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Effect of opioid compounds on feeding and activity of the cockroach, *Periplaneta americana*

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ABSTRACT

Opioid peptides have been implicated in regulation of feeding in invertebrates. Studies have suggested that receptors for opioids are present in cockroaches and that these receptors play roles in affecting both behaviour and feeding. We examined the effect of μ, δ, and κ opioid receptor agonists and antagonists on feeding, mass changes and activity in the cockroach, *Periplaneta americana*. The κ antagonist, nor-binaltorphimine, significantly increased food intake, while naltrexone (general antagonist) and naloxonazine (μ antagonist) both reduced feeding. A large mass loss was observed in cockroaches treated with nor-binaltorphimine, despite the increased food intake. Males did not lose as much mass during the 3 h as females, although drug treatment did have some effect on the loss. Time of activity (%) was not influenced by any drug. Water loss experiments suggested that nor-binaltorphimine increased water loss, accounting for the mass loss despite the increased feeding. We suggest that two populations of opioid receptors are present as previously reported, with one affecting feeding and the other involved with evaporative water loss.

1. Introduction

Opioid peptides are known to be present in several taxa of invertebrates (for reviews, see Harrison et al., 1994; Nagabhushanam et al., 1995). The functions vary, but many studies have demonstrated that both feeding and behaviour are influenced by the presence of these peptides. The mechanisms that control these behaviours are not known, but the evidence suggests that a variety of opioid-like receptors are present. The presence of opioid-like receptors in insects was first described in the brain and midgut of the cockroach, *Periplaneta americana* (Stefano and Scharrer, 1981; Stefano et al., 1982). More recently, Birgul and co-workers (Birgul et al., 1999; Kreienkamp et al., 2002) used reverse physiology, and Santoro et al. (2000) used receptor binding studies, to characterise opioid-like receptors in *Drosophila melanogaster*, indicating that such receptors are present in insects.

In vertebrates, opioid-like peptides are either enkephalins or endorphins. Enkephalins have an amino acid sequence of YGGFM or YGGFL. Although neither of these compounds have been isolated from insects, a number of peptides with a C-terminus of FXRFamide have been isolated (FMRFamide-related peptides or FaRPs) with the X being either methionine or leucine. Interestingly, FaRPs have been shown to have a low affinity for the μ, δ, and κ receptors of vertebrates, and have been shown to inhibit the normal nociceptin responses, although not as a direct result of binding to the opioid receptor (Raffa et al., 1994).

Opioids have been shown to increase the quantity of food ingested in molluscs and insects, yet the mechanisms that cause the increased ingestion have not been investigated in many species. Kavaliers et al. (1987) demonstrated that feeding increased in female *P. americana* following injection of the κ opioid agonist U50,488H, and that the μ opioid antagonist naloxone inhibited the stimulated feeding. Although opioid-like chemicals have been immunolocalised in insects (Duve and Thorpe, 1983; Takeda et al., 1986; Schols et al., 1987; Duve and Thorpe, 1990), the causes and mechanisms of changes in feeding have not been further explored.

In this study, we have extended the work of Kavaliers et al. (1987) examining the role of various opioid agonists and antagonists known to bind to the vertebrate μ, κ and δ opioid receptors. We have examined how these compounds affect cockroach feeding, mass change and activity in relation to gender, light level and water content of food. As an increase in activity could cause a change in either food intake or mass loss, we were only interested in variation in gross motor activity with respect to administered drugs in this study.

2. Methods and materials

2.1. Subjects and chemicals

The methods for studying the responses to the various opioid agonists and antagonists were typically organised to study the

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responses according to a single agonist: antagonist and mixture of agonist and antagonist compared to a saline-injected control in a block design. Cockroaches (*P. americana*) that were reared on lab chow were removed from their colony in the morning and held for 2 h without food prior to experiments. Animals were weighed, then split into groups by allocating the four heaviest to be randomly placed into one of the four blocks. This assignment by mass was repeated until all 16–32 animals per experiment were assigned to one of the four treatments. Although the animals were distributed into groups by mass, the lightest animals were at least 90% of the mass of the heaviest animals so as to limit any possible effects of mass on the measured variables. We used 434 cockroaches in the feeding studies, 46 males and 388 females, with 326 of these same cockroaches (46 males and 280 females) used in the activity study. Exact numbers for each of the treatments are shown on the figures. The mean mass ($x \pm$ s.d) of males was 1.1 ± 0.13 g and the mean mass of females was 1.3 ± 0.21 g.

