) with repeated measures. The factors of variation were group (HR or LR; between subjects), treatment (vehicle, morphine 1.5mg/kg and morphine 3mg/kg; between subjects), and observation session (Test 1 to 4; within subjects).

Data for the effects of the selective μ opioid receptor antagonist β-funaltrexamine on morphine-induced analgesia in experiment 2 were analyzed using a two-way ANOVA. The factors of variation were group (HR or LR) and treatment (vehicle or β-funaltrexamine).

Data for μ opioid receptor mRNA levels in LC, vPAG, NRM and NRPG were compared in HR vs. LR animals using a Student's unpaired *t* test.

Finally, for the analysis of the effects of acute and chronic morphine administration on tail-flick latency in HR vs. LR animals in experiment 1, the assumption of normality was violated because of the cut-off limit in the tail flick test, and data were analyzed using the procedure of Conover [4,22]: a two-way ANOVA was performed on error values as well as ranks. Since the two procedures gave nearly identical results, the parametric analysis was considered to be valid.

Where ANOVA indicated significant main effects and/or significant interactions, post-hoc comparisons were conducted using the Newman-Keuls test.

Results

Experiment 1

The results of the locomotor activity test followed a unimodal distribution. The average locomotor activity for all rats in experiment 1 (n=75) was 148 locomotor counts, with locomotor counts of 232±74 and 74±34 for HR and LR rats respectively. Intermediate rats (IR), had locomotor counts of 137±20.

LR animals had significantly higher latency than HR when tested for baseline response in the tail-flick test (*t*=-2.137, p<0.05; Student's *t-test*; n=25 per group)(figure 1A). ANOVA for the acute analgesic effect of morphine in HR vs. LR animals showed significant effects of treatment ($F_{2,44}$=13.16, p<0.01) and phenotype ($F_{1,44}$=6.171 p<0.05) (n=6-10 per group). Subsequent Neuman-Keuls post-hoc analyses indicated that HR rats in the 3mg/kg morphine group, and LR animals in the 3mg/kg and 1.5 mg/kg morphine groups had significantly higher tail-flick latency than rats in the respective control groups. Moreover, LR animals in the 1.5mg/kg morphine group showed significantly higher tail-flick latency than HR animals in the same treatment group (figure 1B). Interestingly, LR animals in the saline group had similar tail-flick latency than HR animals in the same treatment group, even though latency was significantly higher in these LR animals than in the HR animals assigned to the saline group during testing for baseline tail-flick latency (*t*=-3.7, p<0.01; n=6 animals per group). Since tail-flick latency animals was significantly different in HR vs. LR rats after acute administration of 1.5mg/kg morphine, a group of IR animals (n=8) from the same batch was tested on the tail-flick apparatus to investigate if these animals show an intermediate analgesic response between HR and LR. Tail-flick latency for IR animals after acute 1.5mg/kg morphine administration was 7.29±1.363s.e.m. seconds, in-between HR (5.75±0.373s.e.m. seconds) and LR (10.58±1.604 s.e.m. seconds) rats in the same treatment group. Moreover, there was a significant negative correlation (r= - 0.458, p<0.01) between novelty-induced locomotion and tail-flick latency for the animals in the 1.5mg/kg morphine group.

When data for morphine-induced tolerance were analyzed, ANOVA with repeated measures revealed a significant effect of treatment in both HR and LR animals ($F_{2,66}$=5.986, p<0.01 and $F_{2,66}$=10.949, p<0.01 respectively), and a significant effect of time only in LR animals ($F_{3,66}$=3.822, p<0.05) (n=6-10 per group). Subsequent post-hoc analyses showed significantly higher tail-flick latency in the LR 3mg/kg morphine group on the first day than in any of the following days of testing (figure 2). The above analysis suggests higher morphine-induced tolerance in LR than in HR animals. However, a direct comparison of the effects of chronic morphine treatment on tail-flick latency in rat groups with similar morphine-induced acute anti-nociception, i.e. HR rats in the 3mg/kg morphine group vs. LR animals in the 1.5mg/kg morphine group, yielded a significant effect of time ($F_{3,51}$=3.194, p<0.05; n=9-10 per group) with no significant group effect or significant group and time interaction (Figure 3).

Finally, statistical comparison of animals' weight during chronic morphine administration showed significant effects of treatment ($F_{3,66}$=3.822, p<0.05) and time ($F_{6,264}$=3.822, p<0.01) with no significant effect of group or significant interactions (n=6-10 per group). Subsequent post-hoc analyses yielded significantly higher weight gain for the rats in the 3mg/kg morphine group than in the 1.5mg/kg morphine group on days 2 and 3, and than in the vehicle group on days 2 through 5 of the experiment, independent of the rats phenotype.

