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Honors Tutorial

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Running head: TRANSPLANTATION AND LEARNING

Abstract

It has long been acknowledged that sex differences occur in the performance of learning tasks. Specifically, it has been found that males typically outperform females in spatial learning tasks such as maze tasks. Recently, evidence has emerged which directly links sex differences in behavior to specific regions of the central nervous system (CNS). This evidence indicates that sexually dimorphic behaviors may be altered by the neonatal transplantation of opposite sex brain tissue. This research sought to extend these findings by examining the effects of neonatal transplantation of male hippocampal tissue on the spatial learning ability of adult females in three different spatial learning tasks. It was expected that such transplants would enhance the spatial learning ability of the host animals. The results indicate such enhancement may occur, however more research may be necessary to add strength to these findings. Possible explanations for the observed results are discussed.

Sex differences in rats occur different types of behavior. Sex differences have appeared in many different forms of behavior, including sexual behavior, aggression, and parental behavior (see Goy & McEwen, 1980 for a review). Another sex difference can be found in the performance of learning tasks. Numerous studies have demonstrated that males consistently outperform females on spatial learning tasks such as maze learning tasks (Barrett & Ray, 1970; Kransnoff & Weston, 1976; McNemar & Stone, 1932; Tryon, 1931). These sex differences in spatial learning ability may stem from the presence or absence of specific sex hormones, specifically the presence or absence of androgens (Goy & McEwen, 1980). For example, Stewart, Skvarenina, & Pottier (1975) demonstrated that neonatal injections of testosterone propionate (TP) improved the performance of females in the Lashley III maze to the typical level of males.

Recently, evidence has emerged which directly links such sex difference in learning behavior to sexual dimorphisms in specific regions of the central nervous system (CNS), in particular, the hippocampus. Numerous theories have been posited regarding

function of the hippocampus in the acquisition and performance of learned behaviors; although there is considerable disagreement among researchers, all of these theories suggest that this structure is important for the acquisition and storage of certain kinds of new information (Barnes, 1988; Morris, 1983; O'Keefe & Nadel, 1978; Rawlins, 1985).

There exist gross morphological sexual dimorphisms in the structure of the hippocampus (Diamond, 1984). Diamond's research has demonstrated that in male rats, the right hippocampus is significantly thicker than the portion in the left, whereas in females that balance is reversed.

Further research supporting the role of the CNS in sexually dimorphic behavior has come in the form of an experiment by Arendash and Gorski (1982) in which neonatal tissue from the medial preoptic area of males, a region critical for the expression of male sexual behavior (Christensen, Nance, & Gorski, 1977), was transplanted into the same area of female littermates. The effect of these transplants in the females was an increase of male sexual behavior as adults. This indicates that this male tissue was able to

develop functional connections in the host brain that dramatically affected the sexual behavior of the recipients as adults. This is the first instance that such transplants had been reported to produce behavioral changes in the recipient animals that were consistent with the normal functioning of the transplanted tissue (Arendash & Gorski, 1982).

Arendash and Gorski's study raises the question of whether other sexually dimorphic behaviors, such as spatial learning ability and performance, may be equally affected by the transplantation of opposite sex brain tissue. It has been well documented that hippocampal tissue taken either prenatally or early in the postnatal period is able to survive and develop functional synaptic connections in the brains of host animals (Björklund & Stenevi, 1977; Clarke, Gage, & Björklund, 1986; Stenevi, Björklund, & Svendgaard, 1976). Furthermore it is also known that such transplants have been shown to ameliorate spatial learning and memory deficits in animals who have received lesions in various areas of the hippocampus (Gage, Buzsaki, Nilsson, & Björklund, 1987). The current research, therefore, examined whether or not

and to what extent male hippocampal tissue, implanted into the same area of adult females, could affect spatial learning ability.

Method

Subjects:

Eighteen female nulliparous Sprague-Dawley rats (Crl:CD[SD]BR) purchased from Charles River Laboratories Inc. (Wilmington, MA) served as subjects for this experiment. For the duration of the experiment, subjects were housed singly in 20 x 45 x 25 cm polypropylene cages, the floors of which were covered with pine shavings. Food (Purina rat chow) and water were available ad libitum, except when specified in the experimental procedure. Subjects were housed in light (on from 0500-1900 h) and temperature (21-24 ºC) -controlled testing rooms for the duration of the experiment. Animals in this study were maintained in strict accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Richmond, and those prepared by the Committee on Care and Use of Laboratory Animal Resources , National Research Council (DHHS publication No. [NIH] 85-23, Revised, 1985). Subjects were randomly assigned to either of two experimental conditions: Fetal tissue implant (TISSUE) or sham surgery (SHAM) on day 1 of the experimental procedure.

