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Morphine Hyperalgesic Effects on the Formalin Test in Domestic Fowl (*Gallus gallus*)

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HUGHES, R A AND K J SUFKA *Morphine hyperalgesic effects on the formalin test in domestic fowl* (Gallus gallus) PHARMACOL BIOCHEM BEHAV **38**(2) 247–251, 1991 —Preliminary research demonstrated that formalin injected into the foot of leghorn cockerels elicited significantly more footlifts of longer duration than physiological saline The formalin test was subsequently used to examine morphine effects in this species Previous research demonstrated strain-dependent naloxone-reversible morphine hyperalgesia against thermal nociception in cockerels In Experiment 1 herein White Leghorn cockerels were given either 0 0, 0 5, 1 5, or 2 5% formalin SC into the foot 30 min after an IM injection of either physiological saline or 2 5 mg/kg morphine sulfate The frequency and duration of formalin-elicited footlifts increased significantly as a function of formalin concentration Morphine significantly increased footlift frequency and duration at all but the 0 0% formalin concentration Morphine inhibited respiration in these animals In Experiment 2, naloxone (5 0 mg/kg) significantly reversed both the hyperalgesia and the respiratory depression induced by morphine These results demonstrate that morphine hyperalgesia in leghorn cockerels is neither unique to hot plate test procedures nor peculiar to thermal nociception Atypical morphine effects in this model may be specific to nociception, however, because hyperalgesia was not accompanied by atypical morphine effects on respiration

Hyperalgesia Morphine Naloxone Formalin test Opiate Opioid Chicken Pain Nociception Narcotic drugs Narcotic antagonists

A variety of tests are available with which to assess the antinociceptive action of drugs. Such tests involve thermal (hot plate, tail-flick), mechanical (paw pressure, tail pinch), electrical (jump-flinch) and chemical (formalin, acetic acid) stimuli Although these tests are differentially sensitive to antinociceptive drug effects morphine, the prototypical opiate analgesic, has antinociceptive effects on each test (12,14).

Recent research from our laboratory revealed an unusual morphine effect in White Leghorn (WL) and California White (CW) cockerels against a test of thermal nociception Morphine produced a hyperalgesic effect on the hot plate test rather than the more typical hypoalgesic effect that occurs on this test with other species Animals that received morphine appeared sedated (i e., sleep-like posture, eyes closed) before the nociceptive test but jumped from a heated grid more rapidly than control animals that received physiological saline. This unusual morphine effect is strain-dependent, naloxone-reversible, and displays the dose and temporal characteristics of morphine hypoalgesia (7, 8, 15)

The replicability of morphine hyperalgesia on the hot plate test demonstrates the reliability of the phenomenon, its generality across different tests of nociception, however, is unknown Morphine hyperalgesic effects in WL and CW cockerels may be a unique outcome of hot plate test procedures or is perhaps limited to thermal nociception rather than a phenomenon that more generally reflects atypical morphine effects in WL and CW cockerels One purpose of the present research was to determine the generality of morphine hyperalgesic effects on nociception through the use of the formalin test. This test involves chemical nociception and is procedurally different from tests of thermal nociception (1–3).

Respiratory depression is one of the more common and consistent effects of morphine (9, 12, 14, 17). Thus a second purpose of the present study was to examine morphine effects on respiration in order to determine if morphine would produce atypical effects on this measure. The effects of the opioid antagonist naloxone were also evaluated to determine if morphine effects on the formalin test and on respiration involve opioid receptor activity.

EXPERIMENT 1

We were unable to find any reference to formalin test procedures with domestic fowl Thus preliminary research was conducted to determine if this test could be adapted for use with this

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species. The results of that research demonstrated that a small (0.05 ml) amount of 5% formalin injected into the plantar region of the chick's foot elicited discrete and vigorous footlifts and that the number of footlifts elicited differed significantly from that elicited by intraplantar injection of 0.9% saline Procedures defined by this preliminary research provided the basis (details described below) of the present experiment which was designed to examine morphine effects against several concentrations of formalin in WL cockerels.

