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Morphine Potentiation of Tonic Immobility: Effects of Naloxone, PCPA, and 5,6-DHT[1]

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WALLNAU, L B AND G G GALLUP, JR *Morphine potentiation of tonic immobility Effects of naloxone, PCPA, and 5,6-DHT* PHARMAC BIOCHEM BEHAV **10**(4) 499–504, 1979 —The duration of tonic immobility in chickens, a catatonic-like state produced by brief restraint, was greatly potentiated by a single 1 0 mg/kg injection of morphine Naloxone by itself, however, had no effect on tonic immobility, and only an exceptionally large dose of naloxone blocked the morphine potentiation Pretreatment with PCPA and 5,6-dihydroxytryptamine completely eliminated the morphine enhancement The effect of morphine on tonic immobility may be mediated by a central serotonergic-raphe system

Morphine 5,6-DHT Catatonia Raphe neurons Naloxone Endorphins PCPA Tonic immobility Serotonin

TONIC immobility has been reported in insects, crustaceans, fish, amphibians, reptiles, birds, and even mammals [19]. To produce this response one simply holds an animal down on a flat surface Once the struggling subsides the animal typically assumes a motionless, catatonic-like posture which persists in the absence of further restraint. In domestic fowl tonic immobility may last for a few minutes to over an hour. The reaction has been the subject of many diverse interpretations, and recently has been proposed as a laboratory-evolutionary model of catatonic schizophrenia [20]

Tonic immobility shows a striking resemblance to a drug-induced form of catatonia recently reported in rats [7,28] The specific substance, *B*-endorphin, is one of several naturally occurring morphinomimetic brain peptides thought to have analgesic properties [10,31] Shortly after central administration of *B*-endorphin, animals become immobile, showing signs of waxy flexibility, mydriasis, reduced core temperature, exophthalmus, and loss of the righting reflex These effects can last up to one hour. Sudden changes in stimulation cause subjects to resume normal postures, and recovery is abrupt with no apparent aftereffects All of these symptoms also characterize animals in tonic immobility [20]

In support of the possibility that these might be homologous behaviors, mediated by a central opiate receptor system, morphine has been shown to greatly potentiate the duration of tonic immobility in both rabbits and chickens [14,27], and endorphin-induced catatonia in rats is completely reversed by the opiate antagonist naloxone [7,28]

In light of morphine's analgesic properties, it seems paradoxical that pretreatment with painful stimulation, such as electric shock, also serves to prolong tonic immobility [19] It is possible, however, that pain may trigger the release of naturally occurring morphine-like compounds in the brain and serve to potentiate the response in a manner similar to morphine [41]. Such an interpretation is compatible with the frequent but as yet unsubstantiated claim that tonic immobility may be accompanied by analgesia [12, 22, 35]

An alternative neurochemical account of tonic immobility attributes the reaction to central serotonergic mechanisms [41] For example, there appears to be an inverse, bidirectional relationship between drug-induced changes in midbrain raphe electrical activity and drug effects on the duration of tonic immobility Drugs which decrease raphe activity in rats (e.g , LSD, pargyline) prolong immobility in chickens, while drugs which activate raphe neurons (e g , amphet-

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TABLE 1
EFFECT OF DIFFERENT DOSES (MG/KG) OF NALOXONE ON THE DURATION (SEC) OF TONIC IMMOBILITY

	Dose of Naloxone				
	0 0	0 5	1 0	2 0	10 0
Mean	393 50	496 75	292 25	364 38	496 88
Standard Error	141 28	217 94	150 57	267 58	177 70

amine) attenuate the reaction [2, 3, 9, 17, 32] In keeping with the parallel between raphe electrical activity and tonic immobility and the parallel between tonic immobility and *B*-endorphin-induced catalepsy, recent evidence has shown that morphine suppresses raphe electrical activity [11,26]. However, there is one report that naloxone fails to prevent this effect, suggesting that the effect of morphine on raphe neurons is non-narcotic [26] Earlier failures to find an effect of morphine on raphe firing [25,30] have been attributed to the masking effect of chloral hydrate anesthesia [26] To determine if tonic immobility might be related to morphinomimetic rather than serotonergic mechanisms, the effects of naloxone, morphine, p-chlorophenylalanine (PCPA) and 5,6-dihydroxytryptamine (5,6-DHT) on tonic immobility were examined

