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Pavlivian Conditioning to Insulin-Induced Hypoglycemia as Indicated by Glycemic Response Motor Activity and Anxiety

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PAVLIVIAN CONDITIONING TO INSULIN-INDUCED HYPOGLYCEMIA AS INDICATED BY GLYCEMIC RESPONSE, MOTOR ACTIVITY AND ANXIETY

by

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ABSTRACT

PAVLOVIAN CONDITIONING TO INSULIN-INDUCED HYPOGLYCEMIA AS INDICATED BY GLYCEMIC RESPONSE, MOTOR ACTIVITY AND ANXIETY

Jermaine Jones
Old Dominion University, 2004
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The purpose of this experiment was to demonstrate the responses to stimuli predicting the onset of hypoglycemia (HG), specifically the overt behavioral and glycemic conditioned responses to insulin-produced HG. When drug-opposite compensatory responses become associated with situational and environmental cues that predict drug administration, compensatory mechanisms can be elicited solely by drug-associated cues. Several studies have been conducted to determine the conditioned blood-glucose responses as elicited by stimuli predicting hypoglycemia. However, only a few studies have examined the behavioral conditioned responses associated with hypoglycemia. Insulin-induced HG and the elevated plus maze (EPM) procedure were utilized to determine the direction of the conditioned glycemic, motor activity and anxiety responses in rat subjects. Rodents that received insulin prior to EPM testing demonstrated significantly high levels of anxious behavior and decreased activity during the later phases of testing. Although this study was able to demonstrate the unconditioned effects of hypoglycemia on activity and anxiety, it was unable to detect the presence of a conditioned behavioral or physiological response. The level of activity and time in open arms decreased as a function of amount of testing time. Thus, habituation-related changes in open-arm time and activity levels may have prevented detection of conditioned responses as indicated by these behavioral measures.

This thesis is dedicated to B. Medford and Dr. J. Jones

ACKNOWLEDGMENTS

I credit this thesis to all those mentors whom have fostered my intellectual and personal development. I am incredibly indebted for all the guidance and knowledge you've provided which made this manuscript possible. Thank You: Dr. A. Adibi, Dr. M. Raggozzino, and Dr. Greg Fisher. I would also like to especially thank Dr. Perry M. Duncan, my research mentor of the past two years and in whose laboratory this research was conducted.

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CHAPTER I

INTRODUCTION

Hypoglycemia is an abnormal physiological state of inadequate blood glucose that can be produced by insulin administration. Hypoglycemia has been studied extensively, and the inadequate levels of the fuel substrate, glucose, are accompanied by abnormal behavior, poor cognition and affect, inc[The]luding anxiety. Conditioned responses to the onset of hypoglycemia have been studied and yielded contradictory results; while the anxiety component of this anticipation of hypoglycemia has not been investigated. Therefore this research proposed to determine the direction of the conditioned behavioral and glycemic responses in anticipation of hypoglycemia.

Compensatory Conditioning

Homeostatic adaptation to physiological disturbance is a ubiquitous characteristic of organisms. The body's internal environment must maintain certain conditions to continue proper functioning. Physiological homeostasis is essentially a corrective mechanism to ensure that internal conditions remain stable, despite varying environment conditions and disruptive intervention. This process is brought about by feedback triggered regulatory responses of the body, which include biochemical, behavioral and physiological responses.

Responsivity to a drug often decreases as a function of repeated exposure to that drug. Regulatory homeostatic mechanisms are one of three processes that mediate this occurrence known as tolerance. The existence of tolerance is seen as evidence that

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The model for this manuscript was Behavioral Neuroscience

pharmacological stimulation leads to natural, adaptive physiological responses, which counteract the drug effects (Haefely, 1986; Ramsay & Woods, 1997; Siegel & Allan, 1998). Commonly accepted homeostatic theory asserts that detection of drug-induced physiological disturbances results in compensatory action which restores the baseline state. For example, ethanol injection results in a dose-dependent decrease in body temperature (Freund, 1979; Kalant & Le, 1984). As a result, correctional mechanisms take steps to reverse ethanol-induced hypothermia and return body temperature to the optimal set point.

The pharmacological aftereffects of compensatory responding often persist after the drug is no longer present. Referred to as withdrawal symptoms, they are a frequent result of a series of drug administrations; particularly with CNS depressants and opiates (Julien, 1994). Repeated drug administration can also lead to drug-preparatory symptoms that seek to adaptively cancel the pharmacologically-induced homeostatic imbalance. However when the pharmacological trigger is not present, the conditioned preparatory symptoms produce an opposite homeostatic imbalance, which could be attenuated by the drug's administration. If the subject learns to associate amelioration of the drug preparatory symptoms with the pharmacological stimuli, this arousal can be interpreted as 'craving' for the pharmacological trigger (Hinson & Siegel, 1980).

In the Pavlovian conditioning situation, a stimulus that reliably precedes the onset of an unconditioned response, may alone become capable of invoking the response (Pavlov, 1927). Prior to any pairings an unconditional stimulus (UCS) elicits a characteristic unconditional response (UCR). The stimulus signaling the immediate onset of the unconditional stimulus is known as the conditional stimulus (CS) and through

repeated pairings with the UCS, the CS becomes capable of eliciting the response, which is now known as a conditioned response (CR). Pavlov first demonstrated pharmacological conditioning by pairing a tone with administration of apomorphine. Apomorphine induces salivation, restlessness and a disposition to regurgitate. After several pairings Pavlov noticed that the tone alone was sufficient to produce the overt symptoms of the actual drug administration (Pavlov, 1927).

In a similar manner, situational and environmental cues that predict drug administration can become associated with drug-opposite compensatory responses. Consequently drug-opposite compensatory mechanisms can be elicited solely by drug-associated cues that have become linked to the drug effects. Such conditioned compensatory responses have been observed in several investigations. For example conditioned hyperthermia has been shown to result from stimuli predicting ethanol-induced hypothermia (Crowell, Hinson & Siegel, 1981; Cunningham, 1994; Le, Poulos & Cappell, 1979; Mansfield & Cunningham, 1980).

Other conditionable physiological compensatory responses have been shown to result from ethanol-induced hyposexuality (Pinel, Pfaus & Christensen, 1988), barbiturate-induced sedation (Wahlstrom, 1968), naloxone-induced hyperalgesia (Greeley, 1987), and morphine-induced analgesia (Kayan, Woods & Mitchell, 1969; Sherman, Proctor & Strubb, 1982; Siegel & Allan, 1998; Tiffany & Baker, 1981).

Conditioned responses to a drug are not always compensatory. Several investigations have demonstrated drug-complimentary conditioned responses. For example, rats that have been repeatedly administered psychostimulating amphetamines in a specific environment often exhibit hyperactivity when placed in that same environment,

without amphetamines (Gold, Swerdlow & Koob, 1988, Tilson & Rech, 1973). Conditioned hypoglycemic responses to cues signaling insulin administration have been observed by numerous researchers (Alvarez-Buylla, 1975; Flaherty, Grigson, & Brady, 1987). It has been hypothesized that stimulant drugs produce less tolerance and an iso-directional conditioned response, while central nervous system depressants result in drug-opposite conditioned responses (Ray & Ksir, 1999).

Glycemic Control Processes

Glucose is the only fuel that the Central Nervous System (CNS) can use to perform a myriad of functions. Complete oxidation of one mole of glucose yields an energy equivalent equal to approximately 38 moles of adenosine triphosphate (ATP) (McCall, Millington & Wurtman, 1982). Because the mammalian brain does not store or synthesize glucose, its function depends upon the maintenance of adequate blood glucose levels. Carrier mediated, facilitated diffusion allows for the delivery of glucose across the blood-brain barrier, while insulin sensitive tissues require insulin-dependent recruitment of intracellular glucose transporters. If the brain does not have access to an adequate amount of glucose it leads to profound CNS dysfunction. For this reason, the CNS is given primary access to the glucose-producing carbohydrate reserve of the liver.

The liver acts to regulate blood glucose (BG) levels under the direction of two peptide hormones; insulin and glucagon. Insulin directs the liver to convert blood glucose into stores of glycogen, and glucagon basically reverses this process. Unlike fat, glycogen is considered an intermediate energy store since it can be readily hydrolyzed into glucose, an immediately available energy substrate. Insulin allows blood glucose to enter the cells

of the body via the insulin sensitive glucose transporter, GLUT-4. Glucose transport into insulin sensitive cells increases by more than thirty-fold when stimulated by insulin.

When the body detects a low level of blood glucose (hypoglycemia) the brain signals the pancreas to halt the secretion of insulin and increases the production of glucagon. The brain directs the action of the pancreas via the vagus and splanchnic nerves, experimental stimulation of these nerves has been reported to change the insulin and glucagon output of the pancreas (Daniel & Henderson, 1967; Frohman, Ezdinli & Javid, 1967). This shift in pancreatic function, along with reduced insulin sensitivity in the peripheral tissues and reduced insulin receptor gene expression, essentially reserves the remaining glucose for the CNS (Balage, Grizzard, Sornet, Simon, Dardevet & Manin, 1990; Briata et al., 1990). Although it has been found that insulin does promote glucose utilization in some parts of the brain, the CNS does not require insulin to be able to absorb and utilize glucose, therefore insulin controls the amount of glucose that is available to the CNS by regulating its access to other cells of the body (Cryer, 1981; Woods, Figlewicz-Lattemann, Schwartz & Porte, 1990).

The CNS is dependent on blood glucose levels that are replenished by nutrients (e.g., carbohydrates, amino acids, fats, proteins) being absorbed from the digestive tract. These nutrients are then converted to glucose, the energy substrate that the CNS is able to utilize. During a fasting phase, blood glucose levels are low because there is no food in the digestive tract and therefore no absorption of nutrients. If a fasting phase is prolonged long-term stores of fat cells convert triglycerides into fatty acids and glycerol that can be directly metabolized by all of the cells of the body except those of the CNS. The CNS

must rely on the relatively small carbohydrate reservoir of the liver (≅ 300 calories) while the long-term stores of triglycerides are converted into glucose.

Symptoms of Hypoglycemia

By far the most researched area of blood-brain glucose level modulation is concerned with the effects of a dearth of blood glucose (hypoglycemia). Blood glucose concentrations vary within the normal range of 80-120 mg/dl depending on the amount of physical activity and carbohydrate consumption. Hypoglycemia is diagnosed when blood glucose concentrations fall below 50 mg/dl (Smeeks & Wolf, 2001). Clinically, it is a relevant area of research since hypoglycemia is a frequent side effect of insulin therapy in individuals with insulin-dependent diabetes. Patients practicing conventional insulin therapy experience an average of one to two hypoglycemic episodes per week, and so over a period of forty years, a person could endure as many as four thousand episodes (Cryer et al. 1989).

The introduction of insulin therapy to treat patients with diabetes lead to the first detailed descriptions of the symptoms and signs of acute hypoglycemia (Fletcher & Campbell, 1922; Banting, Campbell, & Fletcher, 1923). Hypoglycemia is known to have many behavioral and cognitive correlates, most likely due to the dependence of the CNS on adequate levels of blood glucose. Subjective reports from diabetic patients who have experienced a hypoglycemic episode attest to a heightened autonomic arousal in response to the onset and early stages of hypoglycemia (Park, 2001). Most frequently reported experiences include sweating, heart palpitations and shaking (Deary, Hepburn, McLeod,

& Frier, 1993). Cryer et al. (1989) attributed these symptoms to autonomic nervous system activation in response to CNS glucose deprivation. These autonomic symptoms are thought to be produced by activation of both the parasympathetic and the sympathetic divisions of the autonomic nervous system, which leads to direct stimulation of end organs such as sweat glands, and hormonal secretions including cortisol from the adrenal cortex (Frier et al., 1981).

