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A locus and mechanism of action for associative morphine tolerance

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Abstract

Repeated administration of an opioid in the presence of specific environmental cues can induce tolerance specific to that setting (associative tolerance). Prolonged or repeated administration of an opioid without consistent contextual pairing yields non-associative tolerance. Here we demonstrate that cholecystokinin acting at the cholecystokinin-B receptor is required for associative but not non-associative morphine tolerance. Morphine given in the morphine-associated context increased Fos-like immunoreactivity in the lateral amygdala and hippocampal area CA1. Microinjection of the cholecystokinin B antagonist L-365,260 into the amygdala blocked associative tolerance. These results indicate that cholecystokinin acting in the amygdala is necessary for associative tolerance to morphine's analgesic effect.

The role of cholecystokinin (CCK) as an anti-opioid peptide is firmly established. CCK suppresses the analgesic effect of morphine and other μ -opioid receptor agonists^{1,2}. CCK antagonists potentiate the analgesic effects of exogenous and endogenous μ -opioid receptor agonists^{3–8} and enhance the inhibitory effect of morphine on nociceptive dorsal horn neurons^{9,10}. Additionally, CCK antagonists impede the acquisition of morphine tolerance yet have no effect on morphine dependence or withdrawal^{3,11–16}. Although CCK antagonists potentiate the effects of opioids, they do not alter nociceptive threshold levels when administered independently^{8,16}. There are two distinct CCK receptors (CCK-A and CCK-B) in the CNS^{17–20}.

Learning can contribute to drug tolerance^{21–27}. When morphine administration is paired with specific environmental cues, these cues can act as conditioned stimuli that elicit a conditioned response opposing the agonist effect of morphine (associative tolerance). Although associative analgesic tolerance following repeated opioid administration is well established, the underlying neurobiological mechanisms are unknown.

The μ -opioid receptor is necessary and sufficient for morphine analgesia²⁸. Animals that become non-associatively tolerant to morphine are selectively tolerant to other μ -opioid receptor agonists. In contrast, associative morphine tolerance generalizes such that the effects of agonists acting at other opioid receptors are also reduced²⁹. This suggests that these two types of morphine tolerance involve different CNS circuitry. However, direct evidence for such circuitry differences has yet to be demonstrated.

Because of its role as an anti-opioid peptide, we tested the hypothesis that CCK is required for associative but not non-associative morphine tolerance. In addition, we investigated potential differences in the CNS circuitry underlying these two types of morphine tolerance.

CCK-A and CCK-B receptor antagonists were administered i.v. following the acquisition of either associative or non-associative analgesic tolerance to morphine. To produce associative tolerance (group A), we used a balanced crossover design in which all rats received i.v. morphine in one of two distinct environments and, on alternate days, i.v. saline in the other environment. After several administrations, morphine was ineffective when given in the environment in which the rat had previously received morphine (Fig. 1a, black circles). However, the same dose of morphine was fully effective when administered in the context previously paired with saline (Fig. 1b). Thus, using this behavioral protocol and method of drug administration, the analgesic tolerance to morphine could be accounted for by learning factors and therefore was associative. To produce non-associative tolerance (group NA), morphine pellets were implanted in rats that then received i.v. saline in either environment used to condition rats in the associative tolerance protocol. Group NA animals were tolerant to morphine in all experimental environments (data not shown), indicating that the morphine tolerance observed in these animals was largely independent of context (non-associative).

Results

Systemic administration of the CCK-B antagonist L-365,260, but not the CCK-A antagonist MK-329, blocked the expression of associative tolerance (Fig. 1c and d). Neither L-365,260 nor MK-329 affected the expression of non-associative tolerance (Fig. 1e and f).

Although the same dose of morphine and same baseline tail-flick latency were used to assess analgesic tolerance, it is possible that the non-associative tolerance was of greater magnitude and thus obscured a possible morphine-potentiating effect of L-365,260. To address this possibility, we studied a second group of non-associatively tolerant rats following three instead of five days of morphine pelleting. These rats were only partially tolerant to morphine, so any potentiating effect of L-365,260 on morphine analgesia should have been easy to demonstrate. However, L-365,260 had no effect on morphine analgesia in these partially but non-associatively morphine-tolerant rats (Fig. 1g and h). These data strongly support the specificity of the CCK-B antagonist in the reversal of associative morphine tolerance.