Experiment 2

Results of the locomotor activity test were similar to the ones described for experiment 1.

In the tail-flick test, two-way ANOVA revealed a significant main effect for rat phenotype ($F_{1,55}$=5.377, p<0.05) and a significant phenotype and treatment interaction ($F_{1,55}$=4.111,

$p<0.05$) (n=14-16 per group). Subsequent post-hoc analyses confirmed the observation in experiment 1 that LR animals in the 1.5mg/kg morphine group have significantly higher tail-flick latency than HR animals in the same treatment group ($p<0.001$), and showed that i.p. injection of 3mg/kg β-funaltrexamine reduces tail-flick latency in 1.5mg/kg morphine treated LR rats to the levels observed in HR animals in the same treatment group (figure 4).

Experiment 3

Results of the locomotor activity test were again similar to the ones described for experiment 1. A direct comparison of μ opioid mRNA levels in HR vs. LR rats in LC, vPAG, NRM and NRPG showed no significant differences in μ opioid receptor expression between the two groups of animals (n=8 per group)(figure 5).

Discussion

Our results show higher basal tail-flick latency in LR than HR animals. These results are consistent with previously published data [25], and suggest that HR animals are more sensitive to heat-induced pain than LR under basal conditions. Interestingly, vehicle-injected HR and LR rats show similar tail-flick latency when tested again two days later, with latency for LR animals decreasing to the level measured in HR. This finding could indicate better learning ability in LR animals. However, previous studies aimed at comparing learning in young adult HR and LR animals have failed to uncover differences between the two groups of animals [5], and spontaneous individual differences in cognitive performances do not correlate with locomotor activity in a novel environment in young adult rats [6]. Possible alternative explanations for our results are that HR animals have reached the lowest possible tail-flick latency under the test conditions and therefore no significant further decrease in latency is possible in these animals, or that saline injections affect tail-flick latency, voiding basal differences between the two groups of animals in the tail-flick test. Finally, our results could simply reflect the fluctuation in basal tail-flick latency seen over time in experiment 1, especially in HR animals.

In our experiments, LR rats also showed significantly higher latency than HR after acute 1.5mg/kg morphine injection, while the two groups of animals were not statistically different after acute 3mg/kg morphine treatment. Our results suggest that a difference in morphine-induced antinociception between HR and LR rats is only present at low morphine doses, and higher doses of morphine are equally effective in the two groups of animals. However, some of the animals in the 3mg/kg morphine group reached the 15 seconds cut-off time set for the test. Therefore, it is also possible that the absence of significant differences in tail-flick latency at this dose of morphine is the consequence of the lower sensitivity of the test once latency gets closer to the set cut-off, and could reflect a limitation of the test itself, rather than a real absence of significant differences between groups. Intraperitoneal injection of the μ opioid receptor antagonist β-funaltrexamine 24 hours before the tail-flick test completely eliminated tail-flick latency differences in HR vs. LR animals after 1.5mg/kg morphine, an indication that different levels of μ opioid receptors in the brain and/or spinal cord could be responsible for the difference in tail-flick latency observed. The dose of β-funaltrexamine used in the experiment is only partially effective in attenuating ethanol-induced place preference [14], and lower than doses previously used to fully antagonize the analgesic effect of morphine in the tail-flick test [24]; however, it completely eliminated the difference in tail-flick latency between HR and LR animals in our experiment. Therefore, higher doses were not tested. The difference in basal tail-flick latency observed between HR and LR rats could also be explained by different levels of μ opioid receptors in these two groups of animals. Endogenous μ opioid receptor agonists such as beta endorphins could bind to the receptors, causing higher basal tail-flick latency in animals with higher μ opioid receptor expression. However, other receptors have also been

shown to modulate tail-flick latency (for review, see [16] and [17]) and it is possible that a combination of different mechanisms and systems is responsible for the higher basal tail-flick latency measured in LR animals.

Because of the effects of β-funaltrexamine on morphine-induced antinociception in experiment 2, in experiment 3 we compared μ opioid receptor mRNA levels in LC, vPAG, NRM and NRPG in HR vs. LR animals. Our results show that μ opioid receptor mRNA levels in these brain regions do not correlate with the behavior observed in the tail-flick test. It is possible that different levels of μ opioid receptors in the spinal cord are responsible for the HR-LR difference in tail-flick latency observed after acute morphine administration. However, previous studies have shown that the effect of morphine on tail-flick latency involves actions at opioid receptors in the brain that override any action that may be caused by combination of morphine with μ opioid receptors in the spinal cord [8]. In our opinion, it is more likely that the phenotype effect observed in acute morphine-induced antinociception is the result of differences in the regulation of μ opioid receptors at the cellular level in the CNS through mechanisms such as receptor phosphorylation [28] that are independent of the absolute level of μ opioid receptor expression.