The adrenergic and sympathetic nervous system hyperactivity produced by hypoglycemia is thought to underlie subjective reports of anxiety during hypoglycemic episodes (Schweizer, Winokur, & Rickels, 1986). Animal models have also identified anxiety and decreased motor activity as affective symptoms of hypoglycemia. Evidence supporting this assumption was provided by Duncan and Grasso (2000) who found that ethanol, a drug with well-documented anxiolytic effects, partially relieves hypoglycemia-related anxiety as indicated by elevated-plus maze (EPM) behavior. Duncan (2001) also demonstrated that in rodent subjects hypoglycemia resulted in decreased activity measured by an automated electronic device.

Later, as hypoglycemia becomes more pronounced other symptoms such as confusion, nausea, incoordination, speech difficulty and odd behaviors emerge. Termed neuroglycopenic, these symptoms are thought to be the caused by CNS malfunction in response to glucose depletion (Deary et al. 1993; Hepburn, Patrick, Brash, Thomson, & Frier, 1991; Towler, Havin, Craft, & Cryer, 1993). Since cerebral function requires a continuous supply of glucose, cerebral glycopenia affects cognitive functioning and particularly information processing and executive decision-making. It is thought that

these two dysfunctions underlie the generation of most neuroglycopenic symptoms (Towler et al., 1993).

Compensatory Conditioning to Insulin-Induced Responses

When receptors in the pancreas detect low blood glucose levels, in order to restore physiological homeostasis negative feedback mechanisms induce the production and release of glucagon. Glucagon promotes the conversion of glycogen into glucose, where the dearth of glucose can be compensated for by the new supply brought about from glycogen.

Previous investigations into conditioned blood glucose response have yielded contradictory results. This earlier research has found that either hyperglycemic or hypoglycemic compensatory responses can result from conditioned stimuli that predict insulin injection (Woods, Makous, & Hutton, 1969; Siegel, 1975). Woods and Kulkosky (1967) identified hypoglycemia as the conditioned response to a placebo insulin administration, while other findings are contradictory. Flaherty et al. (1987) found that the relative novelty of the conditioning context influenced the directionality of the glycemic conditioned response. Their investigation concluded that a hyperglycemic CR could be expected when the conditioning context is different from the housing context, while if the conditioning context is similar to the housing environment a hypoglycemic response will result.

Duncan and Koontz (1994) most recently investigated the direction of the conditioned blood glucose response. After repeated injections 4 units/kg of insulin in a menthol odor-permeated environment, a significant compensatory, hyperglycemic conditioned response was observed when the subjects were injected with water and then

placed in the menthol environment. This finding is consistent with pharmacological tolerance theory which argues that pharmacological conditioned responses are most often antagonistic to the drug effects; hence the conditioned response should act to attenuate the drug's primary effect (Siegel, 1975). Since these animals were not conditioned in their home cages, these results agree with Flaherty's account of the factors determining the direction of BG conditioned responses.

Hypoglycemia and Anxiety

Anecdotal reports and research have identified anxiety as a symptom of early stage hypoglycemia (Deary et al., 1993). This symptom may be a secondary result of the sympathetic nervous system arousal that is thought to underlie all autonomic symptoms of hypoglycemia (Duncan & Grasso, 2000). The elevated plus maze (EPM) paradigm is a widely used methodology to measure behavioral indicators of drug anxiogenic and anxiolytic effects in rodents. Derived from the work of Montgomery (1955), the basic premise of this paradigm is that environmental novelty evokes both fear and curiosity.

The test subject is allowed to freely explore and move about the open and enclosed arms of the maze. The enclosed arms provide security, while the open arms do not. It is assumed that the open areas of the maze evoke a stronger fear response than the enclosed areas (Rodgers & Cole, 1994). This idea is supported by data that has shown that rats display more fear-related behaviors such as immobility, freezing and defecation while in the open arms. Fear-related physiological indicators, such as plasma corticosterone response are also higher with either forced or voluntary entry into open areas of the EPM (Pellow, Chopin, File, & Briley, 1985). Decreased exploration of the maze and time spent in exposed arms of the maze are taken as indicators of decreased

motor activity and increased anxiety, respectively (Rodgers, 1997). Rats treated with anti-anxiety drugs such as Valium tend to spend more time in the open arms compared to normal subjects (Treit, Menard, & Royan, 1993). An opposite effect was found on EPM behavior in subjects treated with cocaine prior to EPM testing (Paine, Jackson, & Olmstead, 2002). In a 1997 review article, Rodgers identifies numerous research studies which have identified these effects of anxiolytic and anxiogenic drugs on EPM behavior.

Duncan and Grasso (2000) utilized the EPM to demonstrate that insulin-injection resulted in increased behavioral indicators of anxiety. An insulin dose of two-units/kg resulted in decreased total entries into the arms of the maze; and reduced total time spent in open arms. Their results also suggest that anxiety and reduced activity both can be conditioned responses in anticipation of hypoglycemia, but lacked sufficient control procedures to make this conclusion without reservation.

The conditioned motor activity response is apparently unlike that of conditioned glycemic response in that previous investigation has shown that conditioned activity changes can resemble the behavioral effects of actual insulin injection. Decreased overall spontaneous motor activity accompanies the hypoglycemia produced by insulin injection and stimuli predicting insulin administration elicit similar behaviors. (Duncan & Koontz, 1994; Siegel, 1975).

In a study not utilizing the EPM Duncan and Koontz (1994) concluded that the conditioned blood glucose response was antagonistic to the effects of insulin injection, but that the conditioned motor activity response was the same as the unconditioned response. Over the course of 5 days of repeated pairings of insulin injections coupled with menthol odor, the researchers observed decreased activity levels accompanying

hypoglycemia and stimuli predicting a hypoglycemic state. After five conditioning days, water was injected into subjects that had previously received insulin. This sequence produced a significant conditioned hyperglycemic response and decreased activity.

The Current Investigation

Living organisms not only respond to survival-related events but also to the stimuli predicting such events. The only two previous investigations that have attempted to examine behavioral conditioned responses that accompany hypoglycemia, Duncan and Koontz (1994) and Siegel (1975), involved some methodological or design inadequacies. Specifically Duncan and Koontz (1994) quantified anxiety as the amount of activity, but since the two behaviors are only somewhat related, it is a less direct indicator anxiety. The current study attempts to credibly demonstrate the direction of the conditioned glycemic response, and utilize the EPM in order to observe for conditioned anxiogenic and motor activity responses to insulin-produced hypoglycemia. It is hypothesized that the conditioned response to hypoglycemia, induced by insulin injection, will be a hyperglycemic blood glucose level. The conditioned motor activity and anxiety responses were expected to mimic that of insulin injection, leading to a decrease in spontaneous motor activity and an increase in anxiety (as indicated by EPM behavior).

Subjects were randomly divided into two conditions based upon whether they were to receive intraperitoneal (IP) administrations of saline or insulin. These injections were provided once a day over the course of four conditioning days. Five minutes after drug administration the rats were tested in the EPM for 15 minutes, with the two behavioral measures being quantified for each of the three, five-minute intervals. For two

days following the conditioning days both groups received IP injections of saline prior to EPM testing, and blood glucose measurements were taken immediately after EPM testing was completed. Glucose reading were obtained using a blood sample taken from the animal's tail.

It was expected that the data from the conditioning days would indicate the presence of the unconditioned effects of hypoglycemia on anxiety and motor activity and to a lesser extent, indicate the emerging conditioned effects of hypoglycemia. For subjects who received insulin, motor activity and anxiety were expected to be respectively lower and higher during the later periods of EPM testing as hypoglycemia develops. This would result in a main effect for 5-minute period and an interaction effect between the period and the injection condition. As conditioning days progressed, as a result of anticipation of the effects of hypoglycemia, it was hypothesized that these effects on the DVs would be observed earlier in the 5-minute periods for the insulin group alone. This anticipation effect would be indicative evidence of the emergence of a conditioned effect, and would have been indicated in the analysis by a significant three-way interaction. During the testing phase after both groups received saline, it was expected to find increased indicators of anxiety and decreased activity in the EPM behavior of those subjects who had previously received insulin. Blood glucose concentrations were also expected to be significantly higher in the insulin group due to conditioned activation of BG level recovery mechanisms.

CHAPTER II

METHODS

Subjects

Eighteen naive male Spraque-Dawley strain rats, were obtained from the Old Dominion University biological sciences animal facility. The subjects were approximately 80-90 days of age and all testing procedures were approved by the Old Dominion University IACUC.

Drugs

Hypoglycemia was induced by intraperitoneal (IP) injections of 3-units/kg of Iletin insulin. Control group animals received IP saline injections of equivalent volume.

Instruments

Plasma glucose measurements were recorded using the Lifescan® One-Touch II™ glucose monitoring device manufactured by Johnson and Johnson®. A single use First Choice® testing strip is placed inside the glucometer and after a single blood drop is applied a blood glucose (BG) reading is provided. System equivalence testing demonstrated that the One-Touch II™ system provided glucose results with 96% accuracy when compared to plasma glucose reference values (Kreuger, Garg & Brink, 2003).

Behavioral measures of anxiety and motor activity were quantified using an elevated plus maze (EPM). Anxiety was quantified as the amount of time the subjects spent in the open areas of the maze, and activity as the number of entries made into each of the four arms. An entry is defined as having all four legs of the maze in a particular arm of the maze. The maze is elevated 64.7 centimeters above the floor, with each arm

measuring 48.3 cm in length, with a width of 12.7 cm. Two arms are open and two are ¾ enclosed by 40.6 cm walls. The arms of the maze are positioned such that the similar arms are directly across from one another, and 90° tangential to dissimilar arms giving the maze its characteristic "plus" design. The entire maze is constructed of wood and painted black.

Procedure

Prior to testing the subjects were handled, weighed, and placed in an adaptation box for approximately 10 minutes a day for five consecutive days. During the weeks prior to testing the subjects were individually housed with continuous access to food (Harlan Tekland rodent diet) and water. The housing environment was temperature controlled (21° *C*, ±3°) and maintained a 12 hour light/dark cycle. All experimentation was conducted between 1400 and 1700 h.

For a complete 24 hours before testing, subjects were deprived of food, but with continuous access to water. Each subject received a 3-units/kg intra-peritoneal injection of either saline or insulin. After a five-minute waiting period behavioral measures were recorded in the EPM for 15 minutes. During this time measures of time spent in open arms (TOA) and number of crossings were recorded in three 5-minute intervals. After the subject completed this procedure, food was provided.

Experimental Design

The 18 subjects were randomly divided into two groups of nine, , with one group receiving insulin and the other receiving saline injections prior to EPM testing. Subjects in the saline group also received 3 units/kg of insulin one hour following EPM testing. This procedure served as a control for the effects of repeated insulin administrations, not

associated with the EPM. An equal number of subjects from each group were tested on each day of experimentation.

The design included four conditioning days on which the subjects were given saline or insulin injections according to their group assignment. The conditioning days were followed by two testing days where both groups received an injection of saline prior to EPM testing. For the testing days only, immediately after the 15-minute testing period, blood was drawn in order to measure the blood glucose (BG) concentration. In order to obtain a blood sample the tail was pricked using a stylet. A blood drop was then placed on the testing strip inside the glucometer. Conditioning and testing sessions were all conducted with 48-hours between each session and an equal number of subjects being tested from each injection condition.

In order to examine the unconditioned effects of hypoglycemia without the interference of extensive amounts of time in the EPM and to observe BG levels before and after EPM testing, a follow-up study was performed one week after initial testing was completed. In this study the same subjects were used, the rats were maintained in their original drug conditions and behavioral measures were recorded in the same manner as during the main study. However in the follow-up study, after receiving their appropriate drug administration a 15-minute time lapse was allowed before the subjects were EPM tested for five minutes. Also during this phase, blood was drawn and BG level determined immediately before drug administration and after EPM testing.