METHOD

Subjects

WL cockerels (*G gallus*, Welp-line 937A White Leghorn commercial stock) were obtained 1 day after hatching (Welp, Inc , Bancroft, IA). The animals were housed in pairs in custom housing (described below) that provided physical separation but not visual or auditory isolation from other chick pairs Animals were maintained under 24-h overhead fluorescent room lighting Room temperature was about 32.0°C for the first week and 29 0°C thereafter. Food (Wayne pullet starter) and water were freely available in the home cage

Apparatus

Four separate 125 × 56 × 30 cm housing enclosures were constructed of 2 × 2 hardware cloth. Each enclosure was divided into 25 × 28 × 30 cm compartments with attached hardware cloth lids (10 compartments per enclosure). The outer perimeter walls of the enclosure were covered by white fabric

The test apparatus consisted of two modified operant chambers housed in fan-ventilated sound-attenuating boxes (LVE). Each operant chamber was modified in the same way. The manipulanda, food delivery trough, and stimulus lights were removed and a 2 × 2 hardware cloth floor was placed over the existing grid floor The interior surface of the end walls and door of the operant chamber were covered with white construction paper except for a 5 × 30 cm opening at the base of the door. The rear wall was covered by onion skin typing paper which permitted illumination of the chamber interior by a 25-W bulb that was positioned behind the rear wall at floor level. Five holes (2 cm dia) in the ceiling vented the chamber's interior. The face of the one-way mirror in each sound-attenuating box was covered with two layers of white cheesecloth. This reduced the likelihood that a chick's behavior would be affected by its reflection but still permitted the experimenter to view the chamber's interior from outside the sound-attenuating box. Rapid door closure of the sound-attenuating box was achieved by replacing the mechanical latches with magnetic latches.

Procedure

The design of this experiment was a 2 × 4 factorial which combined two levels of drug (0 9% saline or 2.5 mg/kg morphine, volumes = 1 ml/kg) with four levels of formalin (0 0, 0.5, 1.5, or 2.5%). At 13 days posthatch, chicks were randomly assigned to one of 8 treatment conditions (n = 10 animals per condition) A chick pair was removed from the home cage, each chick was weighed to the nearest 0.1 gram, given an IM injection of morphine or 0.9% saline, color coded with a felt tip pen, and returned to the home cage. Thirty min after these injections, chick pairs were placed into separate opaque plastic containers and transported to an adjacent room where nociceptive tests were conducted. For the test, one of the four formalin concentrations was injected SC (0.05 ml) into the center pad of the plantar region of the chick's foot. Animals were immediately placed into separate test chambers for a three-min observation period.

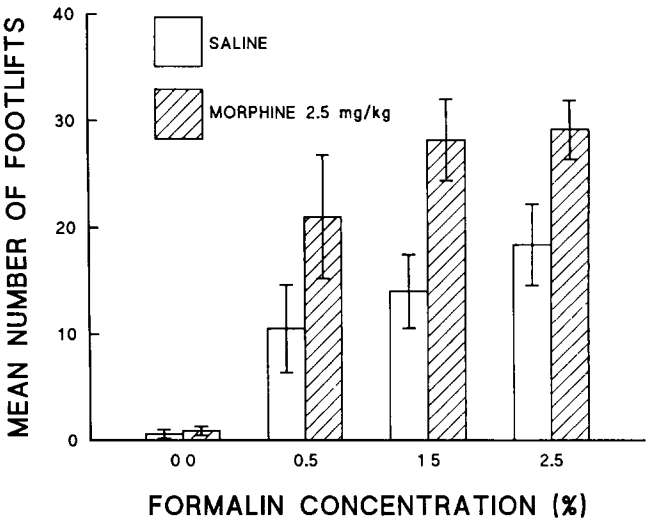

FIG 1 Mean number of footlifts as a function of intraplantar injection of different formalin concentrations 30 min after IM injection of saline or morphine Vertical bars represent SEM