When morphine was administered for one week and rats tested for tail-flick latency every other day, statistical analysis showed an effect of time in LR but not in HR animals. This data suggest higher tolerance to the antinociceptive effects of morphine in LR than in HR animals. However, if morphine doses with similar acute analgesic effect were compared between the two groups of animals, no difference in morphine-induced latency was present. Taken together, our results suggest that the different behavior between groups in the tail-flick test over time is likely the consequence of the different potency of morphine in the two groups of animals, rather than a real difference in tolerance.

Finally, weight monitoring during chronic morphine treatment showed a significantly higher weight gain over time in morphine treated animals. The effect of morphine treatment on weight was limited to the first 48-72 hrs of treatment, after which animals appeared to develop tolerance to this morphine effect. In agreement with our data, a previous report shows tolerance to the inhibitory effects of naloxone on body weight gain and feeding [18]. Our results suggest the absence of phenotype differences in morphine-induced feeding and are consistent with previous observations of little correlation between exposure to environmental novelty and morphine-induced eating [20].

Conclusion

Our data show that the HR-LR model is a useful tool to investigate biological mechanisms underlying between-subjects variability in pain responses to a noxious stimulus and individual differences in acute morphine-induced antinociception. Moreover, our study identifies μ opioid receptors as the receptors responsible for the difference observed in tail-flick latency between HR and LR animals after a low dose of morphine. However, the biological mechanisms responsible for HR-LR differences in basal tail-flick latency and acute morphine-induced antinociception remain to be determined.

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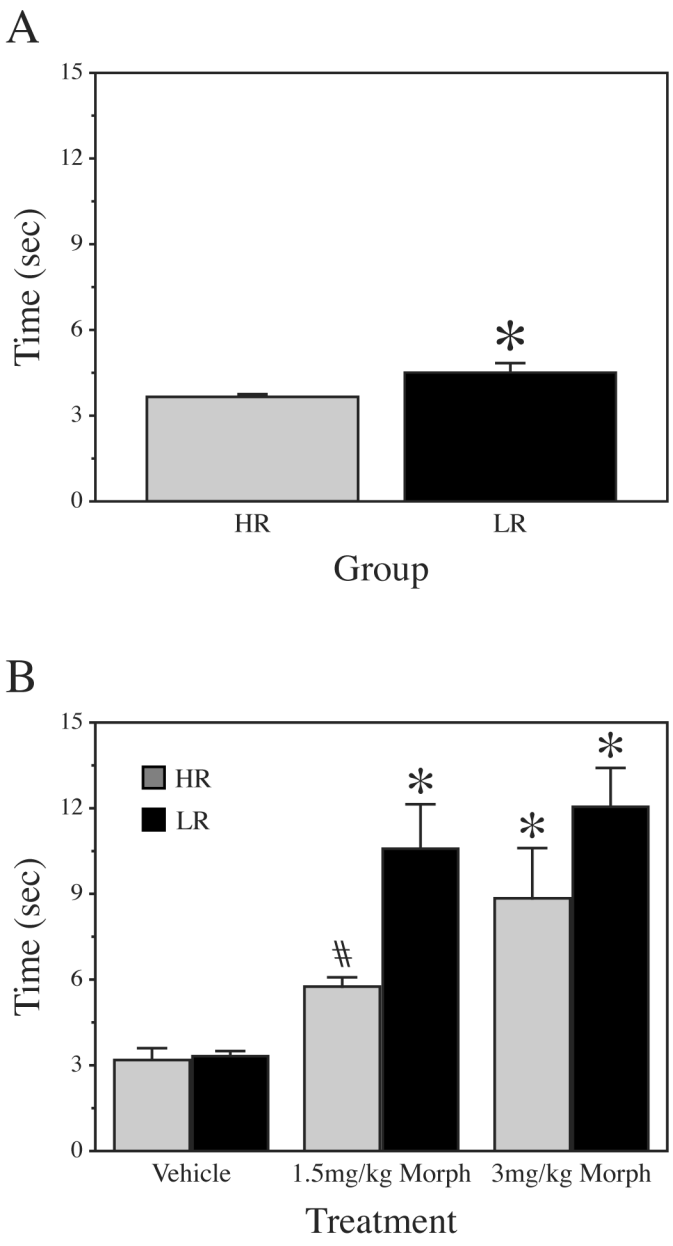


Figure 1.