Chemicals for injection were made up in cockroach saline (115 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 4 mM $CaCl_2$, 10 mM glucose and 90 mM sucrose) and injected at a dose of 1 μg/g animal (based upon the dosages injected in the paper by Kavaliers et al., 1987) between the 6th and 7th abdominal sclerites. The concentration of the solution was varied so that the volume of fluid injected was around 5 μL. When two drugs were injected simultaneously, the dose of both was still 1 μg/g in 5 μL, but that meant the total dose was 2 μg/g. For injection, animals were cooled at 4 °C to minimise movement, injected in less than 1 min with 5 μL of the appropriate dose, and allowed to recover at room temperature. Control animals were handled in exactly the same way except that 5 μL of saline only was injected. Animals were held individually in small plastic boxes with clear lids for observation with preweighed food present. After the experimental period, cockroaches were removed and both the food and insect re-weighed to determine mass changes over time.

The opioid agonists and antagonists (Table 1) were chosen for their specificity for the μ, δ or κ opioid receptors. In addition to the specific receptor antagonists, the general opioid receptor antagonist, naltrexone, was also used to determine the response when all receptors may be blocked. The drugs were made up in insect saline and used immediately. All drugs were obtained from Tocris Bioscience and were the hydrochloride derivative, except the peptide, endomorphin-1.

2.2. Feeding, mass change and activity studies

Feeding periods were for 3 h (similar to times reported in Kavaliers et al., 1987), with periodic examination of activity over 10 min periods within each hour; a total of 30 min per animal. Animals were only classified as active or inactive and the data presented as % time active. Some animals were also videotaped to determine whether these animals were more active when observers were not present. Although these animals were more active, the increase of % activity time was not significant (data not shown). Food was offered to animals as either dry (ground rat lab chow) or wet (mixture 45.5 g lab chow, 5.56 g agar, 207.5 mL water) to ensure that water was available with the food. Composition of dry rat chow was (dry weight) 23% crude protein, 4.7% crude fat, 3.3% crude fiber and 6.5% ash. Light available to the animals was also two levels; either room light (240 lux) or dim light (<1 lux) as a result of turning the lights off and closing the blinds.

Table 1
Opioid compounds used in the feeding experiments and their specificity for receptor type.

Receptor type	Agonist	Antagonist
μ	Endomorphin-1	Naloxonazine hydrochloride
δ	SNC 80	Naltrindole hydrochloride
κ	U50,488H	Nor-binaltorphimine dihydrochloride
μ, κ, δ		Naltrexone hydrochloride

2.3. Water loss measurement

Because body mass loss was highest in cockroaches injected with nor-binaltorphimine, we compared animals injected with those drugs with saline-injected control animals and non-injected cockroaches to determine whether any change in water loss occurred. Male cockroaches ($n = 4$ for each group) were weighed and injected with the drug, control saline or uninjected as described earlier. Male cockroaches were used to avoid potential changes associated with egg development, and for comparison with previous studies on cockroaches, although the mass loss for males was less than for females with this drug (Results).

Animals were individually transferred into a custom-built chamber that consisted of clear, cylindrical Perspex tubing and end caps with gas fittings internally secured with rubber rings. This chamber was incorporated into a flow-through respirometry system with Licor 7100 CO_2/H_2O differential infrared gas analysis equipment (Li-Cor, Lincoln, USA). The chamber was housed in a darkened incubator kept at 20 °C (Binder KB-series incubator; Binder, Germany) and gas was drawn through the system by an Edwards E2M-1.5 high-vacuum pump (BOC-Edwards, BOC group Inc.). The experiments were run for 2 h with 21% oxygen in nitrogen passed through an inline moisture and CO_2 removal column (Glass moisture trap, Model 7214, Alltech, Australia) before passing through the chamber at a rate of 100 mL min^{-1}. The gas mixture was controlled by a Brooks 5878 mass-flow controller with Brooks 5850TR mass-flow meters calibrated at 0–500 mL/min^{-1} (Brooks Instruments, Hatfield, USA).