EXPERIMENT 1

Based on their striking similarities it is possible that tonic immobility and endorphin-induced catatonia might represent homologous responses Endorphin-induced catalepsy is completely blocked by the opiate antagonist naloxone [7,28] If tonic immobility is mediated by naturally occurring endorphins, then naloxone should attenuate the response The first experiment examined the effect of varying doses of naloxone on tonic immobility

METHOD

Animals

Forty straight-run Production Red chickens (*Gallus gallus*) were obtained from a commercial hatchery (Welp, Inc.) at two days of age The animals were housed in brooders and maintained on a starter feed which was prepared locally (Fort Orange Chick Starter, Barber and Bennett, Inc). Artificial illumination was provided from 8 a m to 10 p m each day

Procedure

At 22 days of age, animals were assigned to one of five groups Each animal was first weighed and then received an injection of either distilled water, or naloxone HCl (Endo) in doses of 0 5, 1 0, 2 0 or 10.0 mg/kg. All injections were intraperitoneal Following injection animals were individually placed in cardboard holding boxes for 10 min, and subsequently carried to a test room in the cardboard box Animals were removed from the box and manually restrained on a table for 15 sec on their right side The duration of tonic immobility was timed with a stopwatch from the moment the experimenter released his hands from the subject until the animal righted itself and rose to its feet If the initial induction attempt did not elicit tonic immobility, the experimenter waited 60 sec and repeated the induction procedure Induction attempts were repeated in this manner until the animal exhibited tonic immobility, or until there had been five unsuccessful induction attempts Animals that received five inductions and still showed no response were given a duration score of zero seconds Testing occurred between 10 00 a.m and 4 00 p m To preclude confounding due to a time of day effect, for testing purposes subjects were randomly assigned across groups to different times of day

RESULTS

Table 1 depicts the results of Experiment 1 A square-root transformation was performed to alleviate skew and to minimize heterogeneity of variance [29] Analysis of variance revealed no overall effect for dose, $F(4,35)=0\ 42$ In addition, there were no significant linear, $F<1$, quadratic, $F(1,35)=1\ 02$ cubic or quartic, $F<1$, trend components

EXPERIMENT 2

Since naloxone will block endorphin-induced catalepsy [7,28] but not tonic immobility, it is unlikely that these reactions are related An alternative explanation of the morphine enhancement of tonic immobility is provided by the midbrain-raphe model [41] Drugs which inhibit raphe firing tend to prolong tonic immobility In keeping with this parallel, morphine has been shown to inhibit midbrain raphe neurons [11,26] and enhance immobility duration [14,27] However, the morphine suppression of raphe activity may not be a narcotic effect since in one report naloxone failed to block this effect [26] Furthermore, dextrorphan, the analgesically inactive stereoisomer of the morphine agonist levorphanol, also suppresses electrical activity of raphe neurons [26] However, these findings are contrary to those of Buxbaum and Pamplin [11] in which naloxone did block the morphine inhibition of raphe activity

The second experiment was designed to determine whether naloxone would block the effect of morphine on tonic immobility. In addition, naloxone was tested for its ability to reverse a large hypnotic dose of morphine in chickens

METHOD

Animals

Forty straight-run Production Red chickens were housed and maintained as described in the first experiment

Procedure

At 17 days of age, animals were assigned to one of four groups Subjects received either morphine sulfate (Lilly) or distilled water for their first injection, and either naloxone HCl or distilled water for their second injection. The dose of both morphine and naloxone was 1.0 mg/kg (IM), and the first and second injections were separated buy 10 min. These parameters are comparable to those which demonstrate effective opiate blockade in avians [33] The injection volume for all first injections (morphine or distilled water) was 2 0 ml/kg, while the volume of second injections (naloxone or distilled water) was 2 5 ml/kg Testing began 10 min following the last injection using the test procedures described in

TABLE 2

EFFECT OF MORPHINE (1 0 MG/KG) AND NALOXONE (1 0 MG/KG) ON THE DURATION (SEC) OF TONIC IMMOBILITY

	Drug Condition			
	Morphine/Water	Morphine/Naloxone	Water/Water	Water/Naloxone
Mean	2022 33	1846 78	948 67	1316 78
Standard Error	386 05	555 30	202 35	487 85