After establishing the effect of the systemically administered CCK-B antagonist L-365,260 on associative tolerance, we investigated the potential CNS sites of its action using Fos immunohistochemistry. Animals were again divided into two groups, associative (A) and

non-associative (NA). Following the acquisition of tolerance, animals trained in the associative contexts were given either i.v. morphine in the morphine-paired environment or saline in the saline-paired environment. Non-associatively tolerant (pellet-implanted) rats were given either morphine in the environment in which associatively tolerant rats were given morphine or i.v. saline in the context in which the associatively tolerant rats had received saline.

Associatively tolerant rats receiving morphine in the morphine-paired environment had significantly more Fos-positive cells in the lateral amygdala (LA) than did animals in the three other groups (Figs. 2 and 3; $p < 0.01$). These same rats also had a significantly greater number of Fos-positive cells in the basolateral amygdala than either group of non-associatively tolerant animals ($p < 0.05$). Associatively tolerant animals receiving either morphine in the morphine-paired context (AM) or saline in the saline-paired context (AS) had significantly more Fos-positive cells in area CA1 than did either group of non-associatively tolerant animals (NAM and NAS groups; $p < 0.01$ and $p < 0.05$, respectively). In contrast, no significant differences were found among these four groups for the other brain regions examined (VTA, NAcc core, NAcc shell and Ce amygdala; Fig. 2). These results indicate that activity of neurons in both lateral and basolateral amygdala and in CA1 of the hippocampus was elevated in associatively tolerant animals receiving morphine in a morphine-associated context as compared with activity under conditions of non-associative tolerance. Region CA1 of the hippocampus was also activated in associatively tolerant rats exposed to saline in the saline-paired context.

Two additional control groups of associatively tolerant animals were examined to determine the specificity of the increase in amygdala *fos* activation. One control group received saline in the morphine-associated context, and the other group received morphine in the saline-associated context. When these two groups were included in the overall statistical analysis, there was a significant main effect of treatment, but only within the lateral amygdala ($p < 0.0001$). *Post-hoc* tests revealed significant differences between animals receiving morphine in the morphine context and those receiving either saline in the morphine context ($p < 0.01$) or morphine in the saline context ($p < 0.01$). Specifically, when given saline in the morphine context, animals had 12.6 ± 12.2 (mean $\pm$ s.e.) cells in the lateral amygdala, whereas those given morphine in the saline context had 7.5 ± 4.9 cells. In contrast, rats given morphine in the morphine context had 76.4 ± 24.6 cells in the lateral amygdala. Thus, the appropriate morphine-context pairing was necessary for *fos* activation within the lateral amygdala, and the activation was not a nonspecific effect of morphine administration.

To determine if the reversal of associative tolerance following the systemic administration of the CCK-B antagonist L-365,260 was due to blockade of an action of CCK in either the amygdala or hippocampus, L-365,260 was microinjected into L/BL amygdala and CA1 following the acquisition of associative tolerance. Injections were also made into Ce amygdala, VTA and NAcc core because these nuclei also express the CCK-B receptor^{18,20,30}.

Microinjection of L-365,260 into L/BL and Ce amygdala significantly attenuated associative morphine tolerance ($p < 0.01$ for both nuclei; Fig. 4). However, no significant change in

associative morphine tolerance was apparent following the microinjection of L-365,260 into CA1, VTA or NAcc core. The effect of L-365,260 in L/BL amygdala was not significantly different from that in Ce amygdala and, importantly, was also not significantly different from the effect of systemic L-365,260. In contrast, the effect of L-365,260 in Ce amygdala was significantly different from the systemic effect ($p < 0.05$). This indicates that CCK in L/BL amygdala was required for the expression of associative morphine tolerance and suggests that the attenuation of associative morphine tolerance by systemic administration of L-365,260 was through an action in L/BL amygdala. Given the proximity of the L/BL amygdala to the Ce amygdala and the data from our Fos study, it seems most likely that the effect of L-365,260 in Ce amygdala is due to diffusion of the drug into L/BL amygdala. Taken together, the evidence indicates that neurons in the L/BL amygdala are activated when morphine is given in the morphine-associated context and that CCK acting in this brain region is necessary for the expression of associative morphine tolerance.

