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Differential Sensitivity of Mice Bred for Stress-Induced Analgesia to Morphine and ACEA-1011 in the Formalin Test

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LUTFY, K., L., B. SADOWSKI, P. MAREK, I.-S. KWON, J. F. W. KEANA AND E. WEBER. *Differential sensitivity of mice bred for stress-induced analgesia to morphine and ACEA-1011 in the formalin test.* PHARMACOL BIOCHEM BEHAV **54**(2) 495–500, 1996. – The antinociceptive effect of morphine, an opioid receptor agonist, and ACEA-1011, a novel NMDA receptor/glycine site antagonist, was examined in the formalin test in mice selectively bred for high (HA) and low (LA) swim stress-induced analgesia (SSIA). A subcutaneous (SC) injection of formalin produced a biphasic nociceptive response in both lines. HA mice spent more time licking the injected paw than the LA mice in both phases of the formalin test. Morphine was equally potent in the early phase in both lines, but it was more potent in HA mice than in LA mice in the tonic late phase of the formalin test. Similarly, ACEA-1011 produced an equally potent antinociceptive effect in the early phase in both lines; however, the compound was more potent in LA mice than in HA mice in the tonic late phase of the formalin test. These data suggest that in HA mice antinociception in the tonic late phase of the formalin test is mediated largely by an opioid-mediated mechanism, whereas in the opioid-deficient LA line at least a nonopioid-mediated mechanism involving the NMDA receptor is also implicated.

Antinociception Morphine ACEA-1011 Selective breeding Formalin test

PAIN in clinical situations is usually prolonged and inflammatory in nature, thus, the use of animal models of persistent pain is appropriate to evaluate the potential clinical effectiveness of novel analgesics. The formalin test, which may resemble postoperative pain, is used widely as an animal model for evaluating the antinociceptive effect of mild analgesic drugs (11). Formalin injections into one of the paws in mice produce a biphasic nociceptive response consisting of a transient early phase followed by a tonic late phase (11,12,27,29,32).

Although morphine is a potent analgesic, it produces tolerance following acute or chronic administrations in laboratory animals (14,33) or humans (7,17). Furthermore, it has been reported that opioid analgesics are ineffective in alleviating pain in certain animal models of pain or some pain-suffering patients (1,3,13). These drawbacks of opioid analgesics have instigated numerous studies seeking alternative approaches to pain inhibition.

A growing body of evidence suggests a modulatory role for *N*-methyl-D-aspartate (NMDA) receptors in pain transmission. The coexistence of glutamate with substance P in dorsal root ganglion neurons has been demonstrated (2). There is an increase in the level of glutamate following noxious stimuli in the spinal cord dialysates of freely moving rats (30). Furthermore, the NMDA receptor is involved in the wind-up phenomenon in the spinal cord neurons (6,8–10). Recently, it has been shown that NMDA antagonists can produce antinociception in numerous animal models of pain (4,5,19,21,22,32,34) making NMDA antagonists potential candidates as analgesics. In

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the present study we sought to determine whether NMDA receptor antagonists may produce antinociception in cases of individuals less responsive to opiate-induced antinociception. To this end, we used two mouse lines selectively bred for different levels of swim stress-induced analgesia [SSIA; (23)]. A series of studies done on successive generations of these lines has demonstrated that HA and LA lines differ substantially in the expression of opioid-mediated phenomena (18,24–26,28). The HA mice manifest a high level of opioid-mediated SSIA and morphine-induced analgesia, whereas LA mice manifest low level of nonopioid-mediated SSIA and morphine-induced analgesia (23,24). A tolerance to repeated swimming and a two-way cross-tolerance of SSIA with morphine-induced analgesia develops in the HA line, but not in the LA line (26,28). These differences may result from an altered density of opioid receptors as demonstrated by an enhanced [^{3}H]naloxone binding in brain homogenates of HA mice (20). In the present study the effect of selective breeding was examined on antinociception produced by morphine or 5-chloro-7-trifluoromethyl-1,4-dihydro-2,3-quinoxalindione (ACEA-1011), a novel competitive NMDA receptor/glycine site antagonist (16) in a mouse formalin test.