CHAPTER III

RESULTS

Pretest

The initial experimental design included BG measurements before and after EPM testing on each conditioning and testing day. After completion of the first group of 10 subjects (five from each injection condition) it was noted that all subjects displayed high EPM indicators of anxiety, even as the conditioning days progressed. One of the primary hypotheses of the study was that significantly increased levels of anxiety would be seen in those subjects who received insulin. With the low amounts of time in open arms (TOA) by animals in the saline condition, it was nearly impossible for the insulin condition subjects to spend less time in the open arms ("floor effect") and therefore display more anxiety. It was thought that the pre and post EPM blood draw procedure, which requires a tail prick, might have caused unusually high levels of anxiety. As a result, the data from this group were not used and the testing procedure was modified to perform a post-EPM blood glucose measurement only on the two testing days.

Data Analysis

Results from the four conditioning days were analyzed using Analysis of Variance (ANOVA). A separate 4X3X2 Mixed ANOVA was performed for each of the two dependent variables (number of entries, time in open arms). The independent variables include; conditioning day (1^{st}, 2^{nd}, 3^{rd}, 4^{th}), 5-minute period (1^{st}, 2^{nd}, 3^{rd}), and injection condition (insulin, saline). Follow up analyses included 4X2 Mixed ANOVA comparisons of each dependent variable over the four conditioning days for the 3^{rd} testing period only. Tukey's HSD was performed for all post-hoc comparisons.

To analyze the data from the testing days, a 3X2X2 ANOVA was used for each of the two dependent variables, with independent variables of; 5-minute period (1^{st}, 2^{nd}, 3^{rd}), testing day (1^{st}, 2^{nd}), injection condition (insulin or saline during the conditioning phase).

Mauchly's test of sphericity was performed on all repeated measures analyses. If sphericity was found to have been violated for any particular independent variable, the Greenhouse-Geisser correction was used. The data set had neither missing data nor outliers.

Blood glucose measurements were recorded immediately after EPM testing on each of the testing days. For this dependent variable a 2X2 ANOVA with repeated measures was used with independent variables of; testing day (1^{st}, 2^{nd}), and injection condition.

Conditioning Phase: Time in Open Arms

Figures 1 through 4 present the time spent in open arms of the maze on each conditioning day by rats in each treatment condition, during each five-minute period. Although the figures show that subjects in the insulin condition spent less time in the open arms during several of the 5-minute periods a significant between-subjects main effect for the Injection Condition was not observed, $F(1,16) = 3.19$, n.s. Shown in Figure 5, there was also a decrease in the TOA across the three 5-minute periods especially for the insulin condition but again a 5-Minute Period main effect was not found although it approached significance, $F(2,15) = 3.09$, $p = .06$. ANOVA also found no significant main effect for Conditioning Day, $F(3,14) = 1.44$, n.s. No significant effects for interactions among the independent variables were found, although a significance level of $p=.06$ was determined for the Injection Condition X 5-Minute Period interaction.

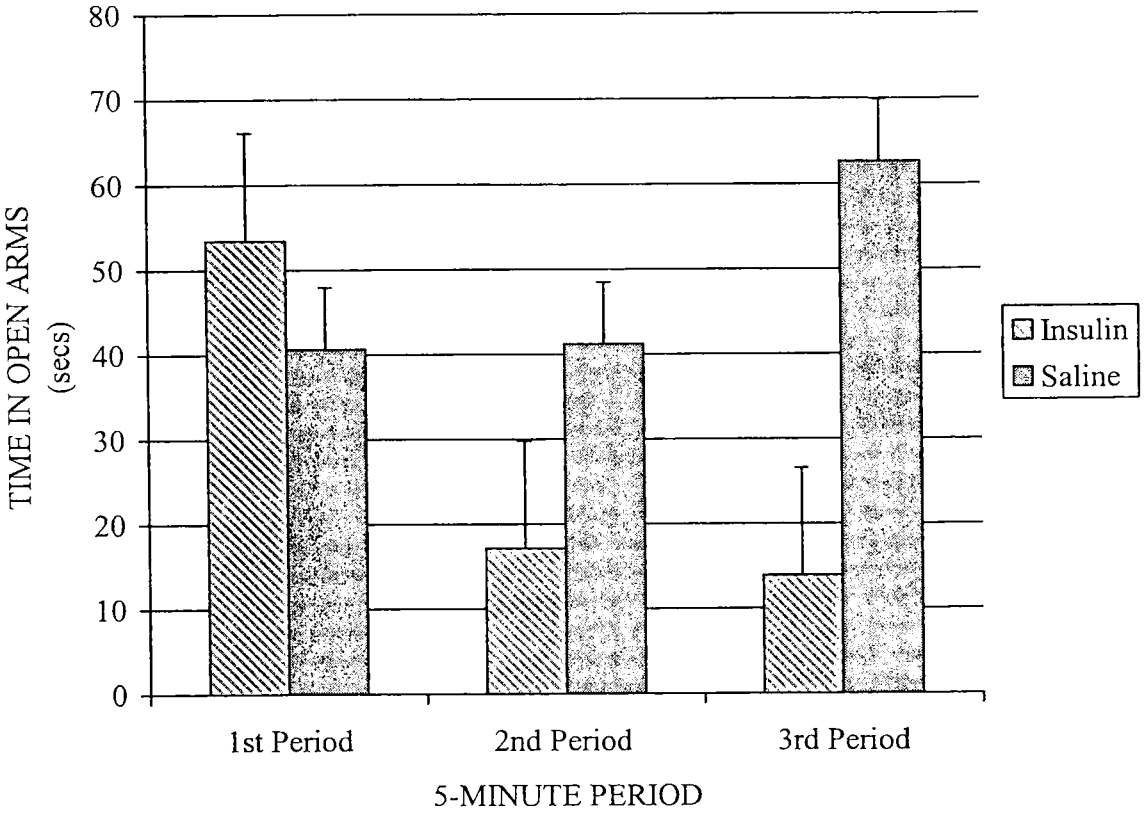

Figure 1. Mean amount of time spent in the open arms of the maze, for each 5-minute period of testing, on the 1st conditioning day.

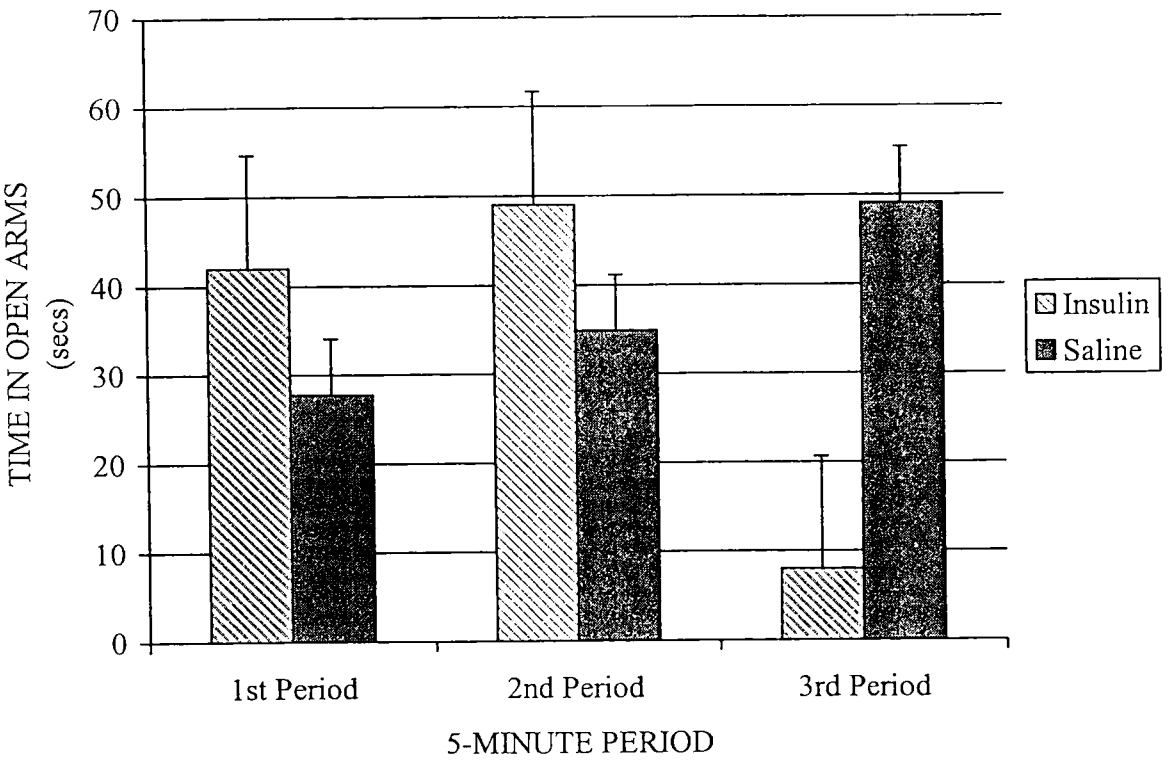

Figure 2. Mean amount of time spent in the open arms of the maze, for each 5-minute period of testing, on the 2nd conditioning day.

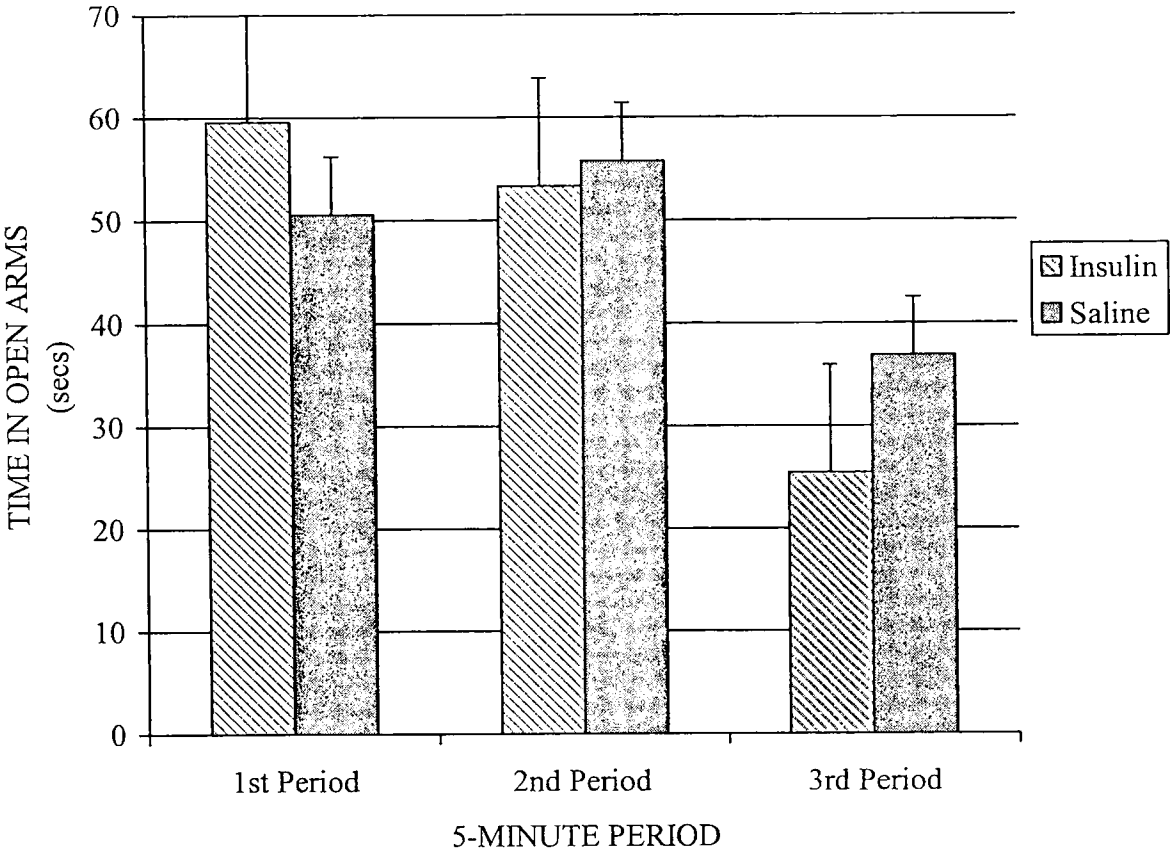

Figure 3. Mean amount of time spent in the open arms of the maze, for each 5-minute period of testing, on the 3rd conditioning day.