To confirm that L-365,260 alone did not have an analgesic effect, control injections of L-365,260 were made into L/BL amygdala in the saline-paired environment. Whereas the administration of L-365,260 into the L/BL amygdala in the morphine-paired environment significantly attenuated morphine tolerance (Fig. 4), injections of L-365,260 into the L/BL amygdala in the unpaired environment had no effect (Fig. 4b). This indicated that L-365,260 in the L/BL amygdala did not have an antinociceptive effect independent of morphine administration.

Discussion

The amygdala is implicated in the encoding of affective memories^{31–33} and in the expression of stimulus–reward associations^{34,35}. Additionally, neurons in the amygdala change their pattern of firing in response to emotionally significant stimuli^{36,37}. Our current results demonstrate that the L/BL amygdala is necessary for the expression of the compensatory response that results from the association between particular environmental cues and the effect of morphine.

When injected into the amygdala, opioid antagonists enhance memory retention³⁸, and the intra-amygdaloid administration of CCK agonists facilitates learning and memory^{39,40}. Furthermore, CCK mRNA expression increases in the amygdala following repeated morphine administration, suggesting that the amygdala is a critical locus for a CCK-mediated compensatory mechanism that contributes to opioid tolerance⁴¹. We have demonstrated that CCK release in the amygdala is required for a contextually triggered compensatory action that maintains pain sensitivity in the presence of morphine. We propose that this compensatory action does not occur in non-associative tolerance because the required temporal relationship between contextual stimuli and drug administration has not been established through conditioning. Our results raise the possibility that the contextual cues that signal impending drug administration are necessary for the release of CCK in the L/BL amygdala. Another possibility is that morphine elicits CCK release in the L/BL amygdala in associatively tolerant rats but that this does not occur in non-associatively tolerant animals.

The contribution of the hippocampus to learning and memory is clearly established^{42–44}. Furthermore, CCK seems to be involved in the acquisition and retrieval of emotionally based memory through an action in the hippocampus⁴⁵. Although we did not establish a contribution of hippocampal CCK, the Fos analysis suggests that CA1 neurons contribute to the expression of associative morphine tolerance. The hippocampus seems to serve as a locus for memory consolidation and to perform the match–mismatch discriminations necessary for memory recognition⁴⁶. Moreover, there are direct, reciprocal connections between the hippocampus and L/BL amygdala^{47,48}. We suggest that the projection from CA1 to the L/BL amygdala contributes to associative tolerance.

Neurons in the hippocampus and amygdala are postulated to participate in a mechanism of learning and memory necessary for processing and assigning value to drug-associated cues, which contributes to drug addiction⁴⁹. It is intriguing that this circuitry overlaps with that involved in associative tolerance. We hypothesize that during the acquisition phase of associative tolerance, CA1 is important for the match–mismatch discrimination that occurs when an animal is exposed to the morphine-associated environment. Therefore, we predict that acquisition of associative tolerance will be impaired when hippocampal connections are disrupted.

In summary, we demonstrated that the CCK-B receptor antagonist L-365,260 reverses associative but not non-associative morphine tolerance. Additionally, our findings indicate that associative and non-associative tolerance involve different patterns of neuronal activity in the L/BL amygdala and in CA1 of the hippocampus. Furthermore, we demonstrated that the learned compensatory mechanism contributing to associative morphine tolerance requires an action of CCK at the CCK-B receptor in the L/BL amygdala.

Methods

Subjects

Male Sprague-Dawley rats (Charles River, Wilmington, Massachusetts) weighing 300–325 g were individually housed. The colony room was kept constant at 21°C and followed a standard 12-h light/dark cycle with food and water available *ad libitum*. Animals were tested at the same time during their light cycle each day.

Drugs

Morphine sulfate powder (supplied by the National Institute on Drug Abuse) was dissolved in physiological saline for micro- and i.v. injections. Subcutaneous morphine pellets (75 mg morphine base) were obtained from the National Institute on Drug Abuse. MK-329 was obtained from Merck Pharmaceuticals (Terlings Park, UK) and was dissolved in distilled water. The CCK-B antagonist L-365,260 (a gift from Les Iversen, Panos Pharmaceuticals) was dissolved in 90% ethanol.

Surgery

Animals were anesthetized with 50 mg per kg Nembutal, implanted with jugular catheters (PE 50), and allowed a one week recovery period. Group A animals in the microinjection

study were implanted bilaterally with stainless steel 26 gauge chronic guide cannula (Plastics One, Roanoke, Virginia). Group NA animals were anesthetized with halothane (1–4%) and administered 2 subcutaneous morphine pellets (75 mg morphine base, NIDA) every 24 h for 4 days following the recovery period.

