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Accumbal opioid receptors modulate cue competition in one-trial overshadowing

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Abstract

The contribution of opioid receptors in the nucleus accumbens to contextual and auditory fear conditioning was examined. Impairment in contextual fear conditioning was found when training occurred under accumbal infusions of the opioid receptor agonist morphine in a dose-dependent and receptor specific fashion, only when shock onset coincided with auditory stimulus offset. Contextual fear conditioning was spared, however when the delivery of shock was not signalled by an auditory stimulus, the auditory stimulus was of low intensity (70dB), or an interval (10s or 30s) was interpolated between auditory stimulus offset and shock onset. These results provide evidence that opioid receptors in the nucleus accumbens regulate competition between contextual and discrete auditory stimuli for association formation.

Keywords

fear; rat; ventral striatum; attention; morphine; learning; memory

1. Introduction

Pavlovian fear conditioning in rodents is widely used to study the substrates of learning and memory in the mammalian brain. In fear conditioning rats receive pairings between a neutral stimulus (e.g., noise) and footshock in a distinctive chamber (context). These pairings establish noise-shock and context-shock associations, which are evidenced in the expression of fear reactions upon re-exposure to the noise conditioned stimulus (CS) or to the conditioning context. These fears interact such that the more fear develops to one, the less fear develops to the other. For example, prior training to fear the conditioning context reduces or blocks the establishment of fear to a noise trained in that context (Iordanova, 2010; Iordanova et al., 2006ab). Similarly, variations in the interval between CS offset and shock onset also results in the establishment of selective fear responses: While co-termination of the CS with footshock onset (delay conditioning) establishes greater fear to the CS compared to the context, the interpolation of an interval between CS offset and

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footshock onset (trace conditioning) results in greater fear to the context compared to the CS (Merlin, 1981; Odling-Smee, 1978).

The nucleus accumbens (Acb) is involved in selecting between two stimuli (a CS and context) for association with shock. Morphine infusions into the Acb remove the blocking of noise conditioning by a pre-trained context (Iordanova et al., 2006a). In delay conditioning, rats with lesions (Riedel et al., 1997) or temporary inactivation of the Acb (Haralambous and Westbrook, 1999) learned to fear a noise CS on par with control rats, but acquired less fear to the context where the shock occurred. In trace conditioning, however, this effect was reversed: rats with accumbal lesions and controls acquired equivalent fear to the context but not the noise CS (Parkinson et al., 1999, Bradfield and McNally, 2010). Thus the Acb can be said to modulate the ability of one stimulus to overshadow learning about another.

The specific role of the Acb in stimulus selection remains unclear. Evidence from blocking suggests that the Acb serves to modulate learned changes in attention (for reviews see Iordanova, 2009; McNally and Westbrook, 2006): while control rats withdraw their attention from the noise because its introduction into the pre-trained context was not accompanied by any change in the already well predicted US, rats infused with morphine in the Acb maintained (rather than withdraw) attention to the noise in spite of the lack of change in the US, thus allowing for noise-shock learning to take place (Iordanova et al., 2006a). In the present series of studies we used a one-trial conditioning procedure to study the effects of accumbal opioid receptors on the allocation of attention to a noise CS and a context in delay and trace fear conditioning. One trial overshadowing removes the effects of prior learning on stimulus selection thus allowing us to examine the role of the Acb on “unlearned” modulation of attention i.e. stimulus processing based on physical as opposed to predictive properties. Experiment 1 confirmed that an Acb infusion of morphine disrupted context but not CS conditioned fear in delay conditioning; Experiments 2 and 3 studied the dose-response function and the receptor specificity of the drug’s action; Experiments 4 and 5 examined the effect of Acb morphine infusion on context and CS fear under conditions which reduced CS conditioning, namely in a trace procedure and by decreasing the CS intensity in a delay procedure.

2. Results

2.1. Experiment 1: Accumbal infusions of morphine disrupt contextual conditioning when shock is signalled by a CS but not when shock is unsignalled

Experiment 1 aimed to examine the involvement of opioid transmission in the Acb in the interplay between conditioning to a context and a discrete auditory CS in a standard delay conditioning procedure. Specifically, this experiment sought to confirm that an infusion of morphine into the Acb impairs contextual conditioning when the shock US is preceded by an auditory CS, and determine whether this impairment is dependent on the presence of a discrete signal for shock delivery. Rats were given either signalled (presence of an auditory CS) or unsignalled (no auditory CS) shock presentations in a conditioning context under saline or morphine infusions in the nucleus accumbens. The rats were tested for conditioned fear to the training context and the CS (in a novel context) in a drug-free state. It was expected that in the signalled groups, rats trained under accumbal infusions of saline would show fear reactions (freezing) to both the conditioning context and the auditory CS (clicker), whereas those trained under accumbal infusions of morphine would show overshadowing of the context by the auditory CS. In the case of the unsignalled groups, there would be no opportunity for overshadowing and therefore contextual fear should proceed equally between the saline and morphine groups.

Figure 1A provides a specific example of the placement of each microinjection cannula tip each for each rat in Experiment 1 and Figure 1B shows a photo micrograph of a representative cannula placement in the Acb for the same experiment. In addition, Figure 1C depicts the area within which the tip of the microinjection cannula for each rat was located in Experiments 1–5. Cannula placements in the Acb ranged from 2.2 mm to 0.48 mm anterior to bregma and as seen in Figures 1A and 1C, the placements were located in the core and shell equally. In Experiment 1, data from four rats were excluded from statistical analysis due to blockage of the cannula or to placement of the cannula outside the Acb.