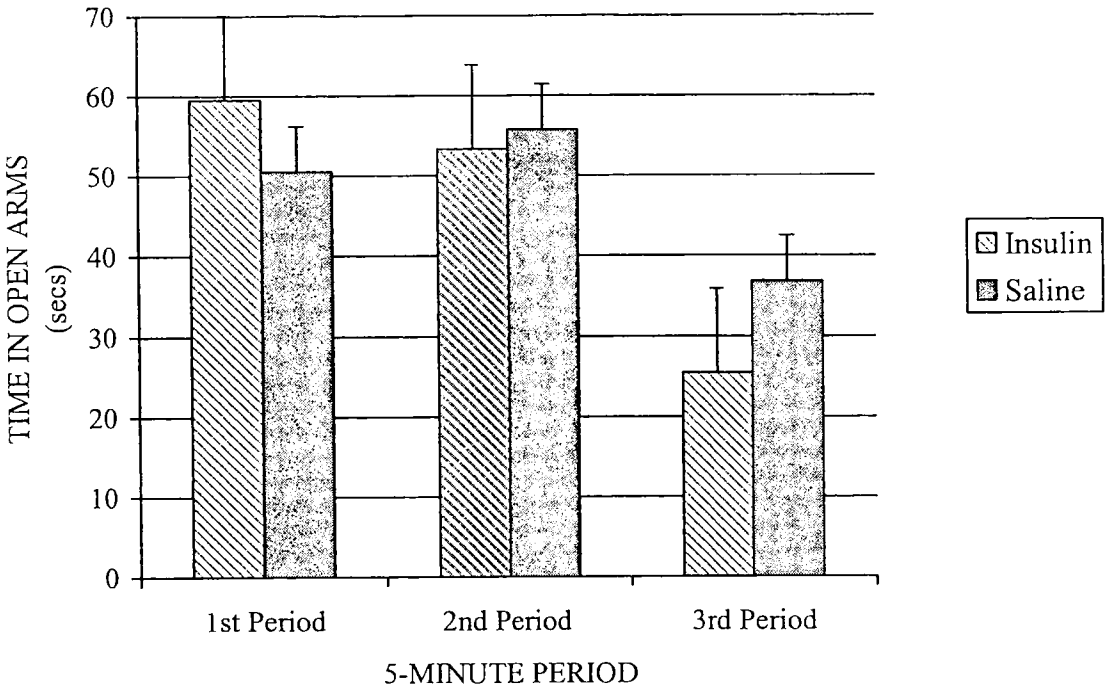

Figure 4. Mean amount of time spent in the open arms of the maze, for each 5-minute period of testing, on the 3rd conditioning day.

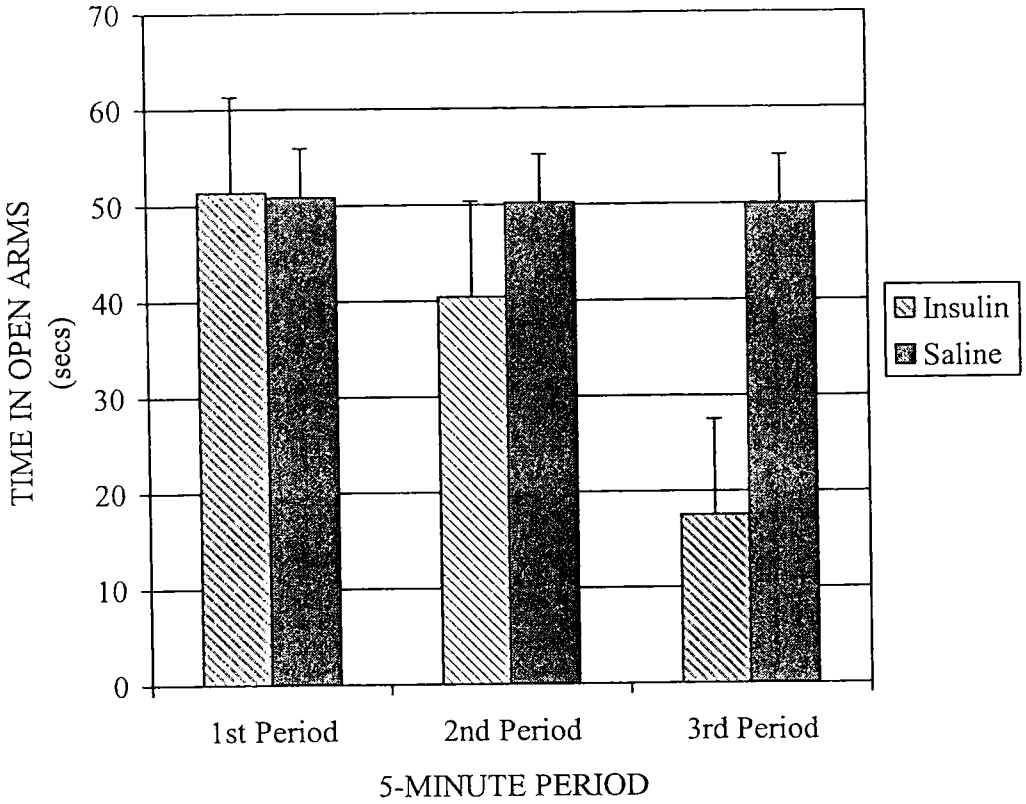

Figure 5. Amount of time spent in open areas of the EPM for each 5-minute period summed over all four conditioning days. TOA was significantly less for subjects in the insulin condition during the 3rd period, $p<.01$.

Table 1 provides a comprehensive list of the analysis results for this dependent variable.

A follow-up 4X2 Mixed ANOVA compared the time spent in open arms between the two injection conditions for the third 5-minute period alone. This analysis found a significant main effect for the Injection Condition as a result of the consistently lower mean TOA for subjects in the insulin group, $F(1,16) = 13.99$, $p < .01$. This difference can be easily observed in Figure 5. No significant main effect for Conditioning Day or interaction was found.

Conditioning Phase: Number of Entries

Figures 6-9 show the mean amount of arm entries for each 5-minute period over the four conditioning days. ANOVA found no significant main effect for the Conditioning Day, $f(3,14) = .39$, n.s. A significant main effect was revealed for the 5-Minute Period, $F(2,15) = 118.43$, $p < .001$. This main effect was a reflection of the significant decrease in the number of entries as the conditioning trial progressed. Displayed in Figure 10, this effect was present in both injection conditions. Pairwise comparisons of the 5-minute periods found a significant ($p < .001$) decrease between each of the periods illustrated in Figure 11. The 5-Minute Period X Conditioning Day interaction, Conditioning Day X Injection Condition and the three-way interactions all proved to be non-significant.

Although no significant Injection Condition main effect was found a significant Injection Condition X 5-Minute Period interaction, $F(2,15) = 9.77$, $p < .001$, was observed. This significant interaction is most likely a result of an exaggerated decrease in the number of entries for subjects in the insulin condition and is illustrated in Figure 11. A follow-up 4X2 Mixed

Table 1
Summary of the results of mixed ANOVA of time spent in open arms during the conditioning phase

Independent Variable	F Value	df	Sig.
5-Minute Period	3.10	2, 32	.059
Injection Condition	3.20	1, 16	.093
Conditioning Day	1.44	3, 48	.243
5-Minute Period * Injection Condition	3.07	2, 32	.060
5-Minute Period * Conditioning Day	1.02	6, 96	.419
Conditioning Day *Injection Condition	.91	3, 48	.445
Conditioning Day* Injection Condition* 5-Minute Period	1.03	6, 96	.411

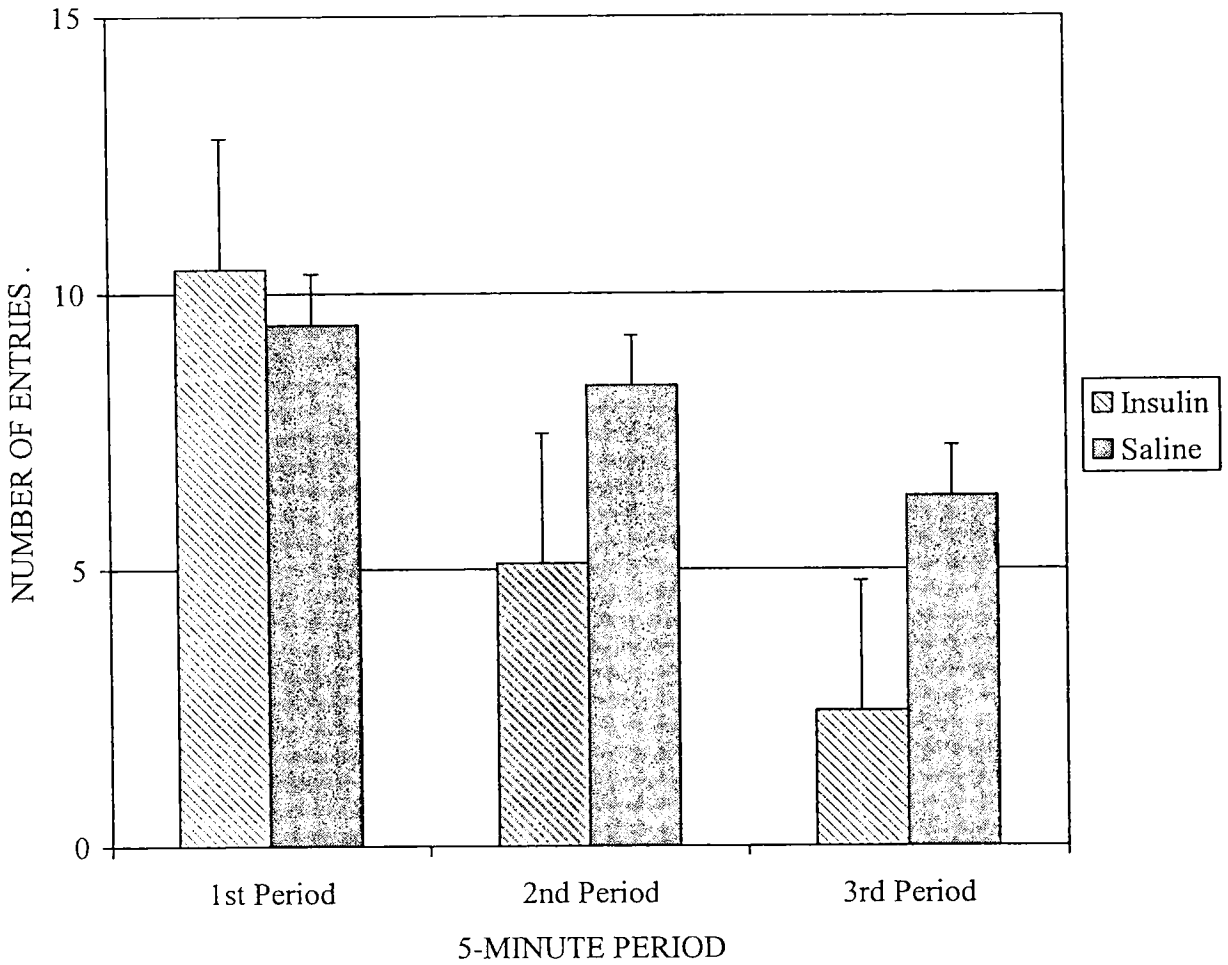

Figure 6. Mean number of entries for each 5-minute period of testing, on the 1st conditioning day.