A) Baseline response in the tail flick test in HR vs. LR animals. Values are mean ± s.e.m. (*n*=25 animals per group). *p < 0.05. Student's *t test*. B) Dose-dependent acute analgesic effects of morphine in HR vs. LR rats. Data are mean ± s.e.m. (*n*=6-10 animals per group). *p < 0.01 compared to vehicle injected animals in the same phenotype group. #p < 0.05 compared to LR rats in the same treatment group. Two-way ANOVA followed by Newman-Keuls test for post-hoc comparisons.

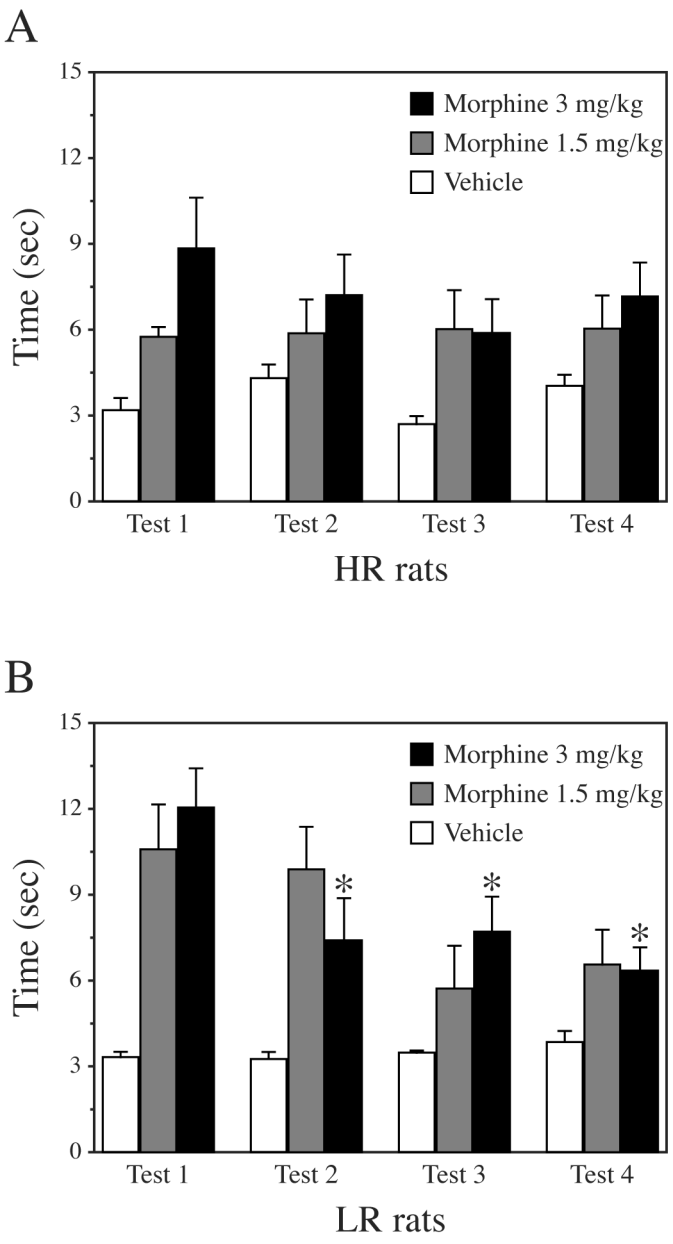


Figure 2.

Effects of repeated morphine injections on tail-flick latency in HR (A) and LR (B) animals. Values are mean ± s.e.m. (*n*=6-10 animals per group). *p < 0.05 compared to latency in the same animals during Test 1. Two-way ANOVA for repeated measures followed by Newman-Keuls test for post-hoc comparisons.