Figure 2 shows the mean percentage of time spent freezing for the remaining rats in Groups Saline-Unsignalled ($n = 9$), Morphine-Unsignalled ($n = 9$), Saline-Signalled ($n = 8$) and Morphine-Signalled ($n = 9$) averaged across the first two minutes of context (left panel) and clicker (right panel) tests. The first two minutes of test were used across all experiments in order to minimise the effect of extinction on test performance. Inspection of these panels suggests that accumbal infusions of morphine spared freezing to the clicker CS but reduced freezing to the context where the clicker-shock pairing occurred. Further, this reduction in contextually-controlled freezing appeared to be specific to rats that had been exposed to a clicker-shock pairing. The 2×2 ANOVA of the freezing levels exhibited during context test confirmed these observations. There was a significant main effect for infusion of morphine versus saline ($F_{(1, 31)} = 14.3$; $p < 0.05$ where $F_c = 4.2$), no main effect for presence versus absence of the auditory stimulus, ($F = 1.8$; $p > 0.05$), and a significant interaction between these factors ($F_{(1, 31)} = 8.1$; $p < 0.05$). Post-hoc analyses on the difference in contextual freezing between the saline and morphine groups revealed a difference in the signalled ($F_{(1, 31)} = 21.35$; $p < 0.05$) but not unsignalled shock condition ($F_{(1, 31)} < 1$). In addition, while the levels of freezing differed in the signalled vs. unsignalled shock presentation in the morphine groups ($F_{(1, 31)} = 9.1$; $p < 0.05$), no such difference was observed in the saline groups ($F_{(1, 31)} = 1.1$; $p > 0.05$).

In this and remaining experiments there was little freezing (less than 5%) when rats were exposed to the second context prior to clicker presentation. The 2×2 ANOVA of the freezing levels during clicker test revealed significant main effect for presence versus absence of the clicker ($F_{(1, 31)} = 205.5$; $p < 0.05$), such that freezing was greatest among rats conditioned with the auditory stimulus. There was no main effect for Acb microinjection of morphine versus saline ($F_{(1, 31)} = 1.3$; $p > 0.05$) nor was there was a significant 2×2 interaction ($F < 1$). In order to check that the failure to detect a difference between freezing to the clicker CS in the morphine- and saline-infused rats was not due to a ceiling effect, we assessed freezing across an additional 6 min of testing (Figure 3). There were no statistically significant differences between the levels of freezing of rats in Groups Saline-Signalled and Morphine-Signalled, $F(1, 15) < 1.0$. There was a significant linear trend, $F(1, 15) = 117.1$ confirming that freezing declined across the test, and there is no significant interaction between linear trend and Groups Saline-Signalled and Morphine-Signalled, $F(1, 15) = 2.0$.

In order to ensure that the order in which rats were tested had not influenced performance to the context and CS, we conducted an additional experiment in which rats were conditioned in the same way as in the signalled groups in Experiment 1 under an accumbal infusion of morphine (7.5 μ g, $n = 8$) or saline ($n = 8$). The following day rats were tested for freezing to the clicker in the second context. There were no differences in the levels of freezing on this clicker test (saline mean = 86.1%, SEM = 4.5%; morphine mean = 73%, SEM = 8.3), $t_{(14)} = 1.48$, $p > 0.05$. On the day following this clicker test, rats were tested for fear to the conditioning context. The accumbal infusions of morphine again significantly reduced freezing in the context where clicker-shock pairings had occurred relative to the freezing elicited by that context in the control rats [saline mean = 35% (SEM = 5.9%); morphine mean = 11.7%, (SEM = 4.6), $t_{(14)} = 3.32$, $p < 0.05$]. Therefore, the impairment in context but

not CS fear in rats trained under accumbal infusions of morphine when the shock was signalled by an auditory CS was not due to the order in which the tests occurred. Rather, the results show that accumbal infusions of morphine disrupted contextual fear conditioning only in the signalled but not unsignalled shock presentation.

2.2. Experiment 2: The impairment of contextual fear conditioning by morphine into the Acb is dose-dependent

The aim of Experiment 2 was to determine whether the deficit in contextual fear conditioning produced by an accumbal infusion of morphine in rats trained in the standard delay procedure was dose-dependent. Four groups of rats were given a single clicker-shock pairing in the conditioning context. Prior to this training the rats received an infusion of either Saline or one of three doses of Morphine (3.75-, 7.5- or 15- μ g). These doses of morphine are similar to ones infused in the Acb previously (e.g., 4-, 8- and 16 μ g/ μ l, Westbrook et al., 1997, Experiment 2a).

The data from four rats were excluded from the statistical analysis due to infection, inaccurate cannula placement or damage to the Acb. Figure 4 shows the mean percentage of time spent freezing for the remaining rats in Groups Saline ($n = 8$), Morphine-3.75 ($n = 9$), Morphine-7.5 ($n = 10$) and Morphine-15 ($n = 9$) averaged across the first two minutes of context (left panel) and clicker (right panel) tests. Inspection of these panels suggests that morphine dose-dependently decreased the levels of freezing to the context. Specifically, the levels of freezing were high and similar for rats trained under accumbal infusions of saline and those trained under the lowest dose of morphine, whereas freezing was markedly reduced for rats conditioned under accumbal infusions of the two higher doses of morphine. There were no such dose-dependent effects on the levels of freezing on the clicker test; the levels of freezing in all groups were high and similar.

Statistical analysis confirmed these observations. Rats conditioned under accumbal infusions of saline (Group Saline) displayed significantly higher levels of freezing to the context than rats trained under accumbal infusions of morphine (Groups Morphine-3.75, Morphine-7.5, and Morphine-15) ($F_{(1, 32)} = 11.6$; $p < 0.05$, where $F_c = 6.4$, $k = 3$). There was evidence for a dose-response effect of morphine; rats conditioned under the lowest dose of morphine (Group Morphine-3.75) froze significantly more than rats conditioned under the two higher doses (Groups Morphine-7.5 and Morphine-15) ($F_{(1, 32)} = 31.1$; $p < 0.05$) whose levels of freezing did not differ ($F_{(1, 32)} = 1.5$; $p > 0.05$).