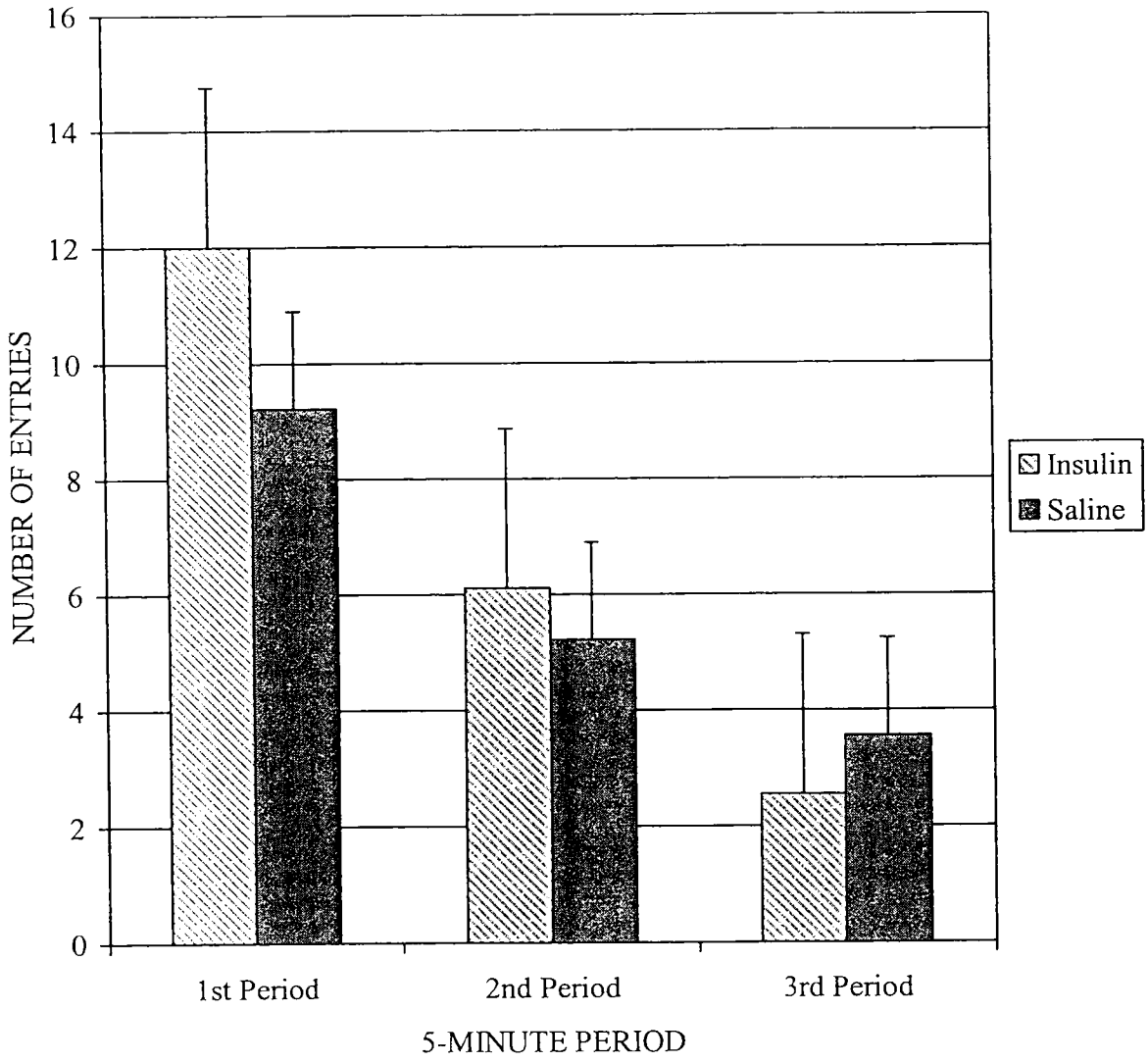

Figure 7. Mean number of entries for each 5-minute period of testing, on the 2nd conditioning day.

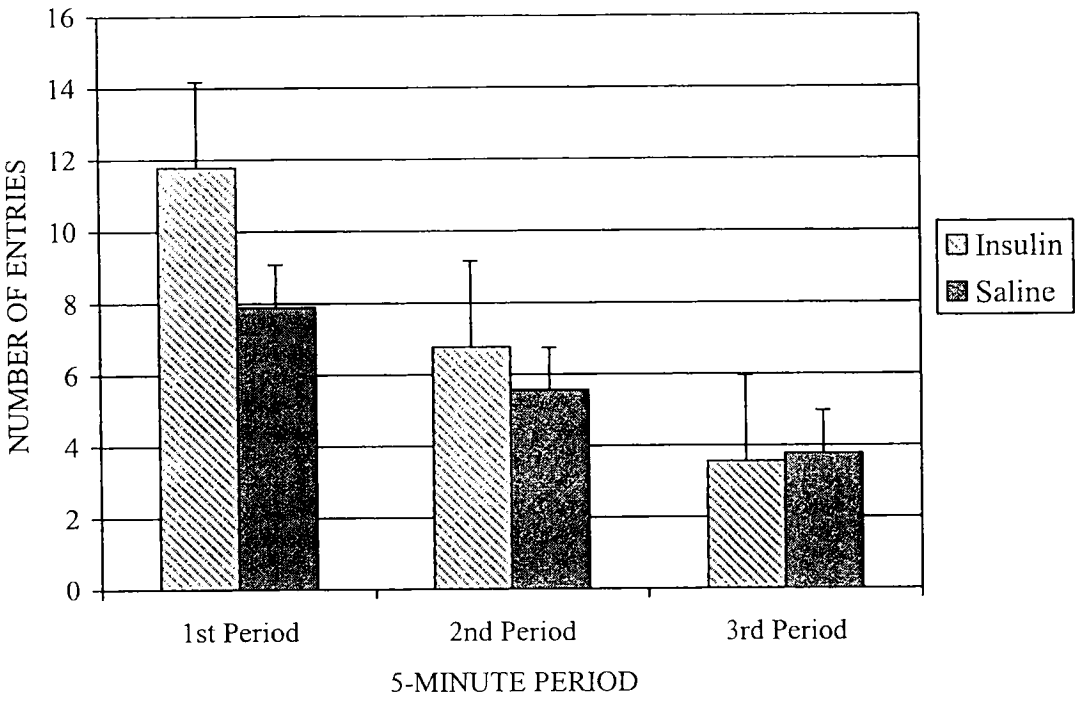

Figure 8. Mean number of entries for each 5-minute period of testing, on the 3rd conditioning day.

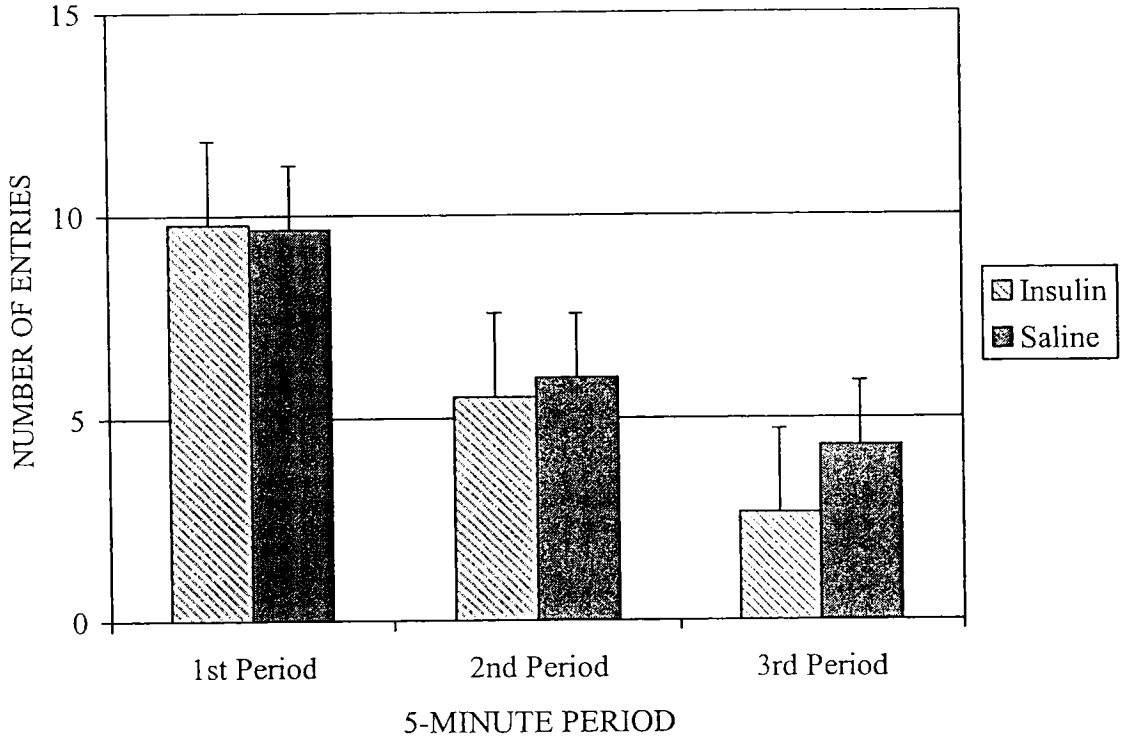

Figure 9. Mean number of entries for each 5-minute period of testing, on the 4th conditioning day.

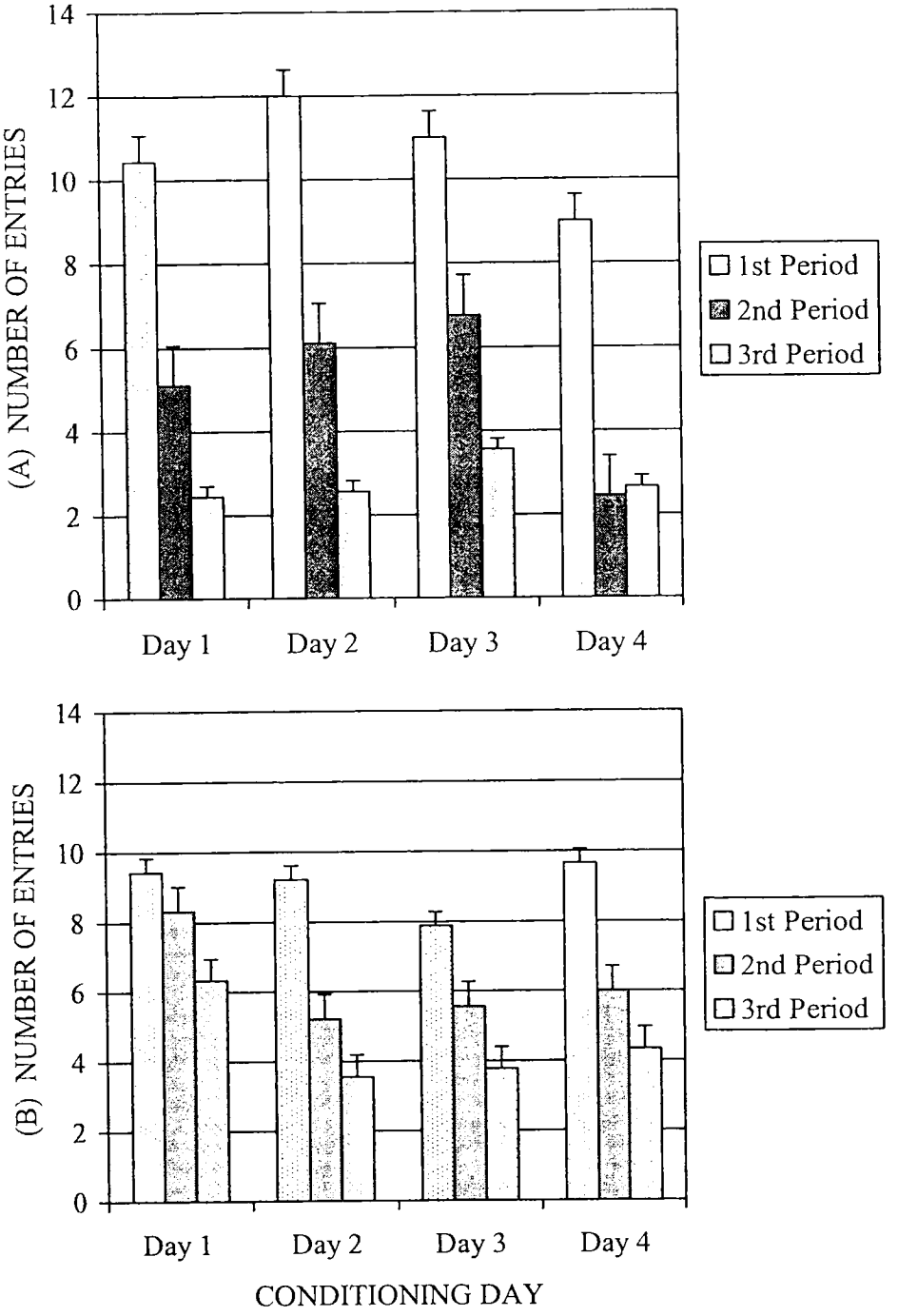

Figure 10. Mean number of crossings for subjects in the insulin (A) and saline (B) groups, for each 5-minute period on each day of conditioning.