METHOD

Subjects

Male HA and LA mice (Institute of Genetics and Animal Breeding, Polish Academy of Science, Jatrzebiec, Poland) bred for 31 and 32 generations for high and low SSIA were used in all experiments. Mice were flown from Poland to Los Angeles at the age of 8–10 weeks. Mice were maintained 4–6 to a cage with free access to food and water under a 12 L : 12 D cycle for at least 10 days prior to any experimentation. All experiments were conducted during the light cycle.

Details of the breeding procedure have been described previously (23). Briefly, Swiss Webster mice (from an outbred stock) were made to swim for 3 min in 20°C water. Mice were then tested for a behavioral end point (flicking or licking of the hind paw) on a hot plate (56°C) 2 min after the completion of the swim. Mice flicking or licking (whichever occurred first) the hind paws by ≥50 s were qualified as progenitors of a HA line, and those responding within 10 s were selected as progenitors of an LA line.

Formalin Test

A modification of a previously described method (11) was used. Mice were placed in Plexiglas jars, and after 1 h of accommodation formalin (20 μl of 5% formaldehyde solution in saline) was injected into the dorsal surface of the right hind paw using a microsyringe (Hamilton Co., Reno, NV) with a 27 gauge needle. The amount of time that each mouse spent licking and/or biting the injected paw was recorded for 1 h in 5-min time intervals. Each mouse was observed by a separate observer who was unaware of treatments and doses.

Effects of ACEA-1011 and Morphine in the Formalin Test

Mice received either ACEA-1011 (5.0–30.0 mg/kg in DMSO, IP; n = 7–16 mice/dose) or morphine (1.0–8.0 mg/kg in saline, SC; n = 4–12 mice/dose) and 30 min later were injected with formalin, transferred to the Plexiglas jars, and immediately observed for licking and/or biting of the injected paw for 1 h. Control animals were injected with DMSO (1 ml/kg; IP) or saline (10 ml/kg; SC), respectively.

Effects of Naloxone on the Antinociceptive Effect of Morphine and ACEA-1011 in the Formalin Test

Due to problems in obtaining HA and LA mice from Poland, Swiss Webster mice obtained from Simonsen Laboratories (Gilroy, CA) were used in this study. Mice were injected with either saline or naloxone (1 mg/kg, IP). Ten minutes later mice were injected with either ACEA-1011 (20 mg/kg, IP) or morphine (4 mg/kg, SC). Control mice were injected with DMSO or saline, respectively. Thirty minutes following the second injections mice were injected with formalin, transferred to the Plexiglas jars, and observed for licking and/or biting of the injected paw.

Drugs

Morphine sulfate and naloxone were purchased from Research Biochemicals International (Natick, MA) and dimethyl sulfoxide (DMSO) from Sigma Chemical Company (St. Louis, MO). ACEA-1011 was prepared in Dr. J. F. W. Keana's laboratory in the Department of Chemistry at the University of Oregon (Eugene, OR).

Data Analysis

Data were analyzed only for the early (0–5 min) and late (15–50 min) phases of the formalin test. The mean time spent licking in the late phase is the average of seven 5-min intervals, i.e., the mean (not the total) time spent licking per 5 (not 35) min. In the pilot studies, data were analyzed by two-way analysis of variance (ANOVA) and by an unpaired two-tailed student's t-test. ACEA-1011 and morphine dose–response, and the effect of naloxone on antinociception induced by ACEA-1011 or morphine data were analyzed using one-way ANOVA followed by Newman–Keuls test to determine significant differences among various group means. For ED_{50} and relative potency estimations due to large differences in nociceptive responses between HA and LA mice in both phases of the formalin test, data were expressed as percent of control and analyzed using the linear regression analysis by the method of Tallarida and Murray (31).

RESULTS

Both lines displayed two distinct phases of licking of the injected paw following an injection of formalin, a transient early phase (0–5 min) followed by a tonic late phase (15–50 min). The amount of time spent licking was minimal from 5–15 min following formalin injection in both lines. Administration of saline or DMSO, used as vehicle in subsequent studies, had no significant effect on either of the phases of the formalin test as compared to naive (untreated) HA and LA mice. An overall two-way ANOVA revealed a significant effect of lines, $F(1, 37) = 7.85$ and $F(2, 37) = 26.85$, for the early and late phases, respectively; $p < 0.01$ or better) but not of treatments, $F(2, 37) = 2.99$ and $F(2, 37) = 0.23$, for the early and late phases, respectively; $p > 0.05$). Therefore, the data from control groups were pooled for analysis. As shown in Fig. 1, HA mice spent more time licking the injected paw than the LA mice in the early ($p < 0.05$) and late ($p < 0.01$) phases of the formalin test.