The test for freezing to the clicker failed to reveal differences between rats trained under accumbal infusions of saline and morphine ($F < 1.0$), or between rats that received the lowest versus the two higher doses of morphine ($F_{(1, 32)} = 1.9$; $p > 0.05$), or between these latter two groups ($F < 1.0$). These results show that a microinjection of morphine into the Acb reduced the level of contextual fear conditioning in a dose-dependent manner but had no effect on the level of fear to the clicker CS.

2.3. Experiment 3: The impairment in contextual fear by infusion of morphine into the Acb is receptor-specific

Experiment 3 aimed to examine the opioid receptor specificity of the effect of accumbal morphine infusions on the contextual conditioned freezing deficit reported in Experiments 1 and 2. If the effect was due to the action of morphine on opioid receptors, then the deficit in contextual fear conditioning should be attenuated (that is, context freezing will be present) when a combination of morphine and the competitive antagonist, naloxone, is infused into the Acb prior to conditioning. Four groups of rats were given one clicker-shock pairing in

the conditioning context. Prior to this pairing rats received either an infusion of Saline, Morphine, Naloxone or Morphine and Naloxone.

Two rats were excluded due to incorrect cannula placements. Figure 5 shows the mean percentage of time spent freezing for the remaining rats in Groups Saline ($n = 9$), Morphine ($n = 9$), Naloxone (Nlx, $n = 10$) and Morphine-Naloxone (Mor-Nlx, $n = 10$) averaged across the first two minutes of context (left panel) and clicker (right panel) tests. Inspection of these panels suggests that the infusion of morphine into the Acb spared fear conditioning to the clicker but reduced conditioning to the context. This reduction in contextual conditioning was spared when both morphine and naloxone were infused into the Acb.

The statistical analysis confirmed these observations. There was significantly less contextual freezing among rats that had been infused with morphine prior to conditioning compared to rats in the remaining three groups pooled together ($F_{(1, 34)} = 42.5$; $p < 0.05$, where $F_c = 4.13$). There were no significant differences between the levels of freezing by rats that had been infused with saline and those that had been infused with naloxone (Group Nlx and Group Mor-Nlx; $F < 1$). Finally, there were no significant differences between the levels of freezing between rats in the naloxone groups ($F < 1$). Similar analyses during the clicker test revealed no differences between the groups ($\max F_{(1, 34)} = 3.2$; $p > 0.05$).

The results of this experiment show that the disruption of contextual fear conditioning by infusion of morphine was due to its action on opioid receptors in the Acb because this reduction in contextual fear conditioning was spared when morphine was combined with the competitive antagonist naloxone.

2.4. Experiment 4: Infusion of morphine into the Acb impairs context conditioning in a delay but not in a trace procedure

The previous three experiments confirmed that agonising opioid receptors in the Acb prior to a single clicker-shock pairing disrupted the establishment of conditioned freezing to the context in which this pairing occurred but not to the auditory stimulus. Experiment 1 showed that this deficit is not due to opioid receptors disrupting contextual fear learning per se. Rather this effect is contingent upon a signalled shock delivery. The aim of the present experiment was to reduce the strength of the auditory CS as a signal for US occurrence and examine the effect of accumbal opioid receptor agonism on contextual fear conditioning. The amount of fear conditioned to the CS is negatively related to the temporal interval between CS offset and US onset (Merlin, 1981) such that stronger conditioning ensues when CS offset co-occurs with US onset (delay conditioning) and weaker conditioning when an interval is introduced between the CS and the US (trace conditioning). Therefore, in Experiment 4 a trace conditioning procedure is employed in order to examine the role of accumbal opioid receptors on contextual and auditory fear conditioning when the strength of the CS-US association is weakened.

The data from one rat was excluded from the statistical analysis due to the removal of a cannula cap. Figure 6 shows the mean percentage of time spent freezing on test for the remaining rats in Groups Saline-Unsignalled ($n = 10$), Morphine-Unsignalled ($n = 10$), Saline-Delay ($n = 9$) and Morphine-Delay ($n = 10$), Saline-Trace10 ($n = 10$) and Morphine-Trace10 ($n = 10$), Saline-Trace30 ($n = 10$) and Morphine-Trace30 ($n = 10$) across the first two minutes of the context (left panel) and clicker (right panel) tests. Inspection of the left panel suggests that rats infused with morphine and shocked immediately after the signal offset (Group Morphine-Delay) froze less to the context than rats in all of the other groups. The statistical analysis confirmed this observation. Rats infused with morphine into the Acb displayed significantly less freezing to the conditioning context than rats infused with saline ($F_{(1, 71)} = 6.7$; $p < 0.05$, where $F_c = 6.0$, $k = 3$). Rats shocked concomitantly with CS offset

(Groups Delay) froze significantly less than rats shocked in the absence of a signal (Groups Unsignalled) or rats shocked after either 10s (Groups Trace-10s) or 30s (Groups Trace-30s) ($F_{(1, 71)} = 11.2$; $p < 0.05$). No other main effect contrasts yielded a significant result.