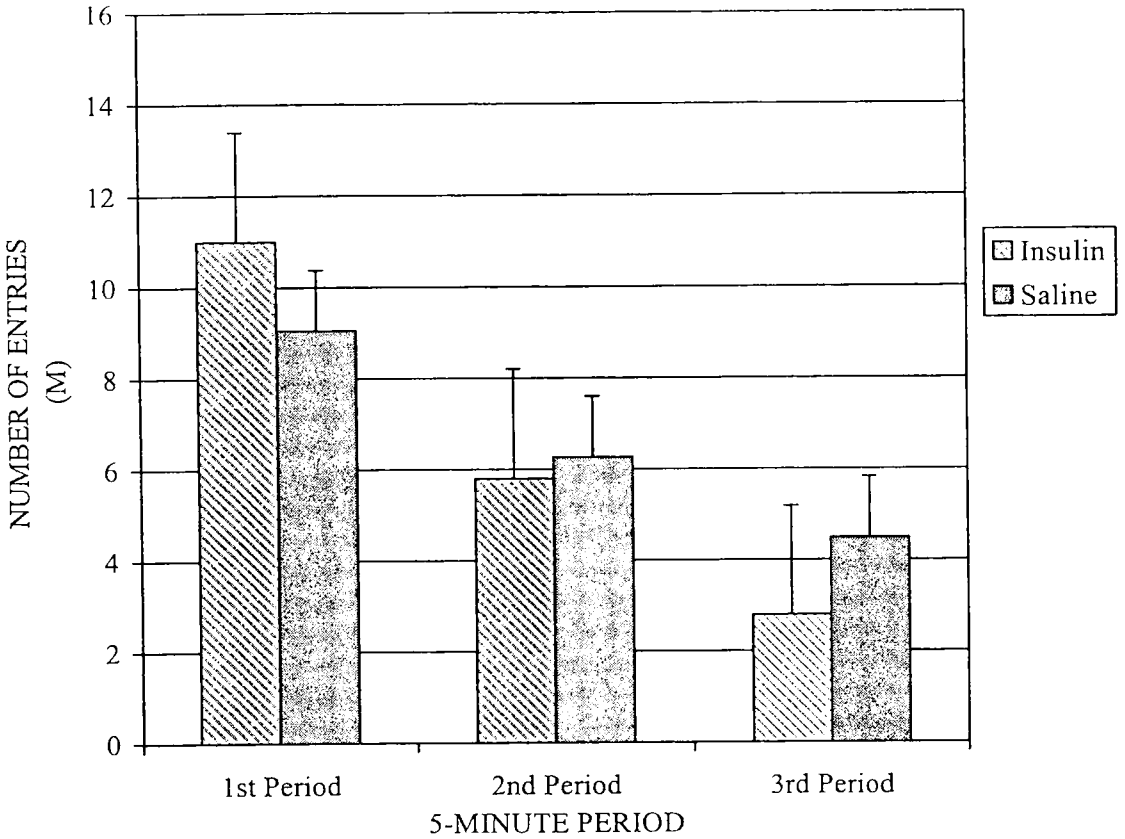

Figure 11. Number of entries for each 5-minute period summed over all four conditioning days. Activity significantly decreased between each five-minute period ($p<.001$) and was significantly lower during the 3rd period for subjects who received insulin ($p<.05$)

ANOVA compared the number of entries between the two injection conditions for the third 5-minute period only and found that the insulin group had significantly lower levels of activity during this period. $F(1, 16) = 6.32$, $p < .05$. Table 2 provides a full list of the analyses for this dependent variable.

Testing Phase: Time in Open Arms

Figures 12 and 13 illustrate the amount of time spent in open arms of the maze during each five-minute period on the two testing days. Testing day ANOVA revealed a significant main effect for the 5-Minute Period, $F(2,15) = 8.97$, $p<.01$ with Pairwise comparisons finding that the amount of time in the open arms significantly decreased between the 1st, and each of the remaining periods (Figure 14). None of the remaining main effects or interactions were significant for this dependent variable. Table 3 provides a list of testing day ANOVA results for this dependent variable. As during the conditioning days a separate analysis of the third-period alone was performed but found no significant difference in the TOA between the insulin and saline groups, $F(1,16) = .07$, n.s.

Testing Phase: Number of Entries

Figures 15 and 16 display the number of entries for each 5-minute period during the two days of testing for each injection condition. The number of entries significantly differed as a function of the 5-minute period, $F(2, 15) = 37.94$, $p < .001$. Activity significantly decreased sequentially from the 1st to 3rd five-minute periods. For the third period, 2X4 ANOVA found no significant difference between the two groups, $F(1,16) = .38$, n.s. No other main effects or interactions were significant. Table 4 provides a complete list of these testing days ANOVA results.

Table 2
Summary of the results of mixed ANOVA of the number of entries during the conditioning phase

IV	F Value	df	Sig.
5-Minute Period	118.28	2, 32	.000
Injection Condition	.02	1, 16	.889
Conditioning Day	.39	3, 48	.756
5-Minute Period * Injection Condition	9.77	2, 32	.001
5-Minute Period * Conditioning Day	.33	6, 96	.137
Conditioning Day *Injection Condition	2.66	6, 13	.058
Conditioning Day* Injection Condition* 5-Minute Period	.25	6, 96	.115

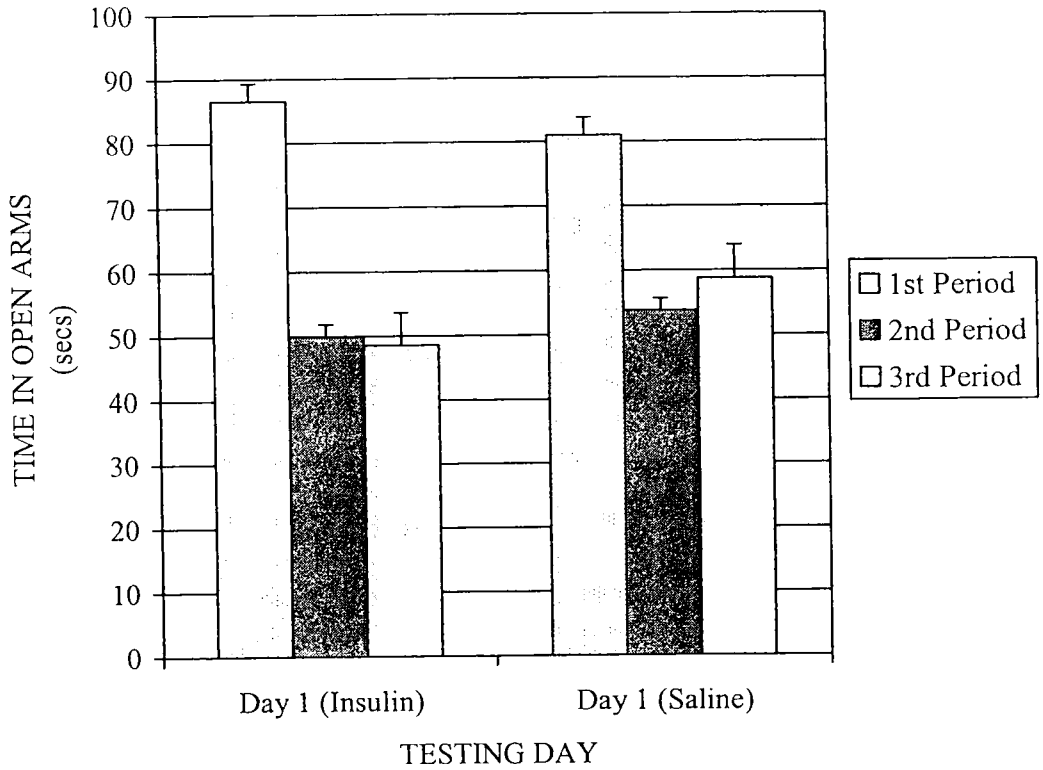

Figure 12. Mean amount of time in seconds subjects spent in open arms of the EPM, on the 1st day of testing. Time in open arms significantly decreased between the 1st and 3rd periods, $p<.001$.

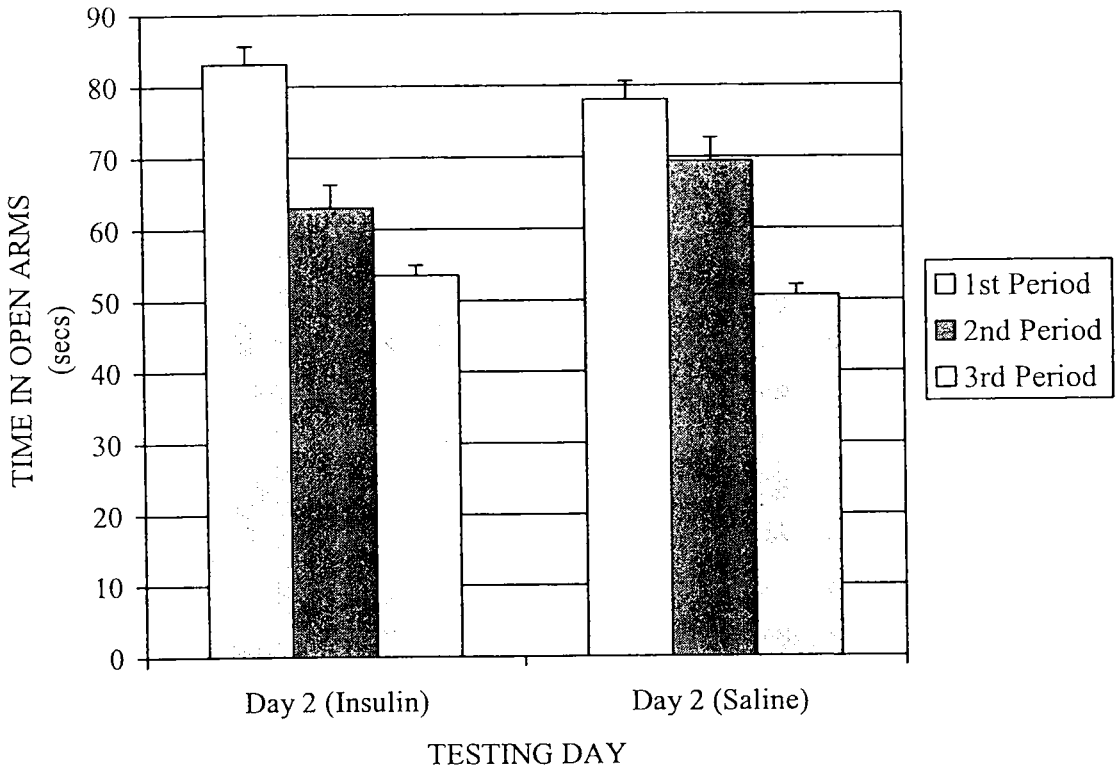

Figure 13. Mean amount of time in seconds subjects spent in open arms of the EPM, on the 2nd of testing. Time in open arms significantly decreased between the 1st, 2nd and 3rd periods, $p<.001$.