TABLE 3

EFFECT OF MORPHINE (1 0 MG/KG) AND NALOXONE (10 0 MG/KG) ON THE DURATION (SEC) OF TONIC IMMOBILITY

	Drug Condition		
	Water/Water	Morphine/Naloxone	Morphine/Water
Mean	1323 42	1414 92	2531 75
Standard Error	247 59	349 08	548 68

the previous experiment. Persons testing birds had no knowledge of the drugs administered to specific animals

To check on the effectiveness of naloxone as an opiate antagonist in chickens, four additional birds were given hypnotic doses of morphine (60 mg/kg). Following the onset of hypnotic and cataleptic reactions, each bird was given 0 8 mg/kg of naloxone intravenously

RESULTS

The results are shown in Table 2. Analysis of variance was performed following a square-root transformation Morphine enhanced the duration of tonic immobility, $F(1,32)=4\ 21$, $p<0.05$ Naloxone, however, failed to block the morphine enhancement as revealed by a nonsignificant orthogonal comparison of the morphine-water and morphine-naloxone groups, $F<1$. In agreement with the findings of Experiment 1, the water-water and water-naloxone groups also did not differ significantly, $F<1$. Control durations of tonic immobility, however, were longer in the present experiment than in the previous experiment. In as much as an injection can be stressful, the use of a double injection procedure may have elevated the baseline in the second experiment This interpretation is in agreement with the finding that aversive events tend to potentiate response duration, and do so as a function of the intensity of the aversive stimuli employed and their temporal proximity to tonic immobility induction [19]

Regarding the effectiveness of naloxone, all animals receiving hypnotic doses of morphine showed cataleptic reactions within a few minutes, and in every case naloxone caused an immediate and striking reversal of these effects

EXPERIMENT 3

Haigler [26] found that both microiontophoretically applied and intravenous injections of naloxone seldom blocked the morphine suppression of raphe firing (e g , 6 of 62 cells and 4 of 62 cells, respectively) Buxbaum and Pamplin [11], however, found that naloxone did block the morphine inhibition of raphe electrical activity A straightforward interpretation of this discrepancy is precluded by the failure of Buxbaum and Pamplin to report dose and route of administration of naloxone, and the relative effectiveness of the blockade While doses of naloxone used in Experiments 1 and 2 range from 25 to 33 times the effective dose (0 03 mg/kg) for opiate blockade in avians [33], perhaps still a higher dose of naloxone would block the morphine enhancement of tonic immobility Such a finding would be consistent with the electrophysiological findings of Buxbaum and Pamplin [11], if indeed they used a dose of naloxone which was considerably higher than Haigler [26] Experiment 3 examined the effectiveness of 10 0 mg/kg of naloxone in combination with morphine

METHOD

Animals

Thirty-six straight-run Production Red chickens were housed and maintained as described in the first experiment.

Procedure

At 28 days of age, subjects were randomly assigned to one of three groups, either water-water, morphine-water, or morphine followed by naloxone The doses of morphine sulfate and naloxone HCl were 1 0 mg/kg and 10 0 mg/kg, respectively. Injection volumes were 1 0 ml/kg (IM). The first injection was followed 10 min later by the second injection, which in turn was followed 10 min later by testing The test procedures were identical to those described in the first experiment, and persons collecting data were not informed of animals' group designation

RESULTS

The results are shown in Table 3 Analysis of variance following a square-root transformation revealed no overall effect, $F(2,33)=2\ 71$, $p<0.10$. However, the planned orthogonal comparisons showed that the morphine-water group differed significantly from the water-water and morphine-naloxone groups, $F(1,33)=5\ 42$, $p<0.03$, but there was no

TABLE 4
EFFECT OF MORPHINE (2 0 MG/KG) AND PCPA (300 0 MG/KG) ON THE DURATION (SEC) OF TONIC IMMOBILITY

	Drug Condition			
	Water/Morphine	PCPA/Morphine	Water/Water	PCPA/Water
Mean	1658 75	405 63	272 50	631 38
Standard Error	386 50	192 30	52 44	390 55

difference between the water-water and morphine-naloxone groups ($F<1$) In fact, the first comparison accounted for 99% of the between-group variability In short, morphine enhanced TI for birds in the morphine-water group but did not when it was followed by 10 0 mg/kg of naloxone.