Procedure:

Surgery took place on post natal days 110-112. All surgery was performed on subjects anesthetized with sodium pentobarbital. Donor tissue was obtained from male pups bred from donor females maintained at the University of Richmond. Males were placed with females and the presence of sperm in the vaginal smear of the female was used to determine embryonic day zero (E_0). On neonatal day 5 (day 0 = DOB) the pups were killed and their brains rapidly removed and placed in a sterile petri dish containing cold phosphate buffered saline (PBS). Dissection of the hippocampus was performed using the procedure described in Shahar, de Vellis, Vernadakis, & Haber (1989). Following dissection, the hippocampus was transferred to a test tube containing cold PBS and disassociated using a glass stirring rod to form a milky suspension. This suspension was placed in a 100 µl Hamilton syringe connected, using Silastic tubing (.10 cc/m), to a 23 gauge needle attached to the arm of the stereotaxic instrument (David Kopf Instruments). Using this apparatus, the tissue was stereotaxically infused bilaterally into the hippocampus using the coordinates -4.5 AP, ±

±3.3 ML, -2.8 DV (Paxinos & Watson, 1986) of the host animals in the TISSUE group by means of a microliter syringe pump (Harvard Apparatus). Subjects were given infusions of 2.5 µl of the tissue suspension (1.26 µl/min. for 2 min.) into each hemisphere. Subjects in the SHAM group underwent the same surgical procedure, but were infused with PBS only. A total of four animals died during or immediately after surgery, two from each group, thus reducing the number of subjects in each group from eight to six subjects.

After a sixty day recovery period, the subjects were placed on a 23 hour food deprivation schedule, with body weight measures taken daily. The subjects remained on this food deprivation schedule throughout the testing period, with each subject's body weight maintained at 80-85% of their body weight prior to being placed on the food deprivation schedule. The first session of maze testing began after all subjects had fallen below 85% of original body weight.

The first spatial learning task consisted of a multiple t-maze through which subjects were trained to run to receive a food reward. The maze consisted of a start box and a goal box connected

by a series of passages 10.5 cm wide x 10.5 cm tall (see Figure 1)

Insert Figure 1 about here

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with black lines marked on either side of the choice points. The maze was constructed entirely of plexiglass, and was mounted on a section of plywood.

Prior to testing the subjects were trained for two days to run to the goal box for food reinforcement in a straight alley, in order to familiarize the subjects with the reinforcement (~.5 g chocolate chips) and to reduce competing behavior once testing began.

During testing, each subject was given five consecutive trials per day for five days with a maximum of five minutes per trial. A subject received two scores for each trial. The first score consisted of the time elapsed from the point when the subject was placed in the start box (S) until the time it reached the goal box (G). The second score consisted of the number of total errors a subject made in traversing from the start box to the goal box. An error was counted each time the subject entered a blind alley far enough to

cross one of the choice point lines.

Two weeks after the last day of testing for the first maze task, the subjects were exposed to a second spatial task This second task consisted of a Morris water maze (Morris, 1984). The maze consisted of a cylindrical pool (105 cm in diameter, 25 cm deep) filled to a depth of 20 cm with room temperature water made opaque with the addition of powdered milk. A transparent platform (14 cm in diameter) was placed into the pool 1-2 cm below the surface of the water (see Figure 2). Subjects were placed into the

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Insert Figure 2 about here

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water at the same location for each trial and were allowed to swim freely for a maximum of one minute or until they reached the platform. At the end of each trial, the subjects were either led to or left on the platform for 15 seconds. The time to reach the platform was recorded for each subject on each trial. Testing consisted of four consecutive trials per day for five consecutive days.

After a period of two weeks, the subjects were exposed to the

third and final spatial task. The final spatial task consisted of an open-field box (122 x 122 x 55 cm tall). The box was constructed of plywood and painted gray. The floor of the box was divided into 36 separate squares (20 x 20 cm) by black lines.

Subjects were given two consecutive trials per day for five consecutive days with a maximum time of five minutes per trial. Subjects were placed in the center of the box at the beginning of each trial. Subjects received two scores for each trial. The first score consisted of the amount of time it took for the subject to reach a food reward placed approximately 56.5 cm diagonal to the subject's start position. The subject's second score consisted of the number of squares that the subject entered before reaching the food reward (recorded as squares/minute).

Histology

At the cessation of testing in the open field box, subjects were killed by an overdose of sodium pentobarbital and were perfused with formalin to fix the nervous tissue (Hart, 1976). The brains were removed and placed in a formalin solution. At a later date, the brains will be paraffin embedded (Armed Forces Institute of

Pathology, 1960) and sliced into coronal sections (50 μm). These sections will be slide mounted and stained with Thionin, for later examination to determine the extent to which the transplanted hippocampal tissue was able to survive and develop functional connections within the host.