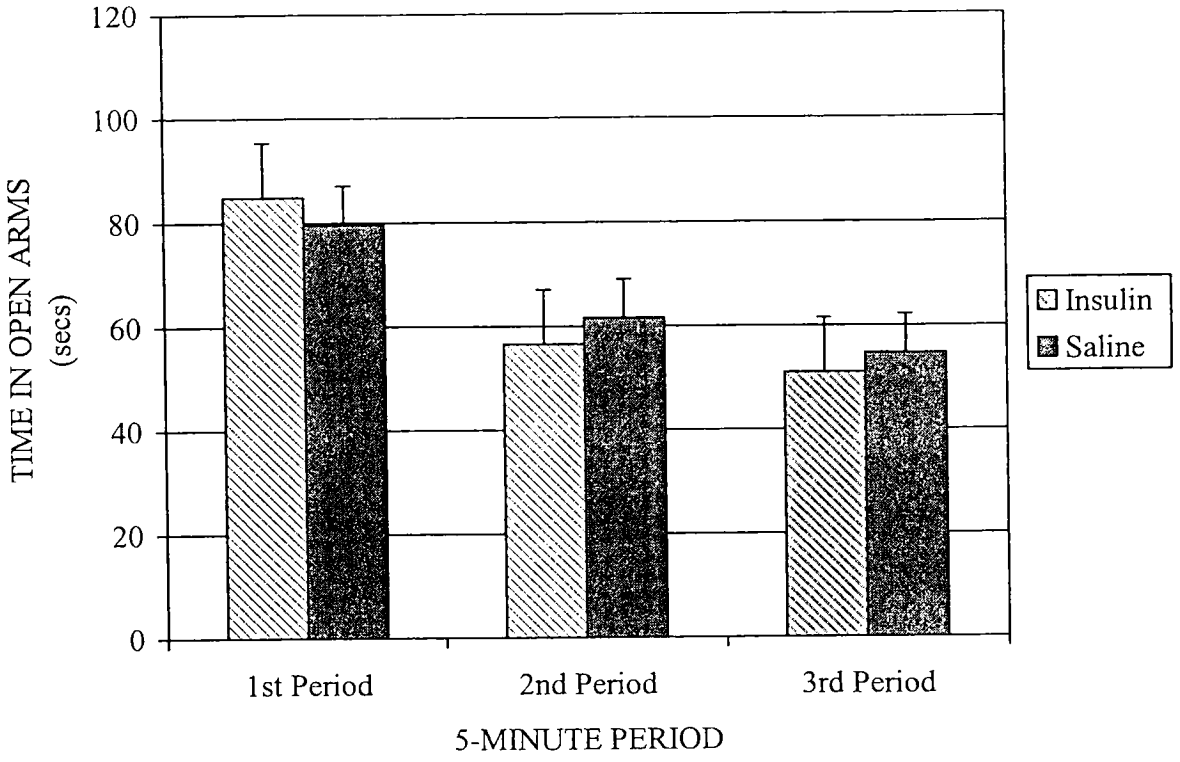

Figure 14. Amount of time spent in open areas of the maze during each 5-minute period, summed over the two days of testing. TOA significantly decreased between the 1st and 2nd ($p<.01$) and the 1st and 3rd ($p<.001$) periods. No significant difference was found between the insulin and saline groups or between the two days of testing.

Table 3
Summary of the results of mixed ANOVA of time spent in open arms during the testing phase

Independent Variable	F Value	df	Sig.
5-Minute Period	8.97	2, 32	.001
Injection Condition	.01	1, 16	.909
Testing Day	.09	1, 16	.758
5-Minute Period * Injection Condition	.29	2, 32	.749
5-Minute Period * Testing Day	.709	2, 32	.500
Testing Day *Injection Condition	.03	1, 16	.871
Testing Day* Injection Condition* 5-Minute Period	.14	2, 32	.874

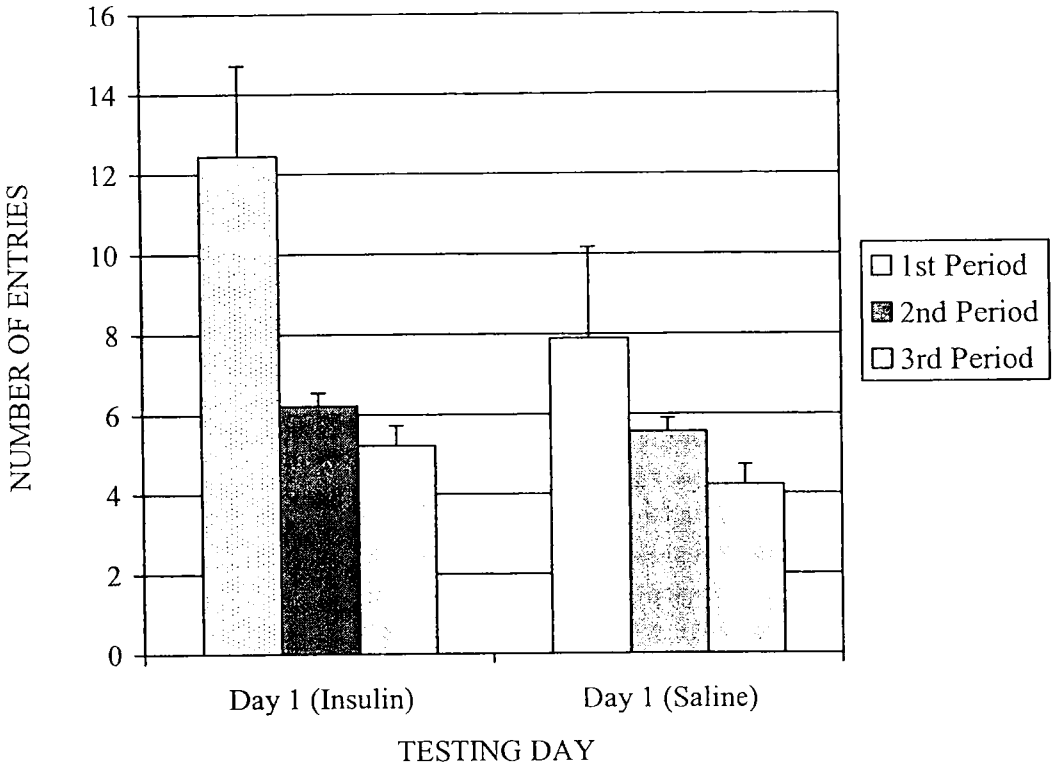

Figure 15. Mean number of entries for subjects in the insulin and saline groups during the 1st day of testing. Number of crossings significantly decreased from the 1st, 2nd and 3rd periods ($p<.001$). No significant differences were found between insulin and saline groups or between the two test days.

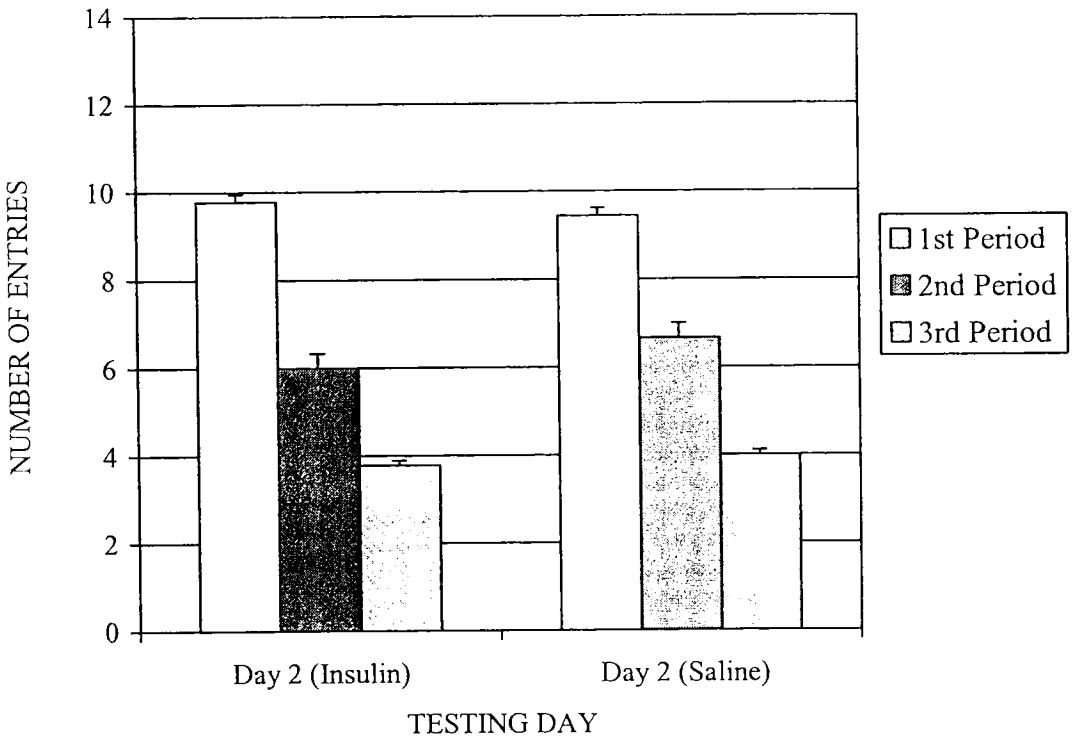

Figure 16. Mean number of entries for subjects in the insulin and saline groups during the 2nd day of testing. Number of crossings significantly decreased from the 1st, 2nd and 3rd periods ($p<.001$). No significant differences were found between insulin and saline groups or between the two test days.

Table 4
Summary of the results of mixed ANOVA of the number of entries during the testing phase

Independent Variable	F Value	df	Sig.
5-Minute Period	37.94	2, 32	.000
Injection Condition	2.50	1, 16	.133
Testing Day	.165	1, 16	.690
5-Minute Period * Injection Condition	2.02	2, 32	.150
5-Minute Period * Testing Day	.58	2, 32	.567
Testing Day *Injection Condition	2.12	1, 16	.164
Testing Day* Injection Condition* 5-Minute Period	.92	2, 32	.407

Blood Glucose Analysis

In Figure 17 the blood glucose measurements taken on each of the testing days are presented by injection condition. Glucose concentrations did not differ significantly as a function of the testing day F (1,16) = .38, n.s or injection condition F(1,16) = .01, n.s. Over the two testing days the mean BG concentrations for the insulin group was 54.45 mg/dl (SD ±5.49) and saline group 52.61 mg/dl (SD ±7.87) The interaction between these two variables was also not significant F(1, 16) = .19, n.s.

Follow-up Study

During the follow-up testing the procedure was the same as during each day of conditioning, except after receiving their appropriate drug administration a 15-minute time lapse was allowed before the subjects were EPM tested for five minutes. During this study BG readings were recorded immediately before drug administration and after EPM testing. For the analysis, the effects of the drugs on BG levels were expressed as the percentage change from the pre-injection baseline. Therefore, if the pre and post injection BG levels were the same, the percent value for the analysis was 100, and a decrease would produce a value less than 100%. Shown in Table 5, the results of Independent t-test revealed that insulin administration resulted in significantly less time spent in the open arms, t (16) =5.66, p< .001, fewer number of entries, t (16) = 2.84, p< .05, and lower blood glucose concentration, t (16) = 9.18, p< .001.