Importantly, there was a significant interaction: the difference between the levels of freezing in saline- and morphine-infused rats subjected to delay conditioning (Groups Saline-Delay vs. Morphine-Delay) was greater than the difference in the levels of freezing between saline- and morphine-infused rats subjected to unsignalled shock or trace conditioning ($F_{(1, 71)} = 13.9$; $p < 0.05$). There were no other significant interactions ($F_s < 1.0$). A post-hoc analysis revealed that rats in Group Morphine-Delay displayed significantly less freezing than rats in Group Saline-Delay, ($F_{(7, 71)} = 20.1$; $p < 0.05$). There were no significant differences between morphine- versus saline-treated rats in any of the other conditions ($F_s < 1.0$). These results confirm that an accumbal morphine infusion reduced context fear conditioning in a delay procedure, when shock co-occurred with the offset of the signal, relative to when shock was unsignalled. They have also shown that such an infusion fails to reduce context fear conditioning in a trace procedure, when the shock occurred some time after the offset of the signal.

Inspection of the right panel of Figure 6 suggests that an accumbal infusion of morphine did not influence the levels of freezing controlled by the CS in either the delay or trace conditions. The levels of freezing for both saline- and morphine- infused rats decreased as the temporal interval between the signal offset and the shock onset increased, but there were no differences between saline- and morphine- infused rats within any of the conditions. The statistical analysis confirmed these observations. There were no statistically significant differences between the levels of freezing of morphine and saline infused rats, $F < 1.0$. Rats that had been exposed to unsignalled shock (Groups Saline-Unsignalled and Morphine-Unsignalled) displayed negligible levels of freezing when tested with the clicker in comparison to the substantial levels by rats in the remaining groups, all of whom that had been exposed to the clicker at conditioning ($F_{(1, 71)} = 99.9$; $p < 0.05$, where $F_c = 6.0$ ($k = 3$). Rats in the delay procedure (Groups Saline-Delay and Morphine-Delay) displayed significantly higher levels of freezing than rats in the trace procedure (Saline-Trace10, Morphine-Trace10, Saline-Trace30 and Morphine-Trace30) ($F_{(1, 71)} = 131.5$; $p < 0.05$). Rats that had been exposed to a 10 s trace interval (Groups Saline-Trace10 and Morphine-Trace10) displayed significantly higher levels of freezing than those exposed to a 30 s trace interval (Groups Saline-Trace30 and Morphine-Trace30) ($F_{(1, 71)} = 15.3$; $p < 0.05$). There were no significant interactions ($F_s < 1.5$; $p > 0.05$, where $k = 3$). These results show that CS fear was unaffected by modulation of opioid activity in the Acb, irrespective of the strength of the CS-shock association.

2.5. Experiment 5: Infusion of morphine into the Acb impairs context conditioning when the intensity of the CS is high but not when it is low

The experiments presented earlier show that accumbal infusions of morphine prior to a single clicker-shock pairing disrupt conditioned fear to the training context only when the shock was immediately preceded by the clicker. The insertion of a temporal interval between the auditory CS and the shock US reduced the conditioned freezing response to the CS and spared that to the context. In other words, when the conditions for association formation between the auditory CS and shock were not optimal, overshadowing of the context by the CS was reduced and thus the detrimental effect of morphine infusions into the Acb was also reduced. The aim of Experiment 5 was to further examine the extent of overshadowing of the context by the auditory CS by reducing the salience of the latter (Rescorla & Wagner, 1972). This was done by creating three CS intensity conditions: Unsignalled (no CS preceded shock), low intensity CS (a 70dB CS preceded shock) and a high intensity CS (a 81dB CS preceded shock). Experiment 5 further examined the effect of

Acb opioid agonism on the interaction between context and CS conditioning across variations in the intensity of the CS.

The data from 11 rats were excluded from statistical analysis because histological examination revealed that their cannulae were not implanted in the Acb or that there was extensive damage to the Acb. Figure 7 shows the mean percentage of time spent freezing for the remaining rats in Groups Saline-Unsignalled (n = 8), Morphine-Unsignalled (n = 9), Saline-70 (n = 10), Morphine-70 (n = 7), Saline-81 (n = 8) and Morphine-81 (n = 7) across the first 2min of context (left panel) and clicker (right panel) tests.

A within-subject (test: context and CS) ANOVA was conducted. The analyses revealed no effect of drug ($F < 1$), no effect of stimulus intensity ($F_{2, 43} = 2.50$), and effect of test ($F_{1, 43} = 23.66$) and the following significant interactions: test x intensity ($F_{1, 43} = 43.66$) and intensity x drug x test ($F_{1, 43} = 4.50$). Post-hoc pairwise comparisons during Context Test revealed no difference between the saline treated rats (min $p = 0.15$). In contrast, the same analyses uncovered differences in context freezing between the morphine treated rats: Groups Morphine-Unsignalled and Morphine-70 exhibited higher levels of fear to the context compared to Group Morphine-81 ($p_s = 0$). But Groups Morphine-Unsig and Morphine-70 did not differ from one another ($p = 1.0$). In addition, the lower level of context freezing obtained for Group Morphine-81 was different to that obtained for the saline treated groups ($p = 0.002$).

Post-hoc pairwise comparisons during CS Test revealed no difference between Groups Saline-70 and Saline-81 ($p = 0.25$), but each one of those groups differed from Group Saline-Unsignalled ($p_s = 0$). Critically, similar analyses revealed differences between Group Morphine-Unsignalled and Morphine-70 compared to Group Morphine-81, $p = 0$, however, no difference was obtained between the former two groups, $p = 0.85$. In addition, Group Morphine-81 did not differ from the Group Saline-81 ($p = 0.74$) nor did Groups Morphine-Unsignalled and Morphine-70 from Group Saline-Unsignalled ($p = 0.60$). These results are consistent with those observed in the previous experiments when a delay procedure was used. Morphine infusion into the Acb impaired context freezing when the shock onset co-occurred with offset of a loud CS, but not when the shock was unsignalled or when its onset co-occurred with offset of a soft CS. They have also revealed that morphine infusions impaired freezing to the soft CS while confirming that they spared freezing to the loud CS.