Dependent measures during the observation period were footlift frequency and duration. A footlift was operationally defined as having occurred when the chick lifted its foot from the floor and replaced it. Lifts that were a component of ambulation were not counted Footlift duration was defined by the interval starting when the chick lifted its foot from the floor and ending when floor contact was reestablished. Respiration was measured immediately after the formalin test session The animal was manually restrained in an upright position with its head covered by the experimenter's palm; rhythmic breast movements were counted for one min. Chicks were then returned to their home cage. All tests were performed by trained observers who were not informed of a subject's pretest or test treatment. Footlift frequency was recorded on electromechanical counters activated by experimenter closure of a hand-held microswitch. Cumulative footlift duration was recorded by silent timers (Hunter Model 120A) that were started by closure of the microswitch and stopped by switch release In our preliminary research (see the Discussion section below) animals adopted a sleep-like posture that we termed separation immobility (SI) Latency to perform this response was recorded in this experiment.

RESULTS

A 2 (Drug Treatment) × 4 (Formalin Concentration) analysis of variance (ANOVA) was performed on body weight data There were no significant differences for main effects or the interaction term for this measure at the adopted $p<0.05$ level of significance Hence, treatment groups were equivalent on this measure

The mean footlift data for each treatment group are presented in Fig 1. Mean number of footlifts increased as a function of increased formalin concentration, and morphine potentiated this formalin effect. A two-way ANOVA performed on these data demonstrated significant main effects of drug treatment, $F(1,72) = 12\ 84$, $p<0\ 001$, and formalin concentration, $F(3,72) = 16.98$, $p<0\ 0001$ The interaction term was not significant Planned pairwise comparisons [*t*-tests using the ANOVA error term, (10)]

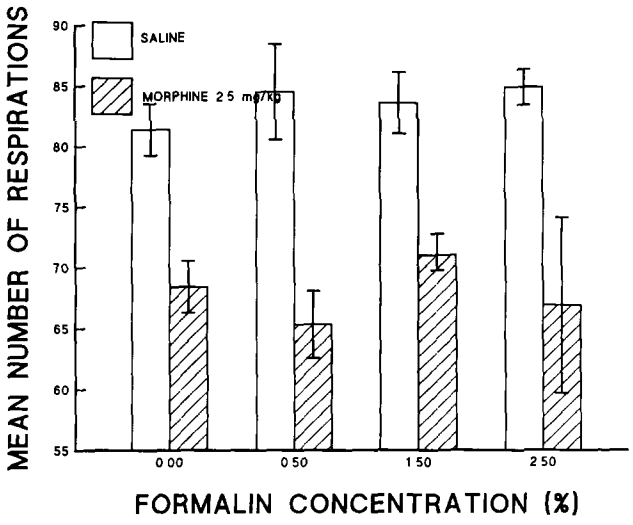

FIG 2 Mean number of respirations in one min as a function of intraplantar injection of different formalin concentrations after IM injection of saline or morphine. Respiration was measured immediately after the formalin test session Vertical bars represent SEM

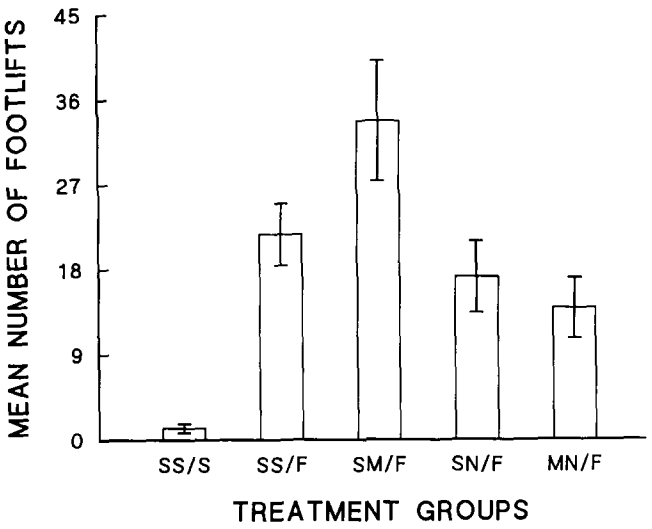

FIG 3 Mean number of footlifts after two successive injections of saline (SS), saline followed by morphine (SM), saline followed by naloxone (SN), or morphine followed by naloxone (MN), tests occurred 30 min later immediately after an intraplantar injection of either saline (S) or 1% formalin (F) Vertical bars represent SEM