Specimens were periodically observed during each trial. Raw data from the gas analyzer was logged on a personal computer using Licor 7100 data acquisition software (Version 1.0.1). The first 20 min of each experiment were excluded from analysis while the chamber was flushed and the animal settled. Data for the following 60 min were imported into Microsoft Excel for examination and analysis.

2.4. Statistical analysis

Data from the feeding studies were normalized (log transform) and analysed using generalised linear mixed models (GLMM, activity) or restricted maximum likelihood models (REML, food intake or body mass changes) (Genstat 5, release 4.2). Component explanatory variables of the models were both added and subtracted from the full model to ensure that significance was present independent of model structure. The random variance terms within the models included date and date·block to account for any possible variation associated with performing the experiments at different times. In the change of body mass model, food intake was included as a covariate to remove possible effects of animals that ate more than other animals from influencing the model. Significance of the various factors was determined by using the Wald statistic (similar distribution to a χ^2 distribution) with a level of significance assumed if $p < 0.05$. Water loss rates (mg h^{-1}) were compared using mass as a covariate and the injection treatments as levels in an analysis of covariance (ANCOVA).

3. Results

3.1. Food intake

Mass of food consumed was significantly affected only by drug treatment (Wald statistic = 50.65, $df = 13$, $p < 0.001$). The drugs differed in their effect on food consumption, with some compounds stimulating, and others inhibiting, food intake (Fig. 1a). Naltrexone, the general opioid receptor blocker, and naloxonazine, the μ receptor