Administration of morphine 30 min prior to the formalin injection produced a dose-dependent antinociception in HA and LA mice in both phases of the formalin test (Fig. 2). A one-way ANOVA revealed that morphine produced significant antinociceptive effect in both lines in the early, $F(5, 41) = 4.67$ and $F(4, 29) = 10.93$, for the LA and HA mice, re-

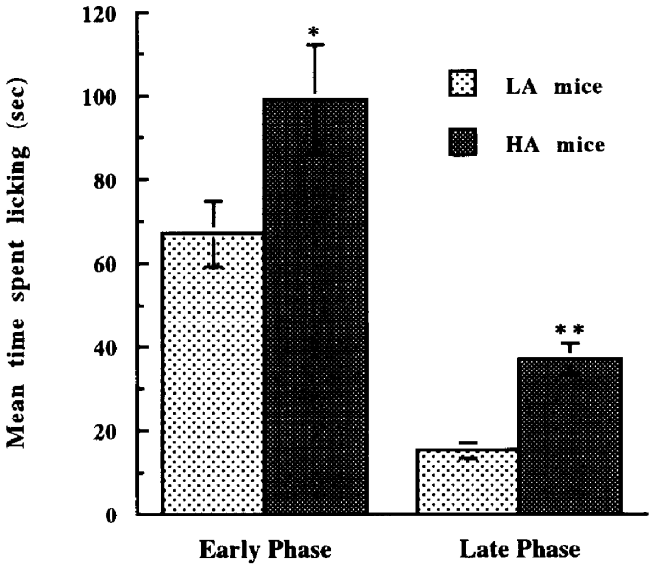

FIG. 1. Formalin-induced biphasic nociceptive response in HA and LA mice. Mice were injected subcutaneously with formalin into the dorsal surface of the right hind paw. The amount of time that each mouse spent licking and/or biting the injected paw was recorded at each 5-min interval for 1 h. The data presented are the means (±SEM) at 0–5 min (early phase) and from 15–50 min (late phase) following formalin injection. Data were analyzed by a two-tailed unpaired student's *t*-test (29). * and ** significantly different from LA line ($p < 0.05$, and $p < 0.01$, respectively).

spectively; $p < 0.01$) and late, $F(5, 41) = 4.53$ and $F(4, 29) = 10.45$, for the LA and HA mice, respectively; $p < 0.01$) phases of the formalin test. The post hoc Newman-Keuls test of the data in LA mice revealed that morphine did not produce any significant antinociceptive effect at doses up to 4 mg/kg in the early phase of the formalin test (Fig. 2, upper panel). However, at 4 mg/kg or higher doses morphine produced significant antinociception (Fig. 2, $p < 0.05$ or better). In the HA mice, morphine produced significant antinociceptive effect at 2 mg/kg or higher doses as compared to either saline or 1 mg/kg morphine-treated group (Fig. 2, lower panel; $p < 0.01$). In the late phase of the formalin test, post hoc test revealed that morphine at lower doses (1 and 2 mg/kg) produced no significant antinociception in the LA mice (Fig. 2, upper panel); even there appeared to be some degrees of hyperalgesic state, yet it was not statistically significant ($p > 0.05$). Morphine produced significant effect at only 8 mg/kg as compared to saline-treated group ($p < 0.05$). In addition, morphine (6 and 8 mg/kg) caused a greater antinociceptive response as compared to 1 mg/kg-treated mice ($p < 0.05$ or better). Furthermore, the antinociceptive effect produced by 8 mg/kg of morphine was significantly greater than the 2 mg/kg morphine-treated group ($p < 0.05$). In the HA mice, morphine produced a significant antinociceptive effect even at 1 mg/kg ($p < 0.05$; Fig. 2, lower panel), the dose that did not produce any antinociception in the LA mice. The ED_{50} and 95% confidence limits (CLs) of morphine are shown in Table 1. When the potency of morphine in the LA mice was compared to that of the HA mice, it was found that morphine was equally potent in the early phase of the formalin test in both lines ($p > 0.05$). An approximately fourfold difference was found in the relative potency of morphine between HA and LA mice in the tonic late phase of the formalin test ($p < 0.05$). Furthermore, morphine was approximately two times more potent in the early than in the late phase in LA line ($p < 0.05$). Although it appeared that HA mice are more sensi-