were computed for saline vs. morphine at each formalin concentration. These two-tailed tests demonstrated significant differences at the 0.5% ($p<0.05$), 1.5% ($p<0.01$), and 2 5% concentrations ($p<0.01$). The saline and morphine groups did not differ significantly at the 0.0% concentration. The mean footlift duration data are not presented because these data displayed the same pattern of effects and significant treatment and group differences as the footlift data Although the morphine groups generally tended to adopt the SI posture sooner than the saline groups (overall mean latency to SI for morphine treatment and for saline treatment was 56 0 and 85.3, respectively) a two-way ANOVA performed on these data did not yield significant effects for drug treatment, formalin concentration or the interaction term.

The data showing treatment effects on mean number of respirations are presented in Fig. 2. These data show only small differences across saline and morphine treatments as a function of formalin concentration. Morphine clearly inhibited respiration and this effect appears to be relatively independent of formalin concentration. In accord with the appearance of the summary data, a two-way ANOVA revealed a significant main effect drug treatment, $F(1,72)=41.73$, $p<0.0001$. Planned pairwise comparisons demonstrated that morphine treatment was significantly different ($p<0.01$) from saline across each formalin concentration.

EXPERIMENT 2

Morphine analgesic effects on the formalin test (2) and morphine inhibition of respiration are naloxone reversible and this result implies opioid receptor mediation (9, 14, 18). The purpose of this second experiment was to determine if the opiate antagonist naloxone would reverse the hyperalgesic effects of morphine on the formalin test. Naloxone effects on respiration were also evaluated.

Subjects and Apparatus

Subject ($N=60$) characteristics, housing, and apparatus were as described in Experiment 1.

Procedure

At 14 days after hatching animals were randomly assigned to one of five independent treatment groups ($n=12$). In this experiment animals received two 1 ml/kg IM injections (one in each thigh; one injection immediately after the other) The injections consisted of 0.9% saline (S), morphine sulfate (M, 2.5 mg/kg), or naloxone hydrochloride (N; 5 mg/kg). Thirty min after the initial injections, animals were given a 0 05 ml intraplantar injection of either 0 9% saline (S) or 1 0% formalin (F) and tested as described in Experiment 1. The five treatment groups were defined by the dual IM injections that were administered 30 min before testing and the intraplantar injection administered immediately before testing The pretest/test injection sequence for each group was Saline, saline/saline (SS/S), saline, saline/formalin (SS/F), saline, morphine/formalin (SM/F), saline, naloxone/formalin (SN/F), and morphine, naloxone/formalin (MN/F).

RESULTS

The footlift data are displayed in Fig 3. A one-way ANOVA performed on these footlift data was significant, $F(4,55)=9.18$, $p<0.0001$. Planned pairwise comparisons demonstrated that all groups that received formalin performed significantly ($ps<0.01$) more footlifts than the saline control group (SS/S) and morphine enhanced this effect (SS/F vs SM/F, $p<0$ 05) Naloxone, by itself, decreased footlifts slightly but not significantly (SS/F vs SN/F, $p>0$ 05) and reversed the increase induced by morphine (MN/F vs SS/F, $p>0.05$) Footlift duration data displayed the same pattern of effects as the footlift frequency data and are, therefore, not presented. Drug treatment effects on respiration are displayed in Fig. 4 A one-way ANOVA was significant, $F(4,55)=15.27$, $p<0.0001$. This difference was a result of the lower respiration in group SM/F compared to all other groups ($ps<0.01$, Scheffe's test) No other comparisons were significantly different Thus prior exposure to formalin did not exert a significant