3. Discussion

Placed into a novel context and exposed to a single pairing of a clicker and foot shock, rats subsequently exhibited fear responses when tested with the now conditioned clicker (CS) in a second different context. They also froze when re-exposed to the context where the shock had occurred but not when tested in the second context. Thus, rats had learned to fear both the CS which signalled the shock and the context where the shock occurred. Experiment 1 confirmed that rats conditioned under an infusion of morphine into the nucleus accumbens (Acb) showed just as much freezing as control rats when subsequently tested with the CS but substantially less freezing than controls when re-exposed to the previously shocked context. In other words, learning about the context-shock relation was overshadowed by the clicker. In contrast to this difference between the levels of context-controlled freezing when the shock had been preceded by the auditory CS, there were no differences between the levels of context freezing when the context was the only signal for shock i.e. when there was no opportunity for the context to be overshadowed. Rats that were shocked in the context under a morphine infusion into the Acb but in the absence of an explicit signal for shock showed just as much freezing as control rats when re-exposed to that context. These results show that rats infused with morphine into the Acb learn to fear a CS which signals shock as

well as a context where shock occurs but are impaired in learning about the context under conditions when a CS signals shock. The presence of the signal for shock overshadows the context in morphine- but not in saline-infused rats.

The intact contextual conditioning obtained in morphine treated rats trained with unsignalled shock presentations in the present set of studies contrasts that reported previously by Westbrook et al. (1997). This discrepancy is most likely due to the difference in dose and cannula gauge between the two sets of investigations. It is conceivable that the higher dose of morphine (15 $\mu\text{g}/\mu\text{l}$ vs 7.5 $\mu\text{g}/\mu\text{l}$) infused into the Acb using a thicker and more damaging guide cannula (22g vs 26g) in the Westbrook et al. (1997, Experiment 1a) studies could have resulted in disruption of conditioned fear to the context.

The overshadowing of context by the CS in morphine-infused rats in the present investigation was 1. independent of the order in which the context and CS were tested (Experiment 1), 2. dose dependent (Experiment 2) and 3. due to the action of the drug on opioid receptors (Experiment 3). This overshadowing of context by the CS in morphine-infused rats was also specific to a delay procedure, when CS offset co-occurred with US onset (delay conditioning, Experiment 4). When an interval was interpolated (10s or 30s) between CS offset and shock onset (trace conditioning), overshadowing of context by the CS in the morphine-infused rats failed to occur. These trace intervals proportionally reduced the amount of CS conditioning relative to the delay procedure in both morphine- and saline-infused rats. The levels of this CS fear were similar between morphine-treated and control rats as were the levels of context fear. Thus, overshadowing of context by CS in the morphine-treated rats was observed when there was a temporally contiguous relation between the CS and US (delay) but not when the CS and the US were separated by an interval of time (trace).

Experiment 5 used a delay procedure to show that an infusion of morphine into the Acb did not just result in overshadowing of context by a CS; it also resulted in overshadowing of a CS by context. Specifically, it confirmed that rats infused with morphine into the Acb showed equivalent levels of fear to the loud auditory CS (81 db) but less fear to the context than control rats. It also found that morphine infused rats showed equivalent levels of fear to the context but less fear to a soft auditory CS (70 db) than control rats. Thus, control rats learned independently about context and CS: context freezing in control rats was similar in spite of variations in the amounts learned about the loud and soft auditory CSs. In contrast, morphine-treated rats learned about context and CS in a dependent manner: the loud auditory CS reduced the amount learned about context and, conversely, the context reduced the amount learned about the soft auditory CS. Thus, the salience of the CS interacts with a temporally contiguous relation to determine whether the CS overshadows the context or the context overshadows the CS.

As noted previously, contemporary models explain stimulus selection effects by proposing that the discrepancy between the actual US and that predicted by all the cues present on a trial regulate associative formation. One type of model (e.g., Pearce and Hall, 1980, LePelley, 2004) holds that the size of this discrepancy or error controls associative formation indirectly by determining subsequent processing of the CSs. A second type of model (e.g., Rescorla and Wagner, 1972) holds that error acts directly on associative formation by determining processing of the US. Both types of model hold that stimulus selection phenomena such as overshadowing are the products of experientially driven changes in predictive value: the subject uses the learned predictive value of a cue to regulate the amount learned about a concomitantly present cue. Overshadowing, therefore, should not occur after the single compound conditioning trial used in the present experiments since each element of the compound (the context and CS) entered that trial with zero predictive

value and, hence, the amounts learned about each element on that trial were independent of each other. All of the data obtained from the saline treated rats in the present experiments are consistent with this assumption: neither of the manipulations used here- variations in the salience of the CS in the delay procedure or in the temporal interval between the CS offset and US onset in the trace procedure- resulted in overshadowing of the context by the CS or of the CS by context in spite of such manipulations resulting in differences in the amount learned about the CS and context. Rats infused with morphine into the Acb sometimes behaved in a manner consistent with this model, for example, they learned about the context and the CS independently of each other in the trace procedure. However, they did not behave in a manner consistent with this independence assumption in the delay procedure: when the offset of the loud CS was contiguous with the onset of the US, they learned about the CS at the expense of context; when the offset of the soft CS was contiguous with onset of the US, they learned about context at the expense of the CS.

A number of distributive processing mechanisms have been proposed in order to account for such cue competition in the absence of prior learning (Sutherland and Mackintosh, 1971; James and Wagner, 1980). At their core is the proposal of a limitation in capacity, either of attention, short-term or working memory. That limitation results in subjects selecting from the array of concomitantly present cues (context or CS) according to their salience and then associating the more salient cue with the US at the expense of the remaining cues. According to these proposals, therefore, the context and CS did not challenge the attentional, short-term or working memory resources of normal rats but did challenge those of morphine-infused rats. This limitation in processing capacity resulted in the selection of either the CS or context for association with the US at the expense of the other. Moreover, this limitation was only present when **both** the context and CS were in simultaneous competition for access to the US, that is, in the delay procedure, but not when the context was present at the time of US delivery and the CS was not (in the unsignalled or the trace procedures).