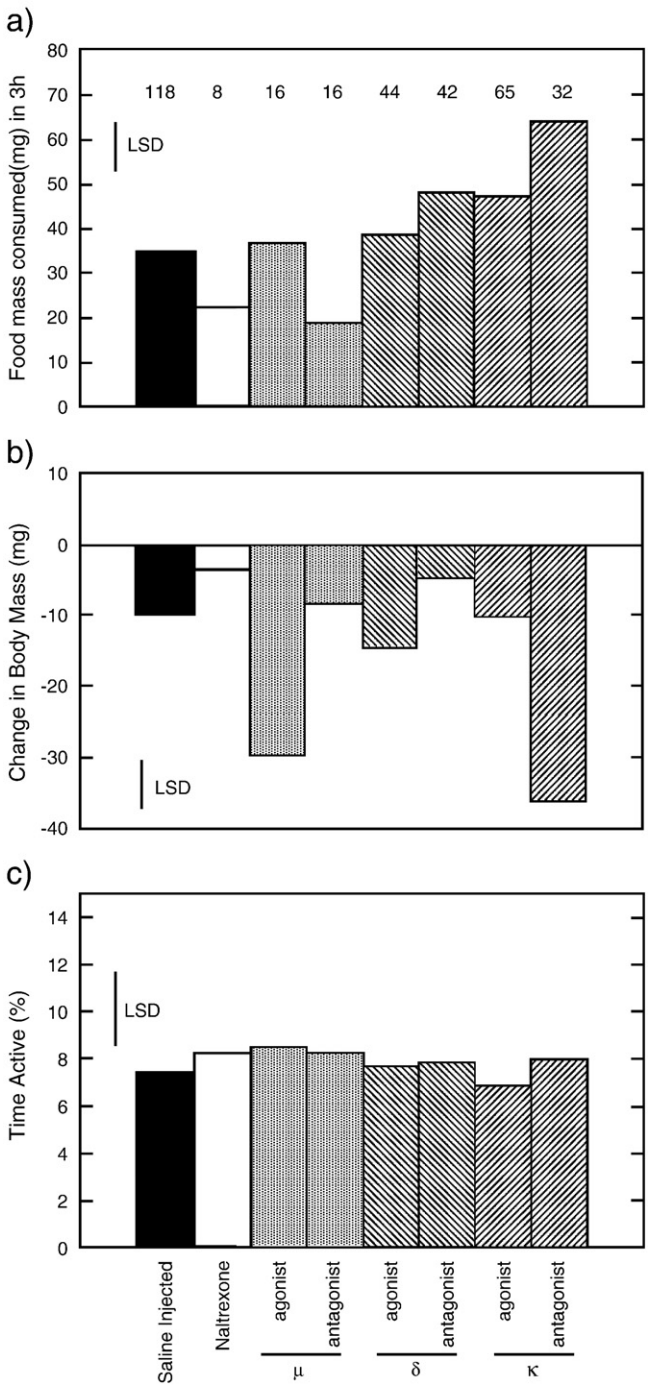

Fig. 1. a) Amount of food ingested by *P. americana* in response to injection of various opioid agonists and antagonists for specific opioid receptors. b) Change in body mass of *P. americana* during feeding periods after injection with various opioid agonist or antagonists. c) Variation in activity during feeding studies of *P. americana* during feeding studies following injection with various opioid agonist and antagonists. LSD is the least significant difference from statistical tests. Number above bar graphs in first panel shows *n* for each drug treatment.

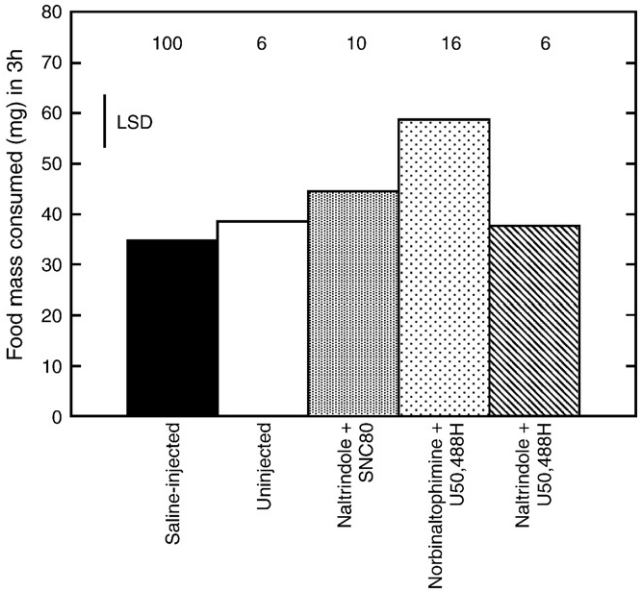

Fig. 2. Effect of combined opioid treatments on food intake of *P. americana*. LSD is least significant difference from statistical tests. Numbers above each column indicate *n* for each treatment.

antagonist, both reduced feeding below control levels. Nor-binaltorphimine, the κ receptor antagonist, and the combined U50,488H + nor-binaltorphimine (Fig. 2) both stimulated feeding, although U50,488H by itself was not stimulatory within the model. Comparison with the control food intake (saline-injected animals) suggests that both naltrindole and U50,488H are marginally stimulatory, but no stimulation was observed when combined (Fig. 2). Diet type (wet or dry food), gender and light conditions had no significant effect on food consumption.

3.2. Body mass changes

In the full model, drug treatment (Wald statistic = 30.50, $df = 13$, $p = 0.004$) and gender (Wald statistic = 8.07, $df = 1$, $p = 0.004$) had significant effects on body mass change, while light level and food type were not significant. Because food intake varied with the drug treatment, food intake could affect the model, so food intake was included as a covariate in the body mass change model. Within this model, not only were drug treatment (Wald statistic = 29.12, $df = 13$, $p = 0.006$) and gender (Wald statistic = 4.81, $df = 1$, $p = 0.028$) still significant, the interaction term (change in food mass × drug treatment) was also significant (Wald statistic = 36.74, $df = 13$, $p < 0.001$) (Fig. 1b). Allowing for the differences in the effects of food intake on body mass changes, some differences can be observed on the effect of various drugs on the mass change of the insects. First, females lost more mass than males, as the predicted mean for females was −25.83 mg compared with males of −5.38 mg (Fig. 3). Second, drug treatments varied in their effect on mass changes, but only nor-binaltorphimine had a clear effect on mass loss when compared to the saline-injected controls (Fig. 1b).