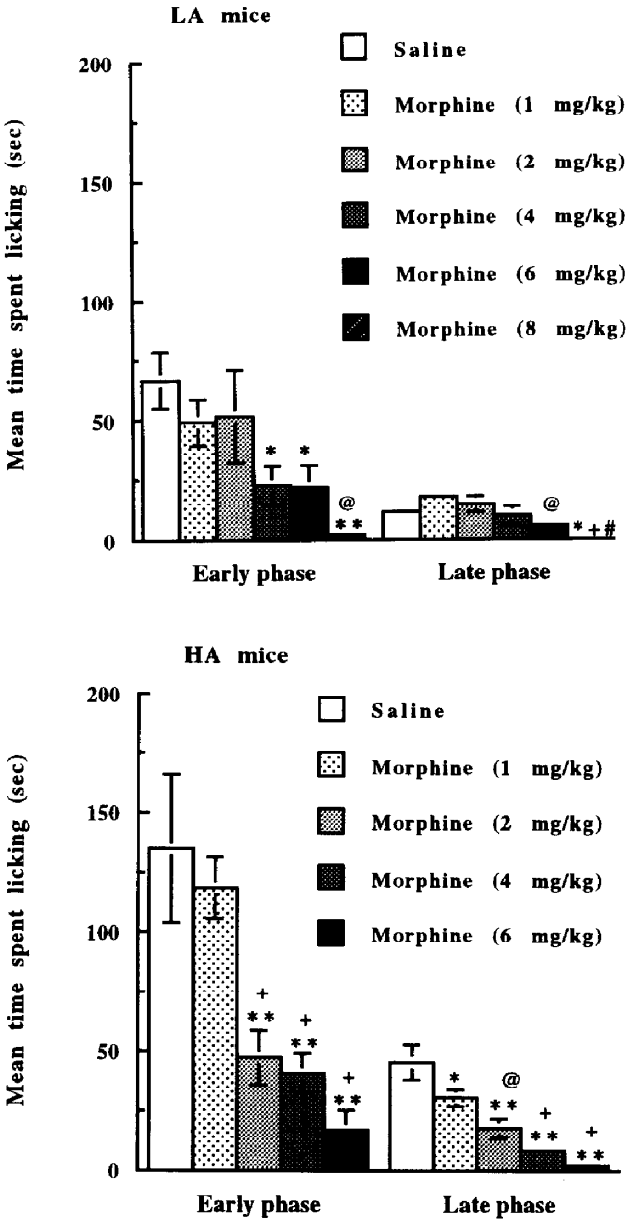

FIG. 2. Morphine dose-response bars in the LA (upper panel) and HA (lower panel) mice in the formalin test. Mice were injected with either morphine (1.0–8.0 mg/kg, SC) or saline. Thirty minutes later, mice were injected with formalin and observed for licking and/or biting of the injected paw for 1 h. The mean time spent licking and/or biting the injected paw (±SEM) is reported here for the first 5 min (early phase) and from 15–50 min (late phase). Data were analyzed by one-way ANOVA followed by Newman-Keuls test. * and ** significantly different from saline-treated mice ($p < 0.05$ and 0.01, respectively). @ and + significantly different from morphine-treated (1 mg/kg) mice ($p < 0.05$ and $p < 0.01$, respectively). # significantly different from morphine-treated (2 mg/kg) group ($p < 0.05$).

TABLE 1

THE ANTINOCICEPTIVE ED_{50} AND 95% CLS OF MORPHINE IN THE EARLY AND LATE PHASES OF THE FORMALIN TEST IN MICE SELECTIVELY BRED FOR HIGH AND LOW SSIA

Genetic Line	Early Phase	Late Phase
HA	2.2 (1.4-3.4)	1.5 (0.8-2.7)
LA	2.5 (1.5-4.2)	5.7 (4.4-7.3)*

Mice were injected with morphine (1-8 mg/kg, SC; n = 4-12 mice/dose). Thirty minutes later, mice were injected with formalin and observed for licking and/or biting of the injected paw.

*Indicates a significant difference in the potency of morphine as compared to the early phase in LA mice or late phase in HA mice ($p < 0.05$).

tive to the antinociceptive effect of morphine in the late phase, there was no significant difference between the potency of morphine in the early and late phases of the formalin test in HA mice.

