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Aversive Properties of Cycloheximide versus Memory Inhibition in Chickens' Formation of Visually Cued Food Aversions[1,2]

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BOLAS, K. C., W. P. BELLINGHAM AND G. M. MARTIN. *Aversive properties of cycloheximide versus memory inhibition in chickens' formation of visually cued food aversions.* PHARMAC. BIOCHEM. BEHAV. **10**(2) 251–254, 1979.—No memory inhibition for a conditioned aversion in chickens, produced by pairing a novel colored food and lithium chloride (LiCl), was found when cycloheximide (CXM) was injected intracerebrally (IC) two or six hr before feeding. Good conditioned aversions were found when CXM alone was injected IC following consumption of the novel food. No aversions were found when CXM alone was injected IC two hr before feeding. Possible reasons for apparent memory inhibition using CXM in some appetitive paradigms are discussed.

Cycloheximide Memory inhibition Conditioned aversions Chickens

AN ACCUMULATING body of evidence suggests that cerebral RNA and protein synthesis may play an important role in memory storage [3]. The results of behavioral experiments with agents that specifically inhibit cerebral protein synthesis seemingly support this hypothesis. The complex effects which may be produced by these inhibitors, however, prevent definite confirmation of the hypothesis that the main behavioral effects are due to memory impairment produced by the inhibition of protein synthesis, and raise a number of theoretical questions.

The present study proposed to determine the effects of cycloheximide (CXM) on the formation of visually cued food aversions in chickens since excellent visual aversions in chickens have been demonstrated [9]. Such a preparation has a number of advantages. The use of chickens as subjects allows easy injection of the drug intracerebrally thereby ensuring that the chemical is reaching the central nervous system. The soft skulls of chickens allow for accurate free-hand injections directly into the forebrain [8]. The chicken forebrain has been studied extensively and research indicates the importance of the telencephalon in terms of learning [16]. In the past, conventional paradigms have limited memory studies to learning situations requiring contiguity of the neutral stimulus and its consequence, thus preventing clear differentiation of the course of action of the inhibitor. In a toxicosis learning paradigm close temporal contiguity is not required [12]. Furthermore, learning is disclosed by reduced consumption on test day as a result of one-trial conditioning which is specific and long lasting [13].

GENERAL METHOD

Animals

The chicks were White Leghorn×Black Australorp obtained as fertilized eggs from BimBimBie Poultry Farm, Melbourne, Australia. They were incubated and hatched according to standard poultry procedures.

Food

The chickens were fed D & R Chick Starter soaked in tap water (100 ml of water added to 100 g of food) to make normal wet mash (NWM). A visually novel red food (RWM) was obtained by adding 6 ml of red Aeroplane food coloring to 94 mls of water and mixing with 100 g of starter. The chickens were sustained on NWM and tap water throughout the experiments except when RWM was presented to the specified groups on either pretraining or training days, and to all groups on the test day. All food consumption data were obtained by weighing each chick to the nearest 0.01 g immediately before and after the 0900–0915 hr feeding period.

Procedure

On Day 1, when the chickens hatched, they were moved to a Multiplo communal brooder. A light-dark cycle with lights on at 0800 hr to 1800 hr was initiated and maintained throughout the experiment with one noted exception. On Day 9 at 1700 hr the birds were moved to individual wire cages (19×16×16 cm) which were covered by paper toweling

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on both sides and the back. Food and water were provided in two plastic containers with a 3 cm hole 3 cm from the base. Food was always placed on the left and water on the right. On Day 9 random assignment to the various groups also occurred.

From Day 1 to Day 10 maintenance was on freely available NWM food and water. Both were changed daily. On Day 11, at 1700 hr, the deprivation schedule was initiated with food available only between 0900–0915 and 1200–1700 hr. Water was continually available except during the 0900–0915 hr feeding session. Food consumption was measured on Days 13 (pre-pretraining), 14 (pretraining), 15 (training), 18 (pretest) and 19 (test). The Day 13 pre-pretraining measure of NWM food consumption was obtained in order to ensure comparability of consumption among the various groups. On Day 14 (pretraining) the conditioned food aversion control groups received the novel RWM while the experimental groups received NWM. For Day 15 (training) the procedure was reversed. The experimental groups received the novel RWM while the conditioned aversion control groups were fed the NWM. All injections either preceded and/or followed the 0900–0915 hr feeding period on the training day. No further access to food was allowed following injections until 1700 hr of the training day when NWM was provided ad lib. Water was provided as usual immediately following the final injection or the end of the feeding peiod, whichever came later. The light on-light off cycle was interrupted with the lights left on until 1800 hr of Day 16. The purpose of providing food and water ad lib and continuous lighting was to facilitate recovery from the injections. Food deprivation was reinstated on Day 17 at 1700 hr. On Day 18 (pretest) consumption of NWM was measured in order to reaffirm group comparability. Therefore 96 hr were allowed for recovery before testing commenced.