3.3. Activity

Cockroaches were mostly inactive (>90% time) during the experiments. Activity was only significantly affected by the food type (wet or dry) (Wald statistic = 17.76, $df = 1$, $p < 0.001$) and by light level (Wald statistic = 17.74, $df = 1$, $p < 0.001$), with animals more active when given dry food or in low light. Drug treatment only marginally affected activity (Wald statistic = 18.95, $df = 11$, $p = 0.062$) (Fig. 1c). No correlation was present between food intake or body mass changes and activity.

3.4. Water loss rates

All cockroaches lost water during the period of measurement, with the highest rates of loss observed in the drug-treated animals (15.7 ±

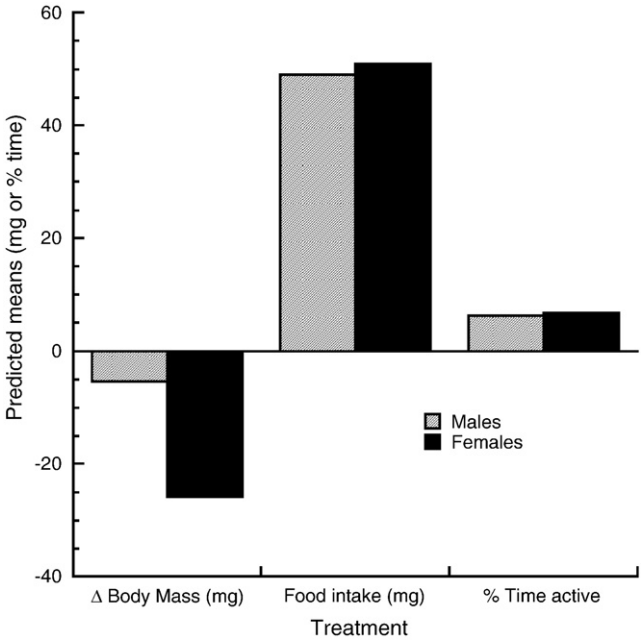

Fig. 3. Differences in predicted means for males ($n=46$) and females ($n=388$) for body mass changes (mg), food intake (mg) and % time active.

2.8 mg h^{-1}) compared with saline-injected (9 ± 2.0 mg h^{-1}) or non-injected animals (5.9 ± 1.6 mg h^{-1}) ($F_{[2,11]}=4.61$, $p=0.05$). Mass had only a marginal effect on water loss ($F_{[1,11]}=4.22$, $p=0.07$). The water loss in the binaltorphimine-injected animals was within the same range as mass loss during the feeding experiments. Surprisingly, however, the mass loss during the experimental period of water loss measurement was not significantly different among the various treatments ($F_{[2,11]}=1.3$, $p=0.3$), although the order of mass loss was the same as water loss.

4. Discussion

Opioids have been implicated in regulation of feeding of many invertebrates, as well as vertebrates. The types of receptors that may be present in insects have not been fully elucidated, although research conducted on *Drosophila* has provided insight. Reverse physiology suggested the existence of least one opioid-like receptor (Birgul et al., 1999) and pharmacological studies suggested that the receptor was similar to a κ opioid receptor (Ford et al., 1986; Kavaliers et al., 1987). Characterization through receptor binding studies further revealed an additional mu receptor in the *Drosophila* head membrane, although the mu specific peptide was inactive (Santoro et al., 2000).