In sum, the evidence presented here suggests that the Acb is critical for the ability to simultaneously process the various cues present, the context and discrete cues, leading to each becoming associated with the US proportional to their respective salience. The evidence presented here also suggests that activation of opioid receptors in the Acb directs processing to the more salient of the cues present at the expense of the less salient, leading to the former becoming associated with the US at the expense of the latter. The present results along with those reported by Iordanova et al. (2006a) provide evidence that the same neural mechanism, namely accumbal opioid transmission, regulates stimulus selection based on the physical and predictive properties of stimuli. Specifically, in the case of Iordanova et al., (2006a), rats infused with morphine in the Acb learned about the predictive relationship between a normally blocked (by the previously conditioned context) auditory cue and shock. This is consistent with the idea that opioid agonism serves to maintain attention toward more (physically) salient cues. Furthermore, the results reported by Iordanova et al., (2006a) show that accumbal morphine agonism prevented the influence of predictive value on learning. In other words, subjects initially select some but presumably not all the cues present for associative formation and then use their predictive value for further selection among these cues. The switch in using predictive value for stimulus selection is likely to involve a reduction in opioid transmission in the Acb. Therefore, opioid transmission in the Acb modulate stimulus selection in learning driven by the physical and predictive values of stimuli, with opioid agonism specifically maintaining attention to cues of high physical salience. Critically, both the initial, unlearned selection and the subsequent, learned selection appear to share the same substrate, namely accumbal opioids.

4. Materials and Methods

4.1. Subjects

The subjects were 251 experimentally naive male Wistar rats weighing between 260 and 320g. They were obtained from the colony of Specific-Pathogen-Free rats maintained by the Combined Universities Laboratory Animal Services. Before surgery, rats were housed in plastic boxes (22 cm high × 65 cm long × 40 cm wide). After surgery, rats were housed individually in rack-mounted wire cages (19 cm high × 24 cm long × 15 cm wide) in Experiments 1–4 and plastic boxes (22 cm high × 40 cm long × 28 cm wide) in Experiment 5. Food and water were continuously available in the boxes and cages that were kept in a colony room under natural lighting. The experiments were conducted between 9.00 a.m. and 5.00 p.m. There were 8–10 rats per group and the groups were weight matched such that the average weight of any group did not differ by more than 20 g from any other group. The use of animals was approved by the Animal Care and Ethics Committee of The University of New South Wales.

4.2. Drugs

Morphine hydrochloride (a generous gift from Glaxo-Wellcome) and naloxone hydrochloride (Sigma) were dissolved in sterile non-pyrogenic saline 0.9% (wt/vol; Astra) to obtain a concentration of 7.5 µg/µl, which was the dose used in all experiments unless otherwise stated. A very similar dose (8 µg/µl) of morphine and naloxone was also used previously in a one-trial overshadowing procedure examining accumbal function (Westbrook et al., 2007, Expt 2a and 2b). Non-pyrogenic saline (0.9% wt/vol) was used for control injections. The pH of each solution was adjusted to 5.0. Solutions were microinjected in a volume of 1.0 µl across a period of 2 min and the microinjection cannula was left in place for a further minute to permit diffusion. During sham microinjections, rats were connected to the infusion pump but no fluids were microinjected.

4.3. Surgery and Histology

Rats were handled for approximately 5 min each day across 3 successive days. Approximately 1 hr prior to surgery, rats were injected intraperitoneally (ip) with a prophylactic 0.3 ml dose of a 300 mg/ml solution of procaine penicillin. They were then injected ip with 1.3 ml/kg of the anaesthetic ketamine (Ketapex; Apex Laboratories) at a concentration of 100 mg/ml, and with 0.3 ml/kg of the muscle relaxant xylazine (Rompun; Bayer) at a concentration of 20 mg/ml. Each rat was placed in the stereotaxic apparatus (Model 900, Kopf) while maintaining the incisor bar at approximately 3.3 mm below horizontal to achieve a flat skull position and a 26-gauge guide cannula (Plastics One) was implanted into the right hemisphere of the brain. The tip of the guide cannula was aimed at the Acb by positioning it 7.0 mm below bregma through a hole drilled 1.4 mm anterior to and 1.8 mm lateral to bregma. The guide cannula was fixed in position with dental cement and anchored by Super Glue (Selleys). Rats were allowed 7 days to recover from surgery, during which time they were handled and weighed daily. A dummy cannula was kept in the guide at all times except during microinjections when a 33 gauge microinjection cannula was inserted into the guide cannula and connected to a 25 µl glass syringe operated by an infusion pump (Harvard Apparatus). The microinjection cannula projected a further 1 mm ventral to the tip of the guide cannula.

At the end of the experiment, rats were given an overdose of sodium pentobarbital and their brains were removed. Unfixed brains were sectioned coronally at 40 µm through the Acb. Every fourth section was collected on a slide and the sections stained with cresyl violet. Cannula placements were verified using the boundaries defined by Paxinos and Watson (1997). The sections were examined under a microscope by a trained observer unaware of

the subjects' group designations. The data of any rat were excluded from the statistical analysis if the cannula tip was more than 0.5 mm outside the Acb, or if the region had sustained extensive damage.