EXPERIMENT 4

Hicks *et al* [27] reported that PCPA pretreatment completely eliminated the morphine enhancement of tonic immobility Serotonin depletion of PCPA has been observed not only to block morphine effects (e g , [16,23]) but also effects of drugs on raphe firing [3] and tonic immobility [9] Yet PCPA itself has no effect on tonic immobility or raphe firing (see [41]) In the fourth experiment an attempt was made to replicate the PCPA blockade of the morphine enhancement of tonic immobility

METHOD

Animals

Thirty-two straight-run Production Red chickens were housed and maintained as described in the first experiment.

Procedure

At 19 days of age, subjects were initially pretreated with either 300 0 mg/kg (IP) of DL-p-chlorophenylalanine methyl ester HCl (Sigma) or an equivalent volume of distilled water (2 0 ml/kg) 72 hours prior to test day On test day, subjects received 2 0 mg/kg of morphine sulfate intramuscularly or an equivalent volume of distilled water (1 0 ml/kg). Animals were tested for tonic immobility 20 min following injection using the test procedures described in the first experiment These parameters are comparable to those employed by Hicks *et al* [27] Again, persons recording data were kept uninformed as to specific drug treatments

RESULTS

The results are depicted in Table 4 Analysis of variance following a square-root transformation revealed that birds receiving morphine and water remained immobile significantly longer than those in all other groups, $F(1,28)=13\ 72$, $p<0\ 01$, while orthogonal comparisons between the remaining groups revealed no significant differences, $F<1$ Thus, PCPA effectively blocked the morphine enhancement of tonic immobility, a result which confirms a previous finding [27]

EXPERIMENT 5

The neurotoxin, 5,6-dihydroxytryptamine (5,6-DHT) has been shown to be a relatively selective, long-lasting depletor of brain serotonin [4, 5, 6] The results of the previous experiment implicate the possible participation of serotonergic mechanisms in the morphine potentiation of tonic immobility To further evaluate this interpretation, Experiment 5 examined the effect of intraventricular injections of 5,6-DHT on the morphine enhancement

METHOD

Animals

Forty-six straight-run Production Red chickens were housed and maintained as described in the first experiment.

Procedure

At three weeks of age, animals were anesthetized with 1 5 ml/kg (IV) of Chloropent (Fort Dodge). A 23 ga stainless steel cannula guide was implanted stereotactically 2 mm anterior to the frontal-parietal suture, on the midline, and 5 mm below the cranial surface. Specific details regarding cannula construction and anchoring have been described in detail elsewhere [42] A 30 ga filler wire was inserted following implantation and extended 0 5 mm beyond the bottom of the cannula guide. All animals were banded during surgery for identification A 24 hr recovery period was provided by individual housing in pigeon cages

Following recovery, the filler wire was removed and a 30 ga cannula was inserted into the cannula guide so that it extended 0 5 mm below the guide Twenty-two subjects received 75 μg of 5,6-dihydroxytryptamine creatinine sulfate (Sigma) in a vehicle of 10 μl saline and 1 0 mg/ml ascorbic acid Twenty-two additional animals received 10 μl of the vehicle The two remaining subjects died following surgery Injections were accomplished with a Hamilton microliter syringe (705 LT) and dispenser (PB 600-1) Following the intraventricular injections, animals were returned to group housing in commercial brooders Seventy-two hr later, half of the animals from each initial pretreatment condition received injections of morphine sulfate (1 0 mg/kg, IM) while the remaining animals received an equivalent volume (1 0 ml/kg) of distilled water Animals were tested using the induction procedures previously described, 20 min following injection

Following data collection, animals were sacrificed and their brains perfused via the carotid artery with a 10% formaldehyde solution Prior to removal of the brain, 15 μl of ink was injected into the cannula. The brains were removed and transected coronally at the cannula tract Verification of cannula placement was based on the absence or presence of ink in the ventricles. The persons who collected immobility data and those who judged cannula placement were kept uninformed of the group designation of each animal