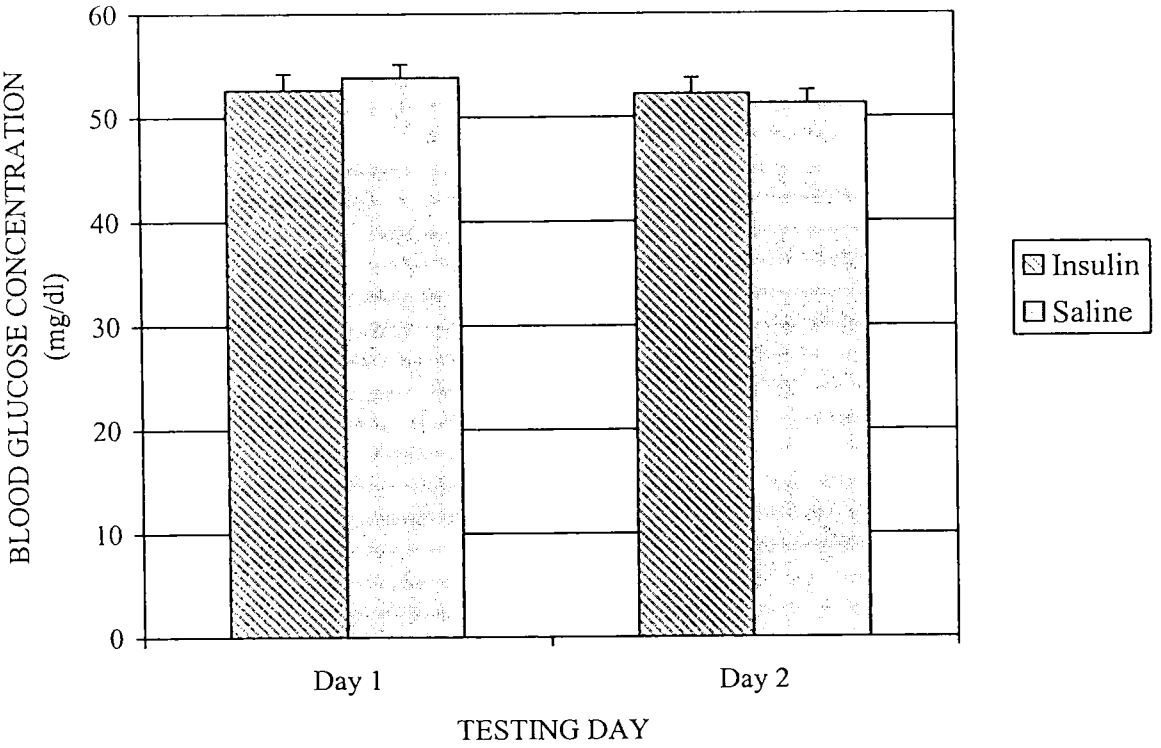

Figure 17. Blood Glucose concentration for subjects in the insulin and saline conditions. No significant difference was observed between the two groups. The interaction effect between the injection condition and testing day was also not significant.

Table 5
Means with standard deviations for the three dependent variables during follow-up analysis

Dependent Variable	Injection Condition	Mean	Std. Deviation
Time in Open Arms (secs)	Insulin Saline	54.33 160.78	±10.74 ±15.59
Number of Entries	Insulin Saline	7.44 11.22	±1.14 ±.68
Blood Glucose Conc. Change (% of baseline value of mg/dl)	Insulin Saline	68.00 115.64	±3.39 ±3.88

CHAPTER IV

CONCLUSIONS

Summary of Unconditioned Responses

Observations from the preliminary study seemed to indicate that repeatedly undergoing the blood draw procedure associated with glucose recording may have lead to increased indicators of anxiety and decreased activity. For this reason, during the primary study BG readings were only taken on testing days. One of the main objectives of the follow-up study was to ensure that during the conditioning phase, the subjects did in fact experience hypoglycemia. As shown in Table 5 the results of the follow-up study clearly demonstrate that blood glucose concentrations did significantly decrease from pre-injection baseline measurements after insulin administration only. This is a reliable effect that has been observed in much previous controlled research using both human and rodent subjects (Deary et al., 1993).

Decreased activity was observed during the later periods of each conditioning session for both the insulin and saline groups. A significant Injection Condition X 5-Minute Period interaction lead to a separate analysis of the 3rd period alone which found that this decrease in activity was greater for the insulin condition. These results indicate that during the last five minutes, the insulin treated subjects showed significantly less activity in comparison to the control. The findings from the follow-up study are also strong evidence of an unconditioned hypoglycemia-induced decrease in motor activity. The follow-up study found that insulin administration, in comparison to saline, resulted in significantly less activity when EPM testing was limited to five minutes. These conclusions agree with the research of Duncan (2001), Duncan and Koontz (1994), and

Siegel (1975) which observed that a significant decrease in activity accompanied hypoglycemia. It also supports the conclusion of Duncan and Grasso (2000) that the behavioral effects of insulin injection require roughly ten minutes to arise, as hypoglycemia develops.

The finding of no main effect of insulin administration on the time in open arms was not unexpected since the analysis summed over the five-minute periods, and the onset of hypoglycemia and its behavioral effects were only expected during the final five minutes of testing (Duncan & Grasso, 2000). The decrease observed between the testing periods during conditioning notes the strength of the unconditioned effects of hypoglycemia to decrease the amount of time spent in the open arms. However this decrease was not strong enough to produced a main effect for 5-Minute Period or Injection Condition, despite the fact that the time in open arms did not vary much between the five-minute periods for the saline group (Figure 5). The separate 3rd period analysis did however reveal that there was a significant decrease in the time spent in open arms only for those subjects who received insulin. This effect was again observed in the follow-up study which observed significantly less TOA for insulin treated subjects. Both of these observations are evidence that hypoglycemia leads to increased behavioral indicators of anxiety. As in the current study, Duncan and Grasso (2000) also utilized the EPM to find that insulin-induced hypoglycemia resulted in increased indicators of anxiety.

The two behavioral correlates of hypoglycemia found in this study and in previous research, are thought to result from increased activity of the autonomic nervous system similar to the "Fight or Flight" physiological response. Both symptoms have been

noted in humans that have experienced hypoglycemia who report anxiety, fatigue and weakness (Deary et al., 1993). Past research has also noted that the anxiogenic effects from drug treatment or other manipulation can be indicated in EPM measures (Paine etal., 2002).

Summary of Conditioned Responses

This study attempted to condition the behavioral and physiological symptoms of hypoglycemia using either the injection procedure or the EPM as a conditioned stimulus predicting the onset of hypoglycemia. Although definite behavioral and glycemic responses to insulin injection were observed, essentially no evidence of conditioned responses was detected.

It was hypothesized that the emergence of the conditioned response would be seen during the conditioning phase, if subjects in the insulin condition began to manifest the behavioral symptoms of hypoglycemia earlier in the 5-minute periods as the conditioning days progressed. The fact that this three-way interaction was not found for either dependent variable is not conclusive evidence that the conditioning did not occur, just that the anticipation of the effects of hypoglycemia, if present, did not significantly change behavior during the earlier five-minute periods. Only the results of from the testing days could conclusively determine the presence of conditioned decrease in activity or increase in indicators of anxiety.

As in the conditioning phase, during the testing phase it was observed that activity significantly decreased between the 1st, 2nd and 3rd periods for both groups. However the lack of a significant group difference or 5-Minute Period X Injection Condition interaction shows that the decrease in activity due to insulin administration found in the

last five-minute periods of the conditioning phase was not present during the testing phase. This suggests that a conditioned decrease in activity was not produced or was not observable in the data. Duncan, Alici and Woodward (2000) observed a conditioned compensatory increase in spontaneous motor activity, but their procedure consisted of 20 conditioning days, and quantified activity using an automated activity meter. In their 1994 study Duncan and Koontz were also able to condition a significant decrease in activity and while their design only employed 5 days of conditioning, it paired hypoglycemia with a menthol odor. It is possible that this current study was not able to condition a response because, due less conditioning days, the pairing between the conditioned stimuli and the unconditioned response was not made. The conditioned stimuli used in this study may not have been sufficiently salient to elicit a conditioned response. Limitations of the EPM as a paradigm for measuring motor activity is another possible reason for the failure to observe a conditioned response.

Data from the testing phase identified a significant decrease in the TOA between the 1st, 2nd and 3rd periods, but as indicated by Figure 14 and a lack of a significant 5-Minute Period X Injection Condition interaction, this decrease is found in both the insulin and saline groups and analysis of the 3rd period found no exacerbated decrease for insulin condition subjects as during the conditioning phase. This finding makes it impossible to conclusively identify a conditioned decrease in TOA (a conditioned increase in anxiety) in the insulin group. Previous research has not identified a hypoglycemia-induced conditioned anxiety response, which leaves the possibility that this behavioral response cannot be conditioned. Again, as with the activity dependent variable, the anxiety testing procedures used here may not have been sufficiently sensitive.

Since similar decreases in the amount of TOA and activity from the 1st to 3rd five-minute periods was observed in both the conditioning and testing phases it cannot be concluded that anxiety and activity responses were not conditioned. It is possible that the decreased number of entries and in the amount of time spent in open arms due to habituation during each 15-minute session, veiled the conditioned effects of hypoglycemia.

It was hypothesized that on testing day one the blood glucose measurement for subjects in the insulin condition would be significantly higher in comparison to the saline condition due to a conditioned hyperglycemic response. If this difference was present on the second day of testing, it would indicate the perseverance of the conditioned response. On the first testing day there was no significant difference in the blood glucose level between the groups. There was also no difference on the second day measurement. These results imply that neither a conditioned hyperglycemic nor hypoglycemic response was produced. Although both hypoglycemic and hyperglycemic BG responses have both been observed by previous investigations, based upon the research of Flaherty, Grigson and Brady (1987) a conditioned hyperglycemic response should have been observed in the current study since the conditioning environment of the EPM varies dramatically from the housing environment of the subjects (Duncan & Koontz, 1994; Siegel, 1975; Woods & Kulkosky, 1976).

The current investigation's failure to detect a conditioned glycemic response is most likely due to differences from procedures used in previous research in which such conditioned responses were seen. Previous research in this area has used larger insulin doses such as much of the work of Siegel (1975, 1989) who often used insulin doses of

20 units/kg or greater. Other investigations have also utilized differing methods of pairing the conditioned stimuli with the unconditioned response. The current study attempted to use the injection procedure and EPM only as conditioned stimuli, so it is possible that the neither of these was a stimulus sufficiently salient to induce a conditioned response.

Future Research

In the primary study a significant decrease in the number of crossings and time in open arms were observed between the 1st, 2nd and 3rd periods for both the insulin and saline groups. This observation was expected for the insulin condition, but is also evidence that continued time in the maze leads to a decrease in activity and TOA without experimenter manipulation. This is most likely due to the decreased exploratory value and interest the maze offers as time progressed. Since there was no 5-Minute Period X Conditioning Day interaction for either dependent variable, it can be concluded that the effects of within-session habituation did not change as a function of conditioning days.

Although the effects of the amount of time in the maze may have made it impossible to identify a conditioned behavioral response in this study, the results do not provide evidence that the effects of hypoglycemia on activity and anxiety cannot be conditioned. In an attempt to utilize the EPM as a conditioned stimulus, this subjects in this study spent much more time in the EPM in comparison to previous related investigations. Numerous other studies utilizing the EPM procedure in various fields of research from endocrinology to behavioral genetics only employ a five-minute maze testing period (Alves, Pinheiro, Motta, Landeria-Fernandez & Cruz, 2004; Bertoglio & Carobrez, 2004; Bohem, Chang, Harris & Blednov, 2003; Frye & Walf, 2004).

Research has also found the repeated sessions of EPM test may have also been problematic for this particular aims of the current study. A majority of studies have found reduced open arms exploration to be a consequence of elevated plus-maze retesting (File, 1990; Lister, 1987; Pellow et al., 1985; Taukulis & McKay, 1992). Altered baselines of anxiety measures and drug responses found in these studies suggest that, when possible, naïve subjects should be used for EPM testing. Attempting to produce a conditioned response using naïve subjects will be difficult and require a somewhat creative experimental design because conditioned subjects by definition are not naïve.

In order for future research to successfully identify the conditioning of anxiety and motor activity, it is recommended that the subjects spend less time in the EPM. In this study the decrease in activity and TOA found to occur with more time in the maze made it difficult to identify the effects of hypoglycemia.

The results of the follow-up research indicated that future research should employ a design in which the researcher waits 15 minutes after drug administration and only tests the subject in the EPM for five minutes. This should attenuate the interference of habituation observed in the current study.

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