4.4. Apparatus

Two chambers (20 cm high $\times$ 23 cm long $\times$ 21 cm wide) were used to shock the rats and to test for contextually controlled freezing. The front and rear walls of these chambers as well as the hinged lid were made of Perspex, and the end walls of stainless steel. The floor in each chamber consisted of stainless steel rods, 2 mm in diameter, spaced 10 mm apart (center to center). The US was a 1.0 s, 1.0 mA unscrambled AC 50 Hz shock from a constant-current generator that was delivered to the floor of each chamber. The current available to each floor could be adjusted using an in-line milliampere meter. Each chamber stood 5 cm above a tray of paper pellet bedding (Fibrecycle) that was changed daily. Upon removal of a rat the floor of each chamber was cleaned with a solution of acetic acid (0.5 %) to eliminate any residue and provide a distinctive odor. These two chambers were located within separate compartments of a wooden cabinet. The door of each compartment was kept open to permit observation of each rat.

A second set of two chambers (35 cm high $\times$ 30 cm long $\times$ 23 cm wide) was used to test performance to the auditory CS. The front of these chambers was constructed of Perspex. The side and rear walls were made of wood and painted with 20 mm wide black and white stripes. The stripes were vertical in one of the chambers and horizontal in the other. The hinged lid of each chamber was made of wood. The floors consisted of stainless steel rods 2 mm in diameter, spaced 13 mm apart (center to center). Each chamber stood 8 cm above a tray of paper pellet bedding. Upon removal of a rat the floor of each chamber was wiped with a solution of vanilla essence (1.0 %) to eliminate any residue and provide a distinctive odor. These chambers were located on the roof of the wooden cabinet that contained the shock chambers. The discrete auditory CS consisted of an 81 dB 10 Hz clicker (rise time $<$ 10 μ s, decay time 250 μ s) delivered from a speaker situated in the ceiling of the experimental room.

Both types of contexts were situated in the same room and were not counterbalanced. The background noise in the room was 69 dB. The stimulus and background intensities were measured with a sound level meter (Type 2235, Bruel-Kjaer Instruments) whose microphone was placed in the center of each test chamber. The room that contained the experimental chambers was illuminated by eight 60 W standard incandescent lights located in the ceiling. All training and test sessions were recorded on videotape via cameras mounted on a wall opposite the chambers. The camera was connected to a monitor and video recorder located in an adjacent room.

4.5. Scoring

Freezing was defined as the absence of all movements, except for those related to breathing (Fanselow, 1980). The behavior of all rats in each experiment was recorded on videotape, and freezing was rated with a time-sampling procedure in which each rat was observed every 2 s and scored as either freezing or moving. A percentage score was calculated for the proportion of the total observation period that each rat spent freezing. Freezing was rated by two observers, one of whom was unaware of the subject's group designation. There was a high degree of agreement between the two observers: the Pearson product-moment correlation between their ratings was above 0.91.

4.6. Statistical Analysis

The data were analysed by analysis of variance (ANOVA) testing sets of contrasts. The familywise error rate was controlled for each family of contrasts tested using the Bonferroni inequality procedure. Post-hoc comparisons were analyzed with the technique described by Scheffe (Harris, 2001). Significance was set at 0.05.

4.7. Behavioral procedures

4.7.1. Experiment 1: Effects of an infusion of morphine into the Acb on the acquisition of fear (freezing) to a context and CS (clicker)—Rats received an infusion of morphine or saline into the Acb twenty minutes prior to placement into a context. Conditioning consisted of a four minute context exposure, where shock was delivered 120 s following placement in the context. For half of the rats infused with morphine and half the rats infused with saline the shock was preceded by a 10 s clicker (Groups Morphine Signalled and Saline Signalled), whereas for the remainder the shock was presented in the absence of any auditory signal (Groups Morphine Unsignalled and Saline Unsignalled). Approximately four hours later in the colony room, rats that had been infused with morphine now received an infusion of saline into the Acb, and those infused with saline now received infusion of morphine. This was done in order to control for any effects of morphine infusion per se on subsequent test performance. On Days 2 and 3, rats were sham infused and exposed to the context to test and extinguish contextually controlled freezing. On Day 4, rats were sham infused and tested for freezing to the CS in the second context. Presentation of the CS occurred 120s following placement into the novel context and lasted for 8min. The duration of the conditioning and test session were identical across all experiments.

4.7.2. Experiment 2: Dose-response effects of morphine on acquisition of context and CS fear—Rats received an accumbal infusion of either 1.0 μ l of saline (Group Saline) or morphine at a concentration of 3.75, 7.5 or 15 μ g/ μ l (Groups Morphine - 3.75, Morphine - 7.5, Morphine - 15, respectively). Twenty minutes after the infusion, rats were placed in the context where shock was delivered 120 s later. For all rats, the shock was preceded by a 10 s clicker. At least 4 hr later in the colony room, rats received infusions into the Acb to control for any effect on subsequent test responding of the morphine: rats in Groups Morphine - 3.75 and Morphine -7.5 were infused with 1 μ l of morphine at a concentration of 11.25 and 7.5 μ g/ μ l, respectively, those in Group Morphine - 15 were infused with saline, and rats in Group Saline received an infusion of 1 μ l of morphine at a concentration of 15 μ g/ μ l. After sham infusions, rats were tested in the previously shocked context (Day 2) and then in the second context for freezing to the clicker (Day 3).