The antinociceptive effect of ACEA-1011 in LA and HA mice in the formalin test is shown in Fig. 3. A one-way ANOVA revealed that ACEA-1011 produced significant antinociceptive effect in the early phase of the formalin test in both HA, $F(4, 35) = 3.12$, $p < 0.05$, and LA, $F(5, 53) = 2.98$, $p < 0.05$, lines. ACEA-1011 displayed approximately similar potency in HA and LA mice. The respective ED_{50} (mg/kg) of ACEA-1011 in the early phase of the formalin test was 29.41 and 39.24 in both lines. The 95% CLs were not possible to estimate due to the distribution of the data. In the late phase of the formalin test, ACEA-1011 produced a significant antinociception in LA mice, $F(5, 53) = 3.49$, $p < 0.05$; Fig. 3, upper panel) with an ED_{50} of 11.46 (8.99-14.69) mg/kg. On the other hand, ACEA-1011 did not produce a significant antinociceptive effect in HA mice at doses that were effective in LA mice. As shown in Fig. 3, ACEA-1011 seemed to attenuate the formalin-induced nociceptive response in HA line in the late phase of the formalin test, but it was not statistically significant, $F(4, 35) = 2.52$, $p > 0.05$. Therefore, we were unable to estimate an ED_{50} for ACEA-1011 in HA mice in the late phase of the formalin test.

The effect of naloxone on the antinociceptive effect of morphine and ACEA-1011 is shown in Fig. 4. In control Swiss Webster mice morphine and ACEA-1011 each produced a significant antinociception in both phases of the formalin test, $F(3, 28) = 7.26$ and 12.64, $p < 0.01$, for morphine and, $F(3, 31) = 8.7$ and 20.48, $p < 0.01$, for ACEA-1011 in the early and late phase of the formalin test, respectively. Naloxone at 1 mg/kg was able to completely block the antinociception effect of morphine in the early phase ($p < 0.01$); however, in the late phase the blockade was not complete (Fig. 4, upper panel). On the other hand, the same dose of naloxone had no effect on the antinociceptive effect of ACEA-1011 ($p > 0.05$). ACEA-1011 produced a significant antinociception in both phases of the formalin test regardless of the pretreatment with either saline or naloxone (Fig. 4, lower panel).

DISCUSSION

The major finding of the present studies is that HA and LA mice differ in terms of responsiveness to the antinociceptive effect of morphine and ACEA-1011 in the tonic late phase of the formalin test. Morphine was found to be more potent in the HA line, whereas ACEA-1011 was more potent in the LA mice. The antinociceptive effect of ACEA-1011 was not blocked by pretreatment with naloxone, which indicates that the effect of ACEA-1011 is not mediated via opioid receptors. These results, together, suggest that in the opioid-sensitive HA mice the tonic late phase of the formalin-induced nociception is largely mediated by opioid-sensitive mechanisms, whereas