TABLE 5

EFFECT OF MORPHINE (1 0 MG/KG) AND 5,6-DIHYDROXYTRYPTAMINE (75 μG) ON THE DURATION (SEC) OF TONIC IMMOBILITY

	Vehicle/Morphine	5,6-DHT/Morphine	Vehicle/Water	5,6-DHT/Water
Mean	1858 86	703 78	532 78	645 44
Standard Error	619 62	391 33	205 27	166 29

RESULTS

The data for 36 animals were used for analysis. Two animals in each group were discarded due to poor cannula placement. The mean and standard errors are depicted in Table 5 Analysis of variance following a square-root transformation revealed that animals in the vehicle-morphine group remained immobile significantly longer than those in the remaining groups, $F(1,32)=5.36$, $p<0$ 03, while orthogonal comparisons among the remaining groups failed to reach statistical significance ($F<1$ in each case) Thus pretreatment with 5,6-dihydroxytryptamine had no effect by itself, but like PCPA, completely blocked the morphine enhancement of tonic immobility.

DISCUSSION

In terms of neurochemistry endorphin-induced catalepsy does not appear to be related to tonic immobility in spite of the striking resemblance between these responses. Naloxone will reverse endorphin-induced catalepsy [7,28], but as shown in the present study it does not alter tonic immobility in chickens Carli [13], however, reported that tonic immobility duration in rabbits was reduced by unusually high doses of naloxone (15 to 25 mg/kg), a finding which conflicts with the results of Experiment 1 and those of Peters and Hughes [34] and Galeano *et al* [18] Furthermore, in large amounts naloxone is not a pure opiate antagonist, producing side effects such as restlessness [8] which might serve to abbreviate tonic immobility due to competing responses. On the other hand, it should be acknowledged that while Davis [14] found morphine to enhance tonic immobility, levallorphan had opposite effects

In Experiment 2, naloxone failed to block the morphine potentiation of tonic immobility These findings parallel those of Haigler [26] in which naloxone failed to block the morphine suppression of raphe firing, and suggest the morphine enhancement is a non-narcotic effect.

It should be noted that the time and dose parameters used in Experiment 2 are well within the range of those which are effective for opiate blockade in avians For example, the behavioral suppression exhibited by pigeons following morphine injections of 10.0 mg/kg (IM) can be reversed by as little as 0 03 mg/kg (IM) of naloxone [33]. Thus, the dose of naloxone employed in Experiment 2 was 33 times the effective dose for opiate blockade in avians As a check on the effectiveness of naloxone as an opiate antagonist in chickens, Experiment 2 also showed that 0.8 mg/kg of naloxone completely reversed the behavioral effects of a hypnotic dose of morphine However, when a dose of naloxone which is more than 300 times greater than the effective dose for opiate blockade in avians was employed in Experiment 3, there was no morphine enhancement This finding replicates the data obtained by Peters and Hughes [34], and would appear to parallel the work of Buxbaum and Pamplin [11] in which naloxone blocked the morphine inhibition of raphe However, it is not clear if differences in naloxone dose can account for the discrepancy between the findings of Haigler [26] and Buxbaum and Pamplin [11]

It is unusual that such a high dose of naloxone was necessary to block the morphine enhancement of tonic immobility It is possible that the morphine effect on TI is not entirely mediated by an opiate receptor system While most evidence suggests that acute morphine administration has no effect on the overall level of brain serotonin in rodents, other data show that serotonin plays a modulating role in at least some of the behavioral effects of morphine [43] Serotonin depletion by reserpine, PCPA, or 5,6-DHT has been found to block morphine analgesia [15, 16, 21, 23, 38, 39] Similarly, serotonin depletion by p-chloro-N-methylamphetamine blocks morphine-induced catalepsy [24]. These findings parallel the results of Experiments 4 and 5 in which PCPA and 5,6-DHT abolished the morphine enhancement of tonic immobility

Lesioning midbrain raphe nuclei blocks the antinociceptive effect of morphine [1,36] and raphe stimulation enhances such effects [37] Recent evidence suggests that morphine competes with serotonin for serotonergic receptor sites [40] and inhibits the firing rate of midbrain raphe neurons [11,26] In light of the apparent relationship between TI and raphe firing [41], we suggest the morphine enhancement of TI may be partially influenced by a central serotonergic system

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