Injections

In all experiments, the dosage of CXM used was 40 μg per 50 μl of physiological saline (SAL). One freehand injection of 25 μl was made into each side of the forebrain of the experimental birds. A rubber stop on the 0.45×13 mm needle ensured that its penetration was not more the 3 mm below the surface of the skull. An equal volume of SAL was injected IC into the CXM injection control birds. The dosage level and injection procedure was identical to that of Mark and Watts [8] and Woolston, Morgan and Hambley [18]. A 0.5 M (2.12 g in 100 ml of distilled water) lithium chloride (LiCl) solution was injected intraperitoneally at 1% of the animal's body weight. LiCl, when used, was always injected 30 min after food removal at the 0900 hr training day feeding session.

The injection levels chosen were those known to produce conditioned aversions and reported to produce memory inhibition. They were also the doses that in combination, and coupled with the recovery procedures noted above, produced acceptable survival for subjects injected with both drugs.

Statistical Analysis

In all experiments, chickens from at least two separate hatches were used and were randomly assigned to one of four groups defined by training day manipulations with exceptions noted. Each group contained at least nine birds. The letters used to code the various groups were R=(RWM); N=(NWM); C=(CXM); S=(SAL); L=(LiCl).The letters were combined according to the order of administration of training day treatments. For example, C-R-L refers to CXM injected prior to presentation of RWM following 30 min after food removal by LiCl, and R-C refers to RWM followed by an injection of CXM. One or two-way analyses of variance were carried out on all data. Significant effects were followed by Scheffé multiple comparisons of means.

EXPERIMENT 1

Memory inhibition effects of CXM injected IC in chickens at the dosage used in the present study (or less) have been reported for one-trial passive avoidance [4,8] and appetitive visual discrimination tasks [14,15]. In order to determine whether CXM had similar effects on visually cued food aversions in chickens, two and six hr CXM pre-feeding injections were chosen as the most reasonable for testing.

Selection of a two hr pre-feeding injection was based on two factors. Firstly, pilot work indicated that two hr was the shortest injection-feeding interval that would consistently produce adequate food consumption due to CXM induced anorexia at shorter intervals. Secondly, Woolston *et al.* [18] have recently shown, using chickens of the same strain and age as well as identical amounts, location and route of injection as the present study, that protein synthesis was maximum at one hr (85%) and fell steadily to about 50% at six hr. Day *et al.* [6] found a similar time course and levels of CXM induced protein synthesis inhibition for IC injected rats.

The six hr pre-feeding CXM injection was chosen because of a recent report by Tucker and Gibbs [17] of apparent memory inhibition for a saccharin cued taste aversion in rats. They found that CXM injected intraventricularly had a maximum inhibitory effect on the taste aversion between 5–7 hr. Shorter CXM-feeding intervals produced no memory inhibition.

Method

Twenty chickens were assigned to a conditioned taste aversion experimental group and 20 assigned to the control group. Experimental chicks were fed the novel RWM during the 0900–0915 hr feeding session of Day 15 (training) and injected IP 30 min later with LiCl. The controls were treated the same except that they were fed the familiar NWM. Equal exposure to the novel RWM was controlled for by feeding the control group the RWM on Day 14 (pretraining) thus preventing any confounding of the test day results by differential neophobia between the groups. All groups were therefore injected with LiCl on the training day. The purpose of this procedure was to ensure that any control-experimental differences on the test day were not contaminated by nonspecific sensitization produced by LiCl and that observed differences could be solely attributed to conditioned aversions to RWM.

In order to assess the memory inhibition properties of CXM on the visually cued aversions, half the controls (N=10) and half the experimental (N=10) chicks were injected IC with CXM 120 min prior to the training day feeding session. The other half were treated identically but injected with SAL.

Another 36 chickens were assigned and treated in the same manner except that CXM and SAL were administered 360 min before food presentation.