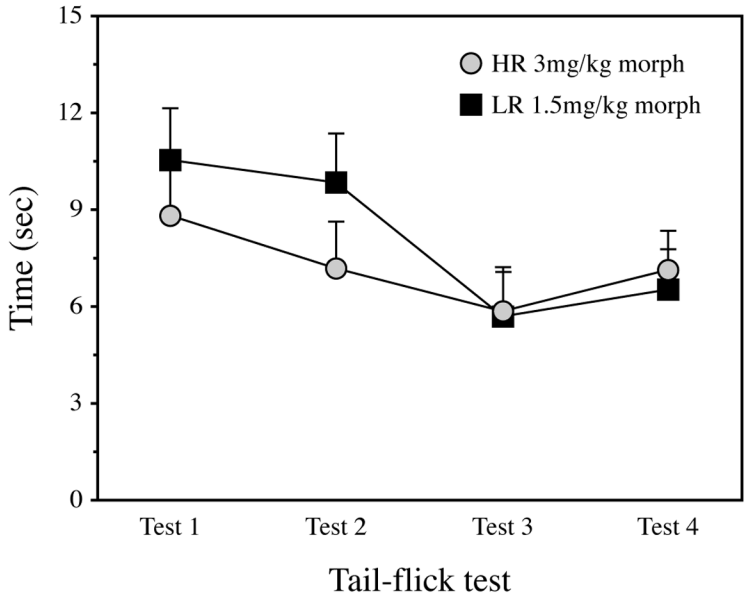


Figure 3.
Comparison of tolerance for morphine doses with similar acute analgesic potency in HR vs. LR animals. Values are mean ± s.e.m. (*n*=9-10 animals per group). Two-way ANOVA for repeated measures.

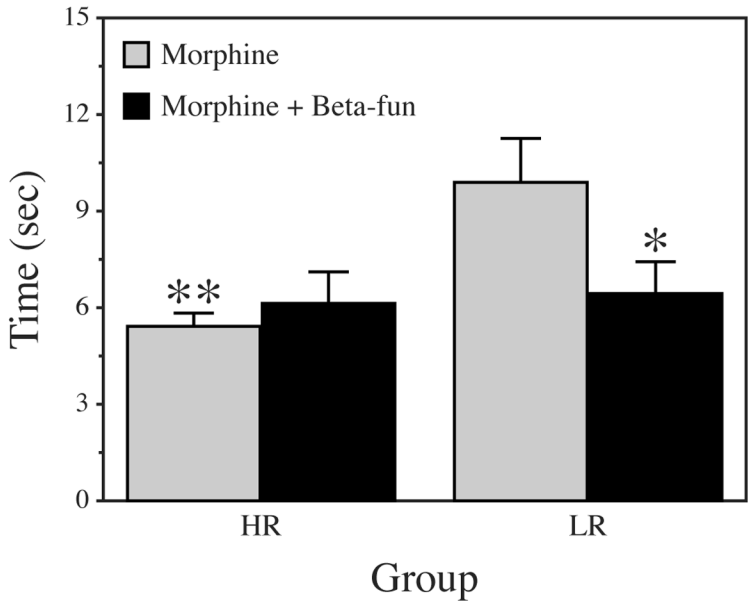
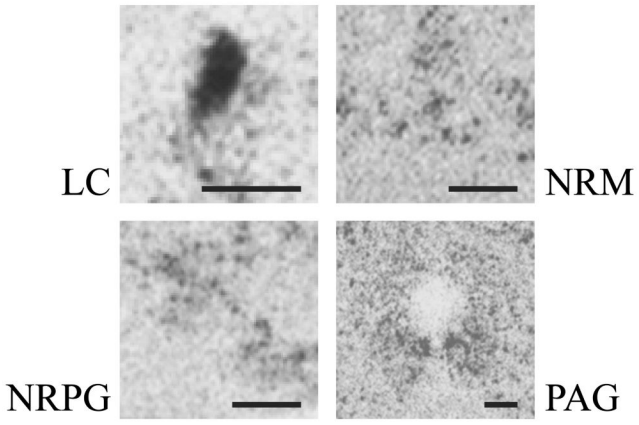


Figure 4.

Effects of the μ receptor antagonist β-Funaltrexamine on tail-flick latency after 1.5mg/kg morphine i.p. injection in HR vs. LR animals. Values are mean ± s.e.m. (*n*=14-16 animals per group). *p < 0.05, **p < 0.01 compared to the LR morphine group. Two-Way ANOVA followed by Newman-Keuls test for post-hoc comparisons.

A



B

	HR	LR
LC	47.27±1.07	44.85±0.72
NRM	9.91±0.31	9.29±0.185
NRPG	13.62±0.28	13.34±0.71
vPAG	13.89±0.81	12.06±0.35

Figure 5.

A) Representative coronal sections showing mRNA signal for the μ opioid receptor in LC (top left), PAG (top right), NRM (bottom left) and NRPG (bottom right) as measured by *in situ* hybridization. Signal intensity was very high in LC, medium in NRPG and vPAG, and low but detectable in NRM. Scale bar=500μm. B) Integrated optical density for μ opioid receptor mRNA levels in the LC, vPAG, NRM and NRPG of HR *vs.* LR animals. All values are mean ± SEM (*n*=8 animals per group). Student's *t*-test.