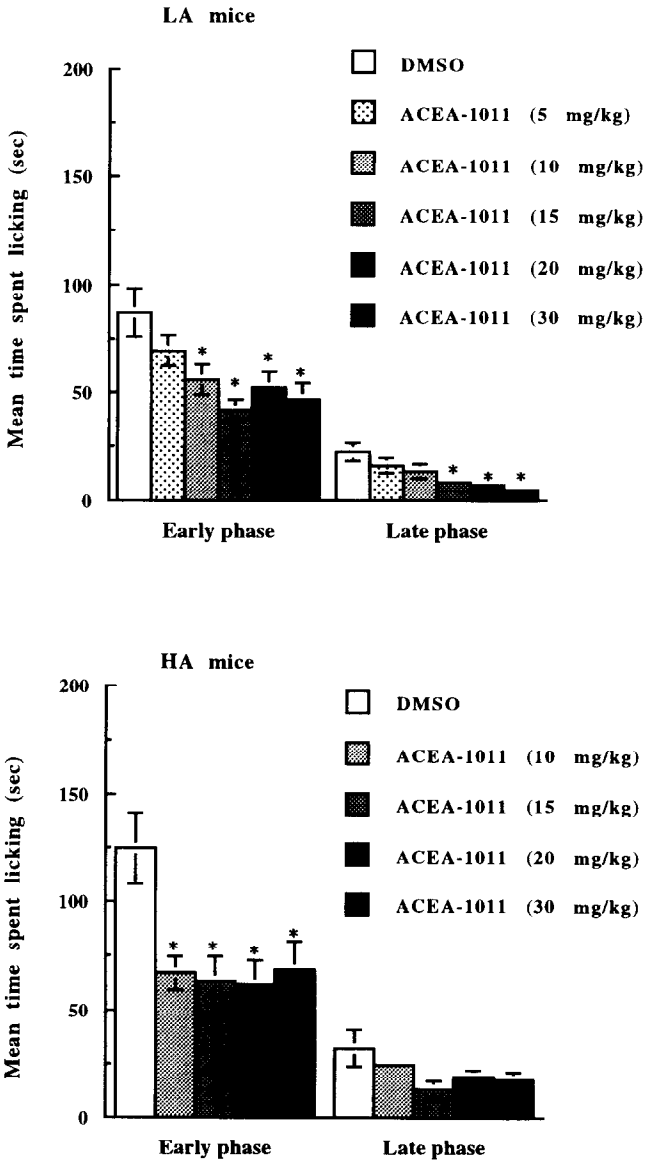

FIG. 3. Antinociceptive effect of ACEA-1011 in the LA (upper panel) and HA (lower panel) mice in the early and late phases of the formalin test. Mice were injected with either ACEA-1011 (5.0-30.0 mg/kg, IP) or DMSO. Thirty minutes later mice were injected with formalin and observed for licking and/or biting of the injected paw for 1 h. The mean time spent licking and/or biting the injected paw (±SEM) is presented here for the first 5 min (early phase) and from 15-50 min (late phase). Data were analyzed by one-way ANOVA followed by Newman-Keuls test. *Significantly different from DMSO-treated mice ($p < 0.05$).

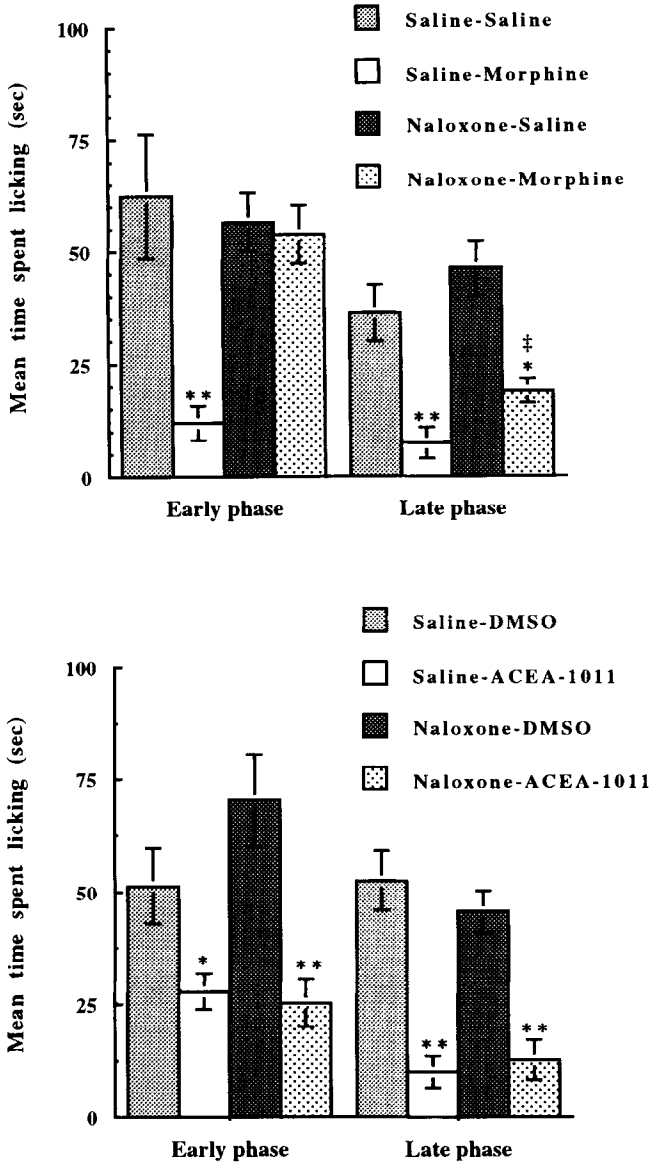

FIG. 4. Effects of naloxone on antinociception induced by either morphine (A) or ACEA-1011 (B). Mice were pretreated with either saline or naloxone (1 mg/kg). Ten minutes later mice were injected with either morphine or ACEA-1011. Controls were injected with either saline or DMSO, respectively. Thirty minutes later mice were injected with formalin and tested. * and ** indicate a significant difference from the respective control group at $p < 0.05$ and $p < 0.01$, respectively. ‡Indicates a significant difference from naloxone-saline treated group ($p < 0.05$).