Behavioral apparatus

Tail-flick latency was measured using a radiant heat source below the glass floor of the experimental chamber. The source was positioned beneath the rat's tail. A photocell was affixed to the radiant heat source so that movement of the tail terminated the trial. A cutoff time of 12 s was used to avoid tissue damage. The behavioral chambers measured 15 × 15 × 15 inches and consisted of either white or black Plexiglas walls and a Plexiglas ceiling.

Behavioral protocol

Group A (associative tolerance) rats were exposed to two distinct environments on alternating days for 16 days. Environments differed in size, color, smell, tactile stimulation and lighting. On the first day in each environment, rats were administered 0.05 ml physiological saline i.v. before being placed in the experimental chamber. Once inside, animals were allowed a 15-min habituation period. Tail-flick latency was then measured every 10 min for 60 min. On alternate days, animals were given 2.5 mg per kg morphine i.v. in 0.05 ml physiological saline in one environment or 0.05 ml physiological saline in the other environment. Following i.v. administrations, animals were allowed a 15-min habituation period; subsequently tail-flick latency was measured as before.

Fos study

On the last experimental day (day 15), animals were divided into four groups of four animals: saline in the saline- or morphine-associated context and morphine in the morphine or saline context. Ninety min after drug administration on the last experimental day, all animals were perfused, and their brains were removed and processed for Fos.

Systemic drug administration

On experimental day 15 or 16, animals received either L-365,260 (0.2 mg per kg, i.v.) or MK-329 (0.1 mg per kg, i.v.) in their home cages 7 min before morphine administration. Following morphine administration, animals were allowed to habituate to the experimental environment for 15 min before tail-flick latency was measured. On experimental day 15 or 16, morphine was administered in the saline-paired environment to confirm that the tolerance that developed was associative. Experimental environments and testing days were counterbalanced. Thus, animals that received L-365,260 or MK-329 plus morphine in the morphine context on day 15 received morphine alone in the saline-paired environment on day 16 and *vice versa*.

Microinjection study

The injection and testing protocol was the same as for the systemic drug administration, but only L-365,260 (15 ng in 0.5 µl per brain site) was used. Animals that received microinjections into the L/BL amygdala participated in one additional experimental day. On

day 17, these animals were divided randomly into two groups. Half of the animals were microinjected with L-365,260 (15 ng in 0.5 μ l per brain site) in the saline-paired environment while the other half received volume-matched microinjections of vehicle (90% ethanol) in the saline paired environment. Tail-flick latency was then measured as before.

Group NA (non-associative tolerance)

On the first experimental day, animals received 0.05 ml saline i.v. followed by a 0.2-ml heparinized saline flush. Animals were allowed a 15-min habituation period in one of the two experimental environments used to condition rats in the associative tolerance study. Tail-flick latency was then measured every 10 min for 60 min. Animals were then returned to their home cages. Sixty min later, animals were implanted with two subcutaneous morphine pellets and returned to their home cages. Thus all non-associatively tolerant animals were exposed to high levels of morphine in their home cages as well as in the two contexts used for saline and morphine conditioning (for the associatively tolerant rats).

Systemic study

Animals were pellet-implanted as above. On the last experimental day, animals were divided into 2 groups. Seven min before i.v. administration of 2.5 mg per kg morphine, group 1 received either MK-329 or L-365,260. Forty-five min following the i.v. administration of 2.5 mg per kg morphine, group 2 received either MK-329 or L-365,260. Following morphine administration, group 1 animals were placed in one of the experimental environments and allowed a 15-min habituation period. Tail-flick latency was measured every 10 min for 60 min. Following morphine administration, group 2 animals were placed in one of the experimental environments and allowed a 15-min habituation period before tail-flick latency was measured every 5 min for 30 min. Group 2 animals were then removed from the experimental environment, administered either MK-329 or L-365,260 and then placed back in the experimental environment. Animals were allowed a 15-min habituation period. Tail-flick latency was measured every 5 min for 30 min. Care was taken to treat NA animals in such a way as to prevent the association of morphine administration with any environmental cues.