4.7.3. Experiment 3: Receptor-specific effects of morphine on acquisition of context and CS fear—On Day 1, rats were infused with 1.0 μ l of either saline (Group Saline), or 7.5 μ g/ μ l of morphine (Group Morphine), or 7.5 μ g/ μ l of naloxone (Group Naloxone), or a mixture of 7.5 μ g/ μ l morphine and 7.5 μ g/ μ l of naloxone (Group Morphine-Naloxone). Twenty minutes after the infusion, rats were then placed into a context where shock occurred 120 s later. Shock was preceded by a 10 s clicker. At least 4 hr later in the colony room, rats received accumbal infusions of either a mixture of morphine and naloxone (Group Saline), naloxone (Group Morphine), morphine (Group Naloxone), or saline (Group Morphine-Naloxone). This was done to equate exposure to the drugs in the accumbans (morphine and naloxone) in all groups, thereby ensuring that any subsequent effects on freezing were not due to the drug per se. After sham infusions, rats were tested in the previously shocked context (Day 2) and then in the second context with the clicker (Day 3).

4.7.4. Experiment 4: Effects of morphine on acquisition of context and CS fear in a delay and trace procedure—On Day 1, 20 min after an accumbal infusion of morphine or saline, rats were placed into the context and shocked 120 s later. Shock was presented either in the absence of the clicker (Groups Unsignalled) or immediately (Groups Delay), 10 s (Groups Trace 10) or 30 s (Groups Trace 30) after the offset of the 10 s clicker. Four hours later in the colony room, morphine treated rats received an accumbal infusion of saline, and saline treated rats an accumbal infusion of morphine. After sham infusions, rats were tested for freezing in the previously shocked context (Day 2) and then in the second context with the auditory stimulus (Day 3).

4.7.5. Experiment 5: Effects of morphine on acquisition of context and CS fear across variations in the intensity of the CS—On Day 1, rats were microinjected with morphine or saline into the Acb. Twenty minutes later, rats were placed in the context and shock was presented after 120 s. For Groups Saline-Unsignalled and Morphine-Unsignalled shock delivery was not preceded by a clicker. For the signalled groups, a 70dB (Groups Saline-70 and Morphine-70) or 81db (Groups Saline-81 and Morphine-81) clicker preceded shock delivery. On Day 2, rats were tested in the previously shocked context, and, on Day 3, in the second context for freezing to the CS with which they were trained. Half of the rats in the No clicker groups were tested with the 81 db clicker and the remainder with the 70 db clicker.

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- Examined the effect of accumbal morphine infusions on overshadowing in one trial fear conditioning
- We examined competition for association between a context and auditory CS with shock
- We manipulated salience using trace vs delay conditioning, auditory cue intensity
- Accumbal morphine infusions disrupted learning about the stimulus of lower salience

Figure 1.

Figure 1A presents the specific placements of the injection cannula tips within the nucleus accumbens for Experiment 1. Figure 1B shows a representative photographic example of a cannula placement from Experiment 1. Figure 1C presents the area in the nucleus accumbens within which microinjection cannula tips were located for all experiments. Atlas templates were adapted from Paxinos and Watson (1998).

Figure 2.

Mean percentage freezing (+SEMs) to context test (left panel) and CS test (right panel) for rats in Groups Saline and Morphine in Experiment 1. An infusion of morphine prior to conditioning impaired freezing to the context but not the CS only when the shock was preceded (signalled) by a discrete CS: Contextual freezing in Group Saline-Signalled differed to that reported for Group Morphine-Signalled.

Figure 3. Mean percentage freezing (+SEMs) to entire 8min CS test for rats in Group Saline-Signalled and Morphine-Signalled in Experiment 1. No difference in the rate of extinction was seen between rats infused with saline and those infused with morphine prior to conditioning.

Figure 4.

Mean percentage freezing (+SEMs) to context test (left panel) and CS test (right panel) for rats in Experiment 2 as a function of dose of morphine. An infusion of morphine prior to conditioning impaired freezing to the context but not the CS in a dose-dependent manner: Group Morphine-3.75 exhibited higher levels of freezing to the context compared to Groups Morphine-7.5 and Morphine-15, whereas the latter two groups did not differ.

Figure 5.

Mean percentage freezing (+SEMs) to context test (left panel) and CS test (right panel) for rats in Groups Sal, Mor, Mor+Nlx, and Naloxone in Experiment 3. The impairment in freezing to the context following accumbal infusions of morphine prior to conditioning was reversed by a concurrent infusion of the opioid receptor antagonist naloxone: Group Mor exhibited lower levels of contextual fear compared to the remaining three groups. Groups Nlx and Mor-Nlx exhibited equivalent levels of contextual fear to Group Sal.

Figure 6.

Mean percentage freezing (+SEMs) to context test (left panel) and CS test (right panel) for rats trained under accumbal infusions of saline or morphine in Groups Delay and Trace in Experiment 4. An accumbal infusion of morphine prior to conditioning impaired freezing to the context only when shock presentation was immediately preceded by a discrete stimulus, and not when an interval of 10s or 30s was interpolated between CS and shock delivery: Group Morphine-Delay exhibited higher levels of contextual fear compared to Group Saline-Delay, however, no differences were obtained between the morphine and saline infused groups in the trace conditions.

Figure 7.

Mean percentage freezing (+SEMs) to context test (left panel) and CS test (right panel) for rats trained under accumbal infusions of saline or morphine in Groups Unsignalled, Signalled –70dB, and –81dB in Experiment 5. An accumbal infusion of morphine prior to conditioning impaired freezing to the context only when shock presentation was preceded by a high intensity CS (81dB) but not when preceded by a low (70dB) or no (Unsignalled) intensity CS: Group Morphine-81 exhibited lower levels of context fear compared to Groups Morphine-Unsignalled and Morphine-70, as well as compared to the saline groups. An accumbal infusion of morphine prior to conditioning impaired freezing to a low intensity CS whilst sparing freezing to the context: Group Morphine-70 did not differ from Group Morphine-Unsignalled in fear to the CS, but these groups exhibited lower levels of CS fear compared to Group Morphine-81, whereas Groups Saline-70 and Saline-81 exhibited higher levels of CS fear compared to Group Saline-Unsignalled.