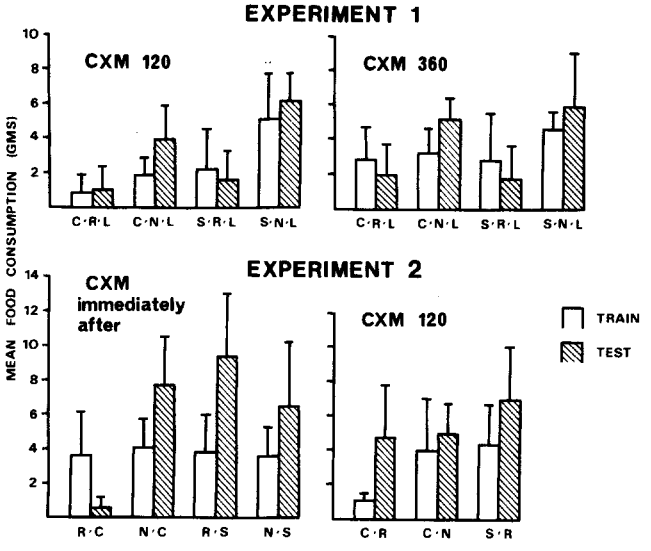

FIG. 1. Means and standard deviations for the training and test days consumption of food for Experiments 1 and 2. The captions at the top of the various groups refer to the pre-feeding injection times of CXM in mins. When LiCl was injected it was always 30 min following feeding. R=red wet mash; N=normal wet mash; C=cycloheximide injected IC; L=lithium chloride injected IP. The letters are combined to indicate the order of administration of treatments on training day. The test measure for all groups was the amount of R consumed. The N control groups were exposed to R on the pre-training day.

Results

A summary of means and standard deviations for all groups is shown in Fig. 1. Statistical analysis for the 120 min groups revealed significantly lower food consumption for the two CXM groups, C-R-L and C-N-L, on the training day, $F(1,36)=15.6$, $p<0.01$, indicating some CXM-induced anorexia. Those groups trained with RWM (C-R-L and S-R-L) had lower food consumption than those trained on NWM (C-N-L and S-N-L), $F(1,36)=48.3$, $p<0.01$. Specifically, these results demonstrate an aversion to RWM regardless of whether the chickens were pre-injected with CXM or SAL.

For the 360 min interval, analysis of test day results also showed significantly lower food consumption for the two groups trained on RWM relative to their NWM controls, $F(1,36)=26.17$, $p<0.01$. These results are similar to those obtained for the 120 min interval and again demonstrated an aversion in spite of the CXM pre-feeding injection on the training day.

It seems clear that no memory inhibition properties of CXM are apparent in this preparation. The fact that there is reduced food consumption at the 120 min pre-feeding injection interval indicates that CXM is producing anorexia. This effect is not present when the injection interval is 360 min. Although significant protein synthesis still occurs [18].

EXPERIMENT 2

The possibility exists that the anorexic properties of CXM are somehow interfering with its memory inhibition properties. That is, memory inhibition by CXM might be intact but CXM, if it is a conditioned aversion agent in its own right, may not appear to produce memory inhibition because it is also producing a conditioned food aversion. The fact that CXM is an anorexic agent cannot be construed as evidence for this argument since it has been shown that anorexia is not a good predictor of the aversion producing capacity of a drug [10].

Such a possibility would require that IC injections of CXM produce aversions when substituted for LiCl injected IP and that chicks would form conditioned aversions when CXM is injected prior to the feeding experience. Booth and Simson [5] injected rats IP with CXM immediately prior to being fed odorized food. A subsequent preference test revealed aversions to the odor by the CXM animals. Similarly, Nakajima [11] injected mice subcutaneously with CXM and found that administrations 30 min prior to training produced significant aversions. In both these experiments CXM was injected at a time interval such that the aversive consequence of the injection began shortly after presentation of the substance to be consumed.

Replication is essential since these experimenters used different dosages of CXM, species of animal, injection times, test procedures and mode of injection than those used in the present experiments.

Method

Forty chickens were randomly assigned to one of four groups: R-C, N-C, R-S and N-S where either CXM or SAL was injected IC immediately after food removal. Another 30 chicks were randomly assigned to three groups: C-R, C-N and S-R where CXM or SAL was injected IC 120 min prior to either presentation of RWM or NWM.