Microinjections

All injection sites were determined based on the atlas of Paxinos and Watson (1986). Bilateral microinjections were made into the following brain structures: VTA ($n = 7$; AP, -5.8 ; ML, ± 0.5 ; DV, -8.5), NAcc core ($n = 10$; AP, 2.5 ; ML, ± 1.2 ; DV, -6.5), CA1 ($n = 12$; AP, -3.6 ; ML, ± 1.5 ; DV, -3.0), CeA ($n = 7$, AP, -2.5 ; ML, ± 4.3 ; DV, -8.3), L/BLA ($n = 12$; AP, -2.8 ; ML, ± 5.2 ; DV, -8.5). Each injection was made using a 1 μ l syringe (Hamilton, Reno, Nevada) attached to 10 cm of PE 50 tubing connected to a 33-gauge injection cannula (Plastics One). Microinjection was conducted at a rate of 0.5 μ l per min. The injection cannula extended 2 mm past the guide cannula and was left in place for 1 min following microinjection to minimize the backflow of drug solution.

At the conclusion of the experiment, animals were anesthetized with pentobarbital, injected with 0.5 μ l pontamine sky blue per brain site and perfused intracardially through the

ascending aorta. Brains were blocked and sectioned coronally at 50 μ m, mounted and stained with neutral red.

Fos study

On days 2 and 4, animals were allowed a 75-min habituation period in one of the 2 experimental environments, whereas the other context was used on days 3 and 5. Animals were then returned to their home cages for 60 min, after which animals were anesthetized and administered 2 subcutaneous morphine pellets as before. On the last experimental day (day 6), half ($n = 4$) of the animals received morphine in the context in which associative animals (group A) had received saline, and half received morphine in the context in which associative animals had received morphine. Ninety min after drug administration on the last experimental day, all animals were perfused, and their brains were removed and processed for Fos. Care was taken to prevent the association of morphine administration with any environmental cues.

Immunohistochemistry

Animals were perfused with 150 ml 0.1 M phosphate-buffered saline followed by 400 ml of 4% formaldehyde in 0.1 M phosphate buffer. Brains were then removed and post-fixed for 2 h at room temperature. Tissue was transferred to a 30% sucrose solution and left overnight. Tissue was cut in 50- μ m sections and washed in a solution of 0.05 M tris-phosphate-buffered saline with 1% normal goat serum and 0.3% TritonX before being incubated for 1 h at room temperature in a blocking solution of 0.05 M Tris-phosphate-buffered saline with 3% normal goat serum and 0.3% TritonX. Primary antibody (rabbit polyclonal, provided by Dennis Slamon, University of California, Los Angeles) was then applied to the tissue. Tissue was incubated overnight at room temperature. Sections were thoroughly washed before the application of secondary antibody (goat anti-rabbit IgG and avidin-biotin-peroxidase complex, Vector Labs, Burlingame, California). Secondary antibody was left on the tissue for a period of 2 h. A nickel/diaminobenzidine (DAB) reaction was used to visualize Fos immunoreactivity⁵⁰. Tissue was processed simultaneously for Group A and NA animals.

Fos quantification

Sections were examined under dark-field illumination to identify the boundaries of each brain region of interest. To simplify quantitation, regional boundaries were defined (NAcc, AP 2.2 to AP 0.48; VTA, AP -5.2 to AP -6.8; CA1, AP -2.3 to AP -4.16; CeA, AP -1.8 to AP -3.0; BLA, AP -1.8 to AP -3.3; LA, AP -2.5 to AP -3.3). Fos-positive cells were quantified at 20 $\times$ magnification using light-field illumination. An observer blind to the experimental treatment counted Fos-positive cells. Once quantitation was completed, the total number of cells for each animal in each brain region was divided by the number of section per region, rendering a mean number of Fos cells for each animal for each brain nucleus.

Statistical analysis: Fos study

A one-way randomized ANOVA was used to analyze the data collected from each brain region. Tukey-Kramer *post-hoc* tests were conducted for pairwise comparisons.

Statistical analysis: systemic and microinjection studies

The mean of the six tail-flick latencies measured for each animal was calculated for each test day. As the data did not follow a standard distribution (due to the use of a 12-s cut-off) non-parametric statistics were used for data analysis. Wilcoxon two-group signed-rank tests were used to compare the mean tail-flick latencies on the different test days. Statistical significance was set at $p < 0.05$. All statistical tests were conducted using GB-STAT, version 6.5. All figures were created using StatView, version 4.0, (SAS Institute, Cary, North Carolina) and Adobe Illustrator, version 5.5.

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Fig. 1.