Statistical Analysis

For the purpose of the overall analysis of the behavioral differences among the two groups in all three learning tasks, a Mann-Whitney U, the nonparametric equivalent of the t-test for independent samples was employed. In all cases an alpha level of .05 was used to determine significance.

Results

Right Angle Maze

Figure 3 displays the mean time to goal responses of the

Insert Figure 3 about here

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subjects in the two conditions (TISSUE, SHAM) on five successive days of right angle maze testing. It appears that the subjects in the tissue transplant condition took less time to traverse the maze than their sham operated counterparts across the five days of testing. The Mann-Whitney U, however, revealed no significant differences between the two groups on day 1 ($U = 16$, $p > .05$), day 2, ($U = 12$, $p > .05$), day 3 ($U = 13$, $p > .05$), day 4, ($U = 11.5$, $p > .05$), or day 5 ($U = 13$, $p > .05$).

Figure 4 displays the mean number of errors committed by the

Insert Figure 4 about here

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subjects in the two conditions in traversing from the start box to the

goal box on five consecutive days of right angle maze testing. It appears that after the first day of testing, the subjects in the tissue transplant condition committed fewer errors in traversing the maze than their sham operated counterparts. The Mann-Whitney U revealed no significant differences between the two groups on days 1-3 (U = 17, p > .05; U = 12.5, p > .05; U = 8, p > .05, respectively). However the Mann-Whitney U did reveal a significant difference between the two groups on days 4, (U = 7, p < .05), day 5 (U = 7, p < .05) with the subjects in the tissue transplant condition committing fewer errors than their sham operated counterparts.

Morris Water Maze

Figure 5 displays the mean amount of time required by the

Insert Figure 5 about here

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subjects in the two conditions to reach the hidden platform on five consecutive days of Morris water maze testing: day 1 (U = 16, p > .05), day 2, (U = 10, p > .05), day 3 (U = 11.5, p > .05), day 4, (U = 15, p > .05), day 5 (U = 15, p > .05).

Open Field

Figure 6 displays the mean amount of time required for the

Insert Figure 6 about here

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subjects in both conditions to find the food reward on five consecutive days of open field testing. On two days of testing, the subjects in the tissue transplant condition took less time to find the food reward than their sham operated counterparts. There were no significant differences between the two groups on days 1 ($U = 9$, $p > .05$), 3 ($U = 8.5$, $p > .05$), or 4 ($U = 9.5$, $p > .05$); there were, however, significant differences present between the two groups on days 2 ($U = 5$, $p < .05$) and 5 ($U = 4$, $p < .05$), with the subjects in the tissue transplant condition taking less time to find the food reward than their sham operated counterparts.

Figure 7 displays the mean amount of squares entered into per

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Insert Figure 7 about here

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minute for the subjects in both conditions on five consecutive days of open field testing. In general, the subjects in the tissue transplant condition were more active across the five days of testing than their sham operated counterparts. There were no significant differences in activity level between the two groups on days 4 ($U = 9$, $p > .05$) or 5 ($U = 8$, $p > .05$). The Mann-Whitney U, however, did reveal significant differences in activity level on days 1 ($U = 7$, $p < .05$), 2 ($U = 5$, $p < .05$), and 3 ($U = 5$, $p < .05$) with the subjects in the tissue transplant condition being more active than their sham operated counterparts.

Discussion

Although the data collected in this experiment are not conclusive, we feel there are trends apparent in the results from both the multiple t-maze and open-field box that support the original experimental hypothesis that the transplantation of male hippocampal tissue would result in improved spatial performance in recipient females. The fact that these differences have manifested themselves with only six subjects in each group attests to the robustness of this effect. Also, the lack of statistical significance where differences were expected was not entirely surprising considering first, the small number of subjects per group and second, the high variability present in both groups in all spatial tasks.

An example that illustrates the problem of the high variability typically found in the performance of spatial tasks is shown in Table 1. This table displays the average times necessary to traverse from

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Insert Table 1 about here

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the start box to the goal in the multiple t-maze for the animals in both conditions across five days. The considerable variability present in both groups on all five days is not surprising considering the findings of other studies of maze learning. These studies have consistently shown that there are significant individual differences in maze learning ability (see Tryon, 1931 for an early review). These individual differences can typically be overcome if the number of subjects in each group is large enough. However, in this experiment, with only six subjects in each condition, it should not be surprising that the typical variability found in maze learning ability should lead to statistically non-significant findings. The use of a non-parametric test for significance helps to reduce this variability, but it still does not eliminate the problem of individual differences in spatial learning ability.