Our results suggest that at least two populations of opioid-like receptors, kappa and mu, may influence feeding in cockroaches. Norbinaltorphimine, a kappa receptor antagonist, increased feeding and mass changes in the cockroaches, but the kappa agonist U50,488H had only a slight stimulatory effect on feeding and no effect on mass changes. The mu-receptor antagonist naloxonazine inhibited feeding, but increased mass loss. In contrast, the general opioid receptor antagonist naltrexone inhibited feeding and also reduced mass loss. This is the first such example of an active role for mu opioids in the control of feeding in insects. Previous work had identified the existence of mu receptors in *Drosophila*, but failed to demonstrate active binding of the peptide (Santoro et al., 2000).

Although the opioid peptides have not been found, peptides that are similar to enkephalins have been reported, the so-called FMRFamide-related peptides. The peptides have been found in all insects examined, although these peptides have diverse C terminals, including FMRFamide, FLRFamide (myosurpressins) and sulfated RFamides (sulfakinins). Receptors for the FMRFamide and FLRFamide peptides have been characterised, however, their relationship to vertebrate receptors is unclear and the receptor sequences have closer affinity to proctolin receptors as well as nematode receptors than to vertebrate receptors (Claeys et al., 2005). Raffa et al. (1994) showed that FMRFamide and similar peptides had low affinity to vertebrate opioid receptors, suggesting that some cross-reaction may occur, but whether the opioids are binding to the insect RFamide receptors is currently unknown.

Kappa opioids have previously been shown to have a role in regulating feeding behaviour in cockroaches. Our finding that the kappa agonist has only a weak stimulatory effect on feeding is contradictory to the earlier work of Kavaliers et al. (1987), who found a very strong stimulatory effect of U50,488H. However, our experimental protocol allowed animals to feed undisturbed for the entire three-hour period, while Kavaliers et al. disturbed the animals each hour to weigh both cockroaches and remaining food. As our experiments minimised disturbance, this technique may account for the variation in food consumption, as well as the reduced activity, observed. The use of an insect activity system, such as Ford et al. (1986), may be more sensitive to movement than our system, so activity may be increased, however, the doses of drugs, volumes of fluids (100 μL) and injection site (brain) were quite different in that study. The main conclusion here is that activity did not appear to influence observed food intake or mass changes during the time of study. How cockroaches varied food intake across the treatments, whether by varying feeding bouts or meal sizes, requires further work.

The reason that we observed a difference between males and females in body mass changes despite no difference in food intake suggests that females may be more susceptible to alternative sources of mass/water loss compared with males. As no difference was found in faecal mass loss, the only two alternatives are either an elevated respiration with a rapid loss of carbohydrate or fat storage in the form of carbon dioxide, or some component of water loss was increased.

The near doubling of the water loss rate between the non-injected and saline-injected control groups can most likely be explained by injection disturbance and associated handling disturbance that may elevate metabolic rate (Machin et al., 1986). However, a further 75% increase between the saline-injected controls and the drug-injected animals is highly suggestive of a drug-induced effect. Importantly, in all experiments faecal output remained unchanged and there were no unexpected increases in CO_2 output that were indicative of bouts of movement activity (data not shown). An increase in CO_2 output in particular has the potential to cause repeated spikes in water loss as a consequence of disrupting resting patterns of gas exchange, which for *P. americana* include periods of tight spiracular constriction and active neuronal control of periodic tracheal ventilation (Machin et al., 1991; Woodman et al., 2008). Mass loss in response to opioid peptide injection may therefore be at least partly explained by disruption of water loss regulation; however further work is necessary to determine how this occurs and whether it is a predominantly transcuticular or respiratory change.

The increased water loss in male cockroaches indicates that opioid drugs have some role in influencing one or more avenues of water loss. A difference in gender in such a response may be associated with the different tracheal arrangements between males and females because of the reproductive structures, such as the elaboration of trachea associated with ovarian development. Alternatively, opioids may differentially influence cockroach behaviour leading to differences in respiratory output, a potential source of variation that requires further study. In any case, the mode of action of these opioid peptides appears multifaceted, acting on at least the digestive and water loss systems simultaneously.

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