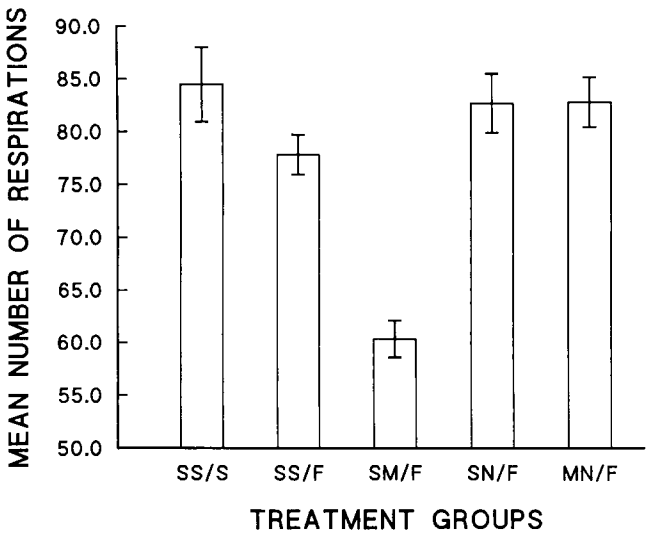

FIG 4 Mean number of respirations in one min after two successive injections of saline (SS), saline followed by morphine (SM), saline followed by naloxone (SN), or morphine followed by naloxone (MN). respiration was measured immediately after the formalin test session in which animals received intraplantar saline (S) or formalin (F) Vertical bars represent SEM

influence on respiration, but morphine inhibited this measure and naloxone reversed this morphine effect. As in the first experiment, groups did not differ significantly in SI latency.

DISCUSSION

The present study was designed to examine morphine effects on chemical nociception and respiration in WL cockerels. In rats, injection of a small amount of dilute formalin into the paw elicits paw-directed responses (lifting, licking, and biting) These responses are inhibited by morphine (analgesic effect) and this inhibition is reversed by naloxone (1–3). A literature search failed to reveal any studies on the use of the formalin test in domestic fowl. Thus to our knowledge, the present study represents the first description of formalin test procedures and responses elicited by formalin in domestic fowl A description of the behaviors elicited by formalin in this species, therefore, seems appropriate

In preliminary research we examined the behavior of WL cockerels after intraplantar injections of 0.9% saline or 5.0% formalin Animals injected with intraplantar saline typically stood in one place in the apparatus, frequently emitted the shrill vocalizations that are common in chicks separated from their social companions (13), and occasionally walked about the observation chamber After varying periods of time (mean = 96 s from the start of the session) these animals would adopt a sleep-like posture, eyes closed, and head down with beak often touching the chamber floor. This posture was maintained for the remainder of the 5-min session and vocalizations were not emitted during this time. We termed this sleep-like response "separation immobility" (SI). The SI response bears a superficial similarity to the restraint-induced response termed tonic immobility (4,6) and we tentatively suggest that it is a fear-related response elicited by separation from familiar social and static home-cage stimuli, and exposure to a novel environment.

Animals given intraplantar formalin reacted immediately with shrill vocalizations and rapid up and down movement (footlifts) of the affected foot. Some lifts were several seconds in duration During these prolonged lift times the animal's foot was held up against its body, sometimes with toes curved down and inward and sometimes with toes flexed outward Footlifts were often followed by short bursts of ambulation These animals sometimes pecked at the affected foot and a chick would occasionally use its beak to grasp the affected foot The footlift response was the most frequent response and occurred in every animal that received formalin To prevent confounding the footlift response induced by formalin with footlifts involved in ambulation, a decision was made to operationally define a "footlift" as those occasions when an animal lifted a foot from contact with the floor and reestablished foot contact with the floor without engaging in ambulation

Animals injected with formalin, like those injected with saline, adopted the sleep-like posture we termed SI and this response occurred at about the same mean time from the start of the session (102 s) as it did in the saline group. Some animals continued to give evidence of formalin effects during the SI phase in the form of holding its foot up against its body It was not possible to observe this response consistently because the animal's position during SI did not always permit observation of the affected foot Most footlift responses occurred within the first minute after formalin injection and rapidly subsided thereafter Few responses occurred in the second minute after injection and rarely in the third to fifth minutes. Subsequent studies used a three-minute observation period We did not formally evaluate the possibility of longer-lasting consequences of formalin (3) but informal observation of animals in their home cage about an hour after tests gave no evidence of long-term effects Higher formalin concentrations may be required for long-term effects Alternatively, domestic fowl may possess endogenous antinociceptive mechanisms that attenuate the long-lasting formalin effects that may occur in other species (3).