Results

There were no significant differences in food consumption on pretraining, training or pretest days for the groups injected immediately after food removal with CXM or SAL. Test day data disclosed a significant drug effect, $F(1,36)=22.98$, $p<0.01$, and a significant interaction effect, $F(1,36)=16.49$, $p<0.01$. Subsequent Scheffé multiple comparisons showed the R-C group to have reliably lower food consumption than the N-C, $F(1,36)=14.11$, $p<0.01$, the R-S, $F(1,36)=39.25$, $p<0.01$, and the N-S groups, $F(1,36)=18.27$, $p<0.01$. That is, the R-C group demonstrated a conditioned aversion due to association between the RWM and the effects of CXM.

For the 120 min pre-injected groups, one-way analysis of variance indicated a significant effect on the training day, $F(2,27)=5.99$, $p<0.01$. Subsequent Scheffé multiple comparisons indicated reliably lower food consumption for the C-R group relative to the C-N group, $F(1,26)=8.45$, $p<0.01$, and the S-R group, $F(1,26)=9.62$, $p<0.01$. Food consumption of all groups was similar on the test day, $F(2,27)=2.30$, $p>0.05$.

GENERAL DISCUSSION

In summary, these experiments indicate that IC injections of CXM into the forebrain of the chicken 120 or 360 min before pairing of a novel colored food with LiCl do not produce amnesia of the conditioned aversion. Furthermore, CXM produced strong food aversions when used as a substitute for LiCl but no aversions were apparent when CXM was injected 120 min prior to feeding. The possibility that CXM's conditioned aversion properties were masking its memory inhibition properties is, therefore, eliminated.

Although it has been shown that CXM injected IP produces conditioned aversions [5,11] it has not been previously demonstrated that IC injections produce conditioned aversions as well. In our experience CXM injected IC, in volumes typically used in memory inhibition studies with chickens, produces conditioned aversions comparable to those found with near fatal doses of IP injected LiCl (i.e., 0.7 M at 1% body weight for the 15 day old chick). This ability to produce aversions is probably attributable to the widespread peripheral "flow-on" documented by Woolston *et al.* [18] for IC injected CXM. They found 40% inhibition of liver protein and distended gall bladders within one hr of IC injection when using chicks of the same strain and age as ours.

There is a discrepancy between these results and the data reported by Tucker and Gibbs [17] using CXM injected intraventricularly in rats. They found that preinjection of CXM from 1–3 hr and beyond nine hr resulted in aversions being displayed for saccharin water which had been followed by LiCl. However, preinjection of CXM from 5–9 hr produced apparent memory inhibition. This memory inhibition interpretation was viewed as being consistent with the time course of CXM induced protein synthesis inhibition found by Barondes and Cohen [2]. In their study CXM injected IC in mice produced protein synthesis inhibition at 90% from 4–8 hr after injection. However, inhibition of protein synthesis was not measured for shorter intervals. Further, dosage levels were considerably higher than those used by Tucker and Gibbs. More importantly recent data demonstrate maximum synthesis inhibition in the first one or two hr in both rats [6], at the dosage used by Tucker and Gibbs, and in chickens [18], at the dosage used in the present study. These latter results are clearly not in accord with the time course for memory inhibition found using taste aversion procedures in rats [17]. Given this difficulty in time course and the absence of memory inhibition found in the present study, it would appear that an alternative explanation is needed for apparent memory inhibition effects produced by CXM in conditioned aversion studies.

The data from our experiment showing that IC injected CXM is an aversive agent and data demonstrating backward conditioning with aversive agents [1,7] provide the basis for a possible explanation. Specifically, CXM followed by LiCl might be more accurately treated as two aversive agents, capable of inducing food aversions, being injected in sequence. An aversion is formed when the first injection precedes the feeding period by an interval sufficient for its effects to dissipate and the second injection is appropriately timed to coincide with the feeding experience because illness, or a significant increment in illness, follows food consumption. An aversion is also formed when the first injection occurs shortly before the feeding period such that the onset of its conditioned aversion properties follow the feeding period and coincide with the second injection. However, when the first injection is at a pre-feeding interval such that its effects have begun, but not yet dissipated, no aversion is formed. This would be due to the fact that the illness produced by the first injection masks the illness produced by the second and consequently there is no discriminable change of internal state appropriately coincidental with the feeding experience. The exact time course for such effects would depend on the agents, their strengths, onset-offset latencies, interactions, as well as the species used. Such an interpretation is verifiable as well as consistent with the extant data and the results of the present study. At a bare minimum it seems quite clear that memory inhibition interpretations involving CXM in appetitive paradigms must be submitted with a great deal of caution.

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