Another possible variable that should be noted is the possibility of an interaction between the normal estrus cycles of the subjects and their activity levels during the testing procedures. Cyclic changes in the activity levels of female rats have been reported by some laboratories (Birke & Archer, 1975; Gray &

Cooney, 1982; Quadagno, Shryne, Anderson, & Gorski, 1972) but not by others (Bengelloun, Nelson, Zent, & Beatty, 1976; Bronstein & Hirsch, 1974; Mullenix, 1981). These inconsistencies suggest that the activational effects of these hormones on general activity levels may, at best, be minor in comparison to the effects of other task related variables (Goy & McEwen, 1980; Slob, Bogers, & van Stolk, 1981).

Despite theses obvious difficulties, the encouraging result of this study is an apparent statistical trend which indicates that the transplantation of the male hippocampal tissue did indeed improve the performance of recipient females in both the multiple t-maze and open field tasks. Given the relatively small number of subjects in each condition, these data should be viewed with caution. However, the fact that this trend is robust enough to manifest itself with only 6 subjects in each condition leads the authors to believe that an increased number of subjects in each condition would help to decrease the variability typically present in the performance of spatial tasks, producing more conclusive results.

There remains, however, the question of exactly what factors

may be mediating these behavioral changes in learning ability due to these tissue transplants. The research that has examined the sexual differentiation of the CNS and its subsequent impact on behavior would appear to indicate that these alterations of learning ability are largely due to alterations of the synaptic organization of the CNS that occurs during the critical period of prenatal and early postnatal development (Goy & McEwen, 1980; MacLusky & Naftolin, 1981, Toran-Allerand, 1978).

It is generally accepted that the pattern of the mammalian brain is inherently female or bipotential, with sexual differentiation toward masculine patterns of gonadotropin secretion and behavior largely determined by the exposure to testicular hormones during a restricted or critical phase of development (Goy & McEwen, 1980; MacLusky & Naftolin, 1981, Toran-Allerand, 1978). This hormonal mechanism is not the only factor involved in the sexual differentiation of the CNS, but there is substantial research which indicates that this early hormonal exposure does play an important role (Goy & McEwen, 1980; MacLusky & Naftolin, 1981; Toran-Allerand, 1978).

The effects of this early hormonal exposure on the structure and function of the CNS are rather extensive and diverse, and the mechanisms involved in the development of sex differences in the morphology of the CNS are largely unknown. However, there is an expanding number of such examples of sex differences in the morphology of the CNS. One such difference that has been found in the hippocampus involves the discovery of differences in its synaptic and dendritic organization which occur during development (Meyer, Ferres-Torres, & Mas, 1978). These findings are similar to the previous findings of Raisman & Field (1971) who found similar differences in the synaptic organization of the preoptic area of males and females. This dendritic organization can be considered as a morphological indicator of functional events occurring within nervous centers. This organization is known to increase during postnatal development (Padilla, 1967), and to diminish with age (Geinisman, Bondareff, & Dodge, 1977) or in such experimental conditions as functional deprivation (Valverde, 1967).

One explanation for these differences in synaptic organization in both the hippocampus as well as other structures of the CNS is

that these morphological sex differences may be an expression of the growth-promoting effects of gonadal steroids, and that these differences in neural connectivity (circuitry) may form the basis for sexual differentiation (MacLusky & Naftolin, 1981; Toran-Allerand, 1978). Evidence for this nerve growth theory has emerged in the research of Toran-Allerand (1976) in a study examining the effects of testosterone and estradiol-17ß on tissue cultures of newborn mouse preoptic area. Toran-Allerand's findings were that the addition of testosterone and estradiol-17ß to such a tissue culture resulted in an acceleration and enhancement of the proliferation of neuronal processes, characterized by extensive neural proliferation. Conversely, the reduction of these hormones produced the opposite effect, characterized by a reduction and retardation of neural outgrowth in those regions previously found to be steroid sensitive.

Such acceleration and enhancement of the development of neural circuitry may have tremendous consequences. Accelerated growth may bring about the survival of neural elements that might otherwise have been eliminated (Goy & McEwen, 1980; Toran-Allerand, 1978). To some extent, the survival of neurons may be

may be dependent on the formation of synaptic contacts, as cells that form only a limited number of synaptic contacts have been found to be eliminated during normal CNS maturation (Purves & Lichtman, 1980).

Another important consequence of gonadal steroid exposure is altered neurotransmitter function (McEwen, 1981). There is evidence to suggest that similar mechanisms may also be operating on neurotransmitter systems during development. Giulian, Pohorecky & McEwen (1977) have presented evidence which implies that prenatal hormone exposures may result in higher levels of the neurotransmitter, serotonin. The serotonergic and cholinergic systems have been directly linked to the acquisition and performance of spatial learning tasks (Nilsson, Strecker, Daszuta, & Björklund, 1988; Richter-Levin & Segal, 1989