The distributions of footlift frequency for animals given saline (mean $<$ 1.0) and those given formalin (mean = 36.0) did not overlap. The distributions of footlift duration data for these groups also did not overlap The formalin test clearly provides a suitable response basis for the purpose of evaluating morphine effects on chemical nociception in domestic fowl

In Experiment 1, the mean number of footlifts displayed by animals given saline increased as a direct and graded function of formalin concentration from a mean of less than 1 0 at 0.0% to nearly 20 at 2 5% concentration. This graded effect provides presumptive evidence that formalin is a noxious stimulus in domestic fowl and indicates that dilute formalin can be used to examine nociception in this species in the same way that it is used in other species (1–3)

In our previous research on thermal nociception in cockerels, morphine produced naloxone sensitive hyperalgesia (7,8). In the present study, morphine produced hyperalgesia on a test of chemical nociception. Leghorn cockerels given 2.5 mg/kg morphine sulfate 30 minutes before tests performed significantly more footlifts of longer duration than saline-injected control animals at all concentrations of formalin (i.e., 0 5, 1.5, and 2.5%) except the 0.0% level. That the 0.0% groups did not differ significantly suggests that the hyperalgesic effect does not reflect a general increase in activity. In Experiment 2, the opioid antagonist naloxone (5 0 mg/kg) reversed morphine hyperalgesia. Morphine hyperalgesia in the intact drug-naive animal is clearly unusual, but the present results demonstrate that this morphine effect, although unusual, is neither unique to hot plate test procedures nor peculiar to thermal nociception. Moreover, hyperalgesia, like morphine analgesia, is naloxone reversible and this result indicates that the effect is mediated by opioid receptors

Naloxone sensitive respiratory depression is a well documented morphine effect (12). Although morphine produced an atypical effect on nociception in WL cockerels, morphine inhibited respiration in this model and this effect was reversed by naloxone

This finding suggests that atypical morphine effects in this strain of domestic fowl are not reflected in all morphine-sensitive systems and may be restricted to nociception. Clear support for this suggestion, however, will require evaluation of morphine effects on respiration unconfounded by prior testing and free of effects related to restraint during the measurement procedure. Nevertheless, the present findings, together with previous results (7, 8, 15–17), indicate that in the present model, morphine produces sedation, hypothermia, respiratory depression, and hyperalgesia. Within this morphine profile, only the hyperalgesic effect is uncommon and paradoxical.

Hyperalgesic morphine effects have been reported to occur in rodents after chronic morphine exposure (11) and at relatively long intervals (e.g , 4 h) after acute morphine administration (5). These hyperalgesic effects are preceded temporally by morphine analgesic effects (5,11). The morphine hyperalgesic effects reported herein and in our previous research with domestic fowl are uncommon and paradoxical in that they occur on first exposure to morphine, display the temporal characteristics of morphine analgesia, and do not appear to be temporally dependent on analgesic effects (15).

Morphine can induce either hyperalgesic or analgesic effects in domestic fowl and this difference appears to be genetically determined (6) Speculation derived from converging evidence suggested (6) that breeding for the high feed efficiency and low body weight characteristics of the WL strain may have produced a shift in the population of mu opioid receptors that subserve morphine analgesic effects (14,18) to kappa receptors that may subserve hyperalgesic effects (19,20). Recent evidence based on the use of selective opioid antagonists, however, suggests that morphine hyperalgesic effects in domestic fowl, like analgesic effects in other species, are subserved primarily by mu opioid receptors at central nervous system loci (17) Monoaminergic systems are known to participate in the modulation of nociception and it is possible that strain-dependent hyperalgesic effects produced by morphine in domestic fowl reflect alterations in these systems This possibility remains to be determined

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