in the opioid-deficient LA mice a nonopioid-mediated mechanism (most likely NMDA receptors) is also involved.

Subcutaneous injections of formalin in HA and LA mice produced a biphasic nociceptive response consisting of a transient early phase (0–5 min) followed by a tonic late phase (15–50 min) consistent with the results of previous studies using other mouse strains (12,27,29,32). Interestingly, HA mice spent more time licking the injected paw than the LA mice in both phases of the formalin test, suggesting that in the HA mice the nociceptive response to formalin is upregulated. This result is surprising in view of the previously demonstrated greater sensitivity of the LA line to noxious stimuli in the phasic pain models, as reflected by shorter baseline hot plate and tail flick latencies in the LA mice than in the HA mice (23,24). So far, we have no satisfactory explanation for this discrepancy. It is only conceivable that the selection has modified differentially the neuronal mechanism(s) controlling phasic and tonic nociception. This may provide an argument that different neuronal circuits are involved in phasic and tonic pain models.

Morphine decreased the mean time spent licking in both HA and LA mice. The antinociceptive effect of morphine did not differ between the lines in the early phase of the formalin test (Table 1). This indicates that similar opioid receptor-mediated mechanisms are involved in the early phase of the formalin test in HA and LA mice. In the tonic late phase of the formalin test, HA mice appeared to be more sensitive to morphine-induced antinociception than the LA mice. The higher potency of morphine in the tonic late phase of the formalin test in the HA mice is congruent with the higher sensitivity of this line to SSIA and morphine-induced analgesia in the hot plate and tail flick tests, and suggests that the selection has similarly modified neuronal mechanisms of opioid-induced antinociception involving phasic as well as tonic pain inhibition. Furthermore, it points to a common genetic makeup of SSIA and opioid-mediated phasic as well as tonic pain inhibitory mechanisms.

It has been demonstrated that the NMDA receptor antagonists produce antinociception (4,5,19,21,22,32,34). ACEA-1011, which is a novel competitive NMDA receptor/glycine site antagonist (16), reduced the mean time spent licking in both HA and LA mice in the early phase and only in LA mice in the tonic late phase of the formalin test. Furthermore, this compound significantly attenuated the mean time spent licking in control Swiss-Webster mice in both phases of the formalin test, and this effect was not affected by pretreatment with naloxone, the treatment that either completely or partially antagonized the antinociceptive effect of morphine. Although we were unable to study the effect of naloxone in HA and LA mice, based on the results of our study in control Swiss-Webster mice it is assumed that the antinociceptive effect of ACEA-1011 is not mediated via the conventional opioid receptors. The antinociceptive effect of ACEA-1011 in the present study suggests that antagonism at the glycine modulatory site associated with the NMDA receptor is another approach to block NMDA receptors and produce antinociception. The higher potency of ACEA-1011 in the late phase of the formalin test in the LA mice than in the HA mice suggests a greater involvement of nonopioid mediated mechanisms in tonic pain inhibition in the opioid-deficient LA line. This may indicate that selective breeding of mice for divergent SSIA has modified both opioid- and nonopioid-mediated neuronal mechanisms controlling tonic pain. However, the relationship between genetic background of SSIA and nonopioid (NMDA receptor-mediated) pain inhibitory mechanism cannot be as yet evaluated. On the basis of the results of present studies the most likely mechanism controlling antinociception in the LA line involves the NMDA receptor.

In summary, our results suggest that in the opioid-rich HA line antinociception in the tonic late phase of the formalin test is mediated largely by an opioid-mediated event, whereas in the opioid-deficient LA line an NMDA receptor-mediated mechanism is also involved. NMDA receptor antagonists could provide an alternative means for pain management in

patients less responsive to opioid analgesics due to individual deficiencies or tolerance to opiate treatment.

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