Associative tolerance is reversed by CCK-B receptor antagonism. **(a)** Acquisition of associative tolerance ($n = 24$) with administration of saline on even-numbered days and morphine (2.5 mg per kg) on odd-numbered days in their associated environments. **(b)** Associative tolerance is context dependent. Tail-flick latency ($n = 19$) in morphine-paired and saline-paired environments before (Baseline) and after (Morphine) the acquisition of associative tolerance. Baseline tail-flick latencies were measured on experimental days 1 and 2, respectively. Animals were tolerant to morphine when tested in the morphine-paired environment by the sixth administration (day 13) but showed no evidence of analgesic tolerance to the same dose of morphine given in the saline-paired environment (** $p < 0.01$). **(c, d)** Associative tolerance is reversed by the systemic administration of the CCK-B

antagonist L-365,260 (0.2 mg per kg, $n = 11$, $p < 0.01$) but not by the CCK-A antagonist MK-329 (0.1 mg per kg, $n = 13$). (**e, f**) Non-associative tolerance is not reversed by the systemic administration of L-365,260 (0.2 mg per kg, $n = 8$) or MK-329 (0.1 mg per kg, $n = 4$). Following the full development of non-associative tolerance, L-365,260 and MK-329 were administered 7 min before systemic morphine. (**g–j**) Neither pre- nor post-treatment with CCK antagonists reversed partial non-associative tolerance. To avoid a ceiling effect, two groups of animals were implanted with subcutaneous morphine pellets every day for three instead of five days. This produced partial analgesic tolerance, which was unaffected by either L-365,260 (0.2 mg per kg, $n = 13$) or MK-329 (0.1 mg per kg, $n = 13$). (**g, h**) L-365,260 given 45 min after an additional challenge dose of morphine (2.5 mg per kg, i.v., $n = 6$, gray bar) does not potentiate morphine in partially tolerant rats (black bar, **g**). As a fall in morphine concentration could account for this failure, we controlled for this possibility by administering L-365,260 7 min before systemic morphine administration (2.5 mg per kg, i.v., $n = 7$; **h**). No potentiation was observed (black bar in **h** not significantly different from gray bar in **g**). (**i, j**) MK-329 had no significant effect on analgesia in partially tolerant rats, whether administered 45 min after (**i**; $n = 6$) or 7 min before (**j**; $n = 7$) a challenge dose of morphine (2.5 mg per kg, i.v.).

Fig. 2.

Mean number of Fos-positive cells per section (50 μm) per brain region for associatively and non-associatively tolerant animals ($n = 4$ animals per group). Increases in Fos expression are observed in amygdala and hippocampus during expression of associative morphine tolerance. NAcc, nucleus accumbens; VTA, ventral tegmental area; CA1, hippocampal area CA1; CeA, central amygdala; BLA, basolateral amygdala; LA, lateral amygdala. (* $p < 0.05$; ** $p < 0.01$).

Fig. 3.

Photomicrographs of Fos-positive cells in the amygdala of associatively and non-associatively tolerant animals. AS, associatively tolerant animals administered saline in the saline-paired environment on experimental day 15; AM, associatively tolerant animals administered morphine in the morphine-paired environment on experimental day 15; NAS, non-associatively tolerant animals administered saline on experimental day 6; NAM, non-associatively tolerant animals administered morphine on experimental day 6.

Fig. 4.

Microinjection of the CCK-B receptor antagonist L-365,260 into CNS sites. Abbreviations as in Fig. 2. **(a)** Comparison of tail-flick latencies following systemic administration (SYST; $n = 11$) or microinjections of L-365,260 into the L/BLA ($n = 12$), CeA ($n = 7$), VTA ($n = 7$), NAcc core ($n = 10$) or CA1 ($n = 12$). White bars, baseline in the morphine-paired context on experimental day 1 or 2; black bars, L-365,260 administered 15 min before morphine on experimental day 15 in the morphine-paired context (** $p < 0.01$, * $p \pm 0.05$). **(b)** Comparison of tail-flick latencies following the administration of L-365,260 or vehicle in either the morphine-paired or saline paired-context. White bars, baseline in the saline-paired and morphine-paired contexts; black bars, L-365,260 administered either 15 min before morphine in the morphine-paired context on experimental day 15 or 15 min before saline in the saline-paired context on experimental day 17; gray bar, vehicle (90% ethanol) administered 15 min before saline in the saline-paired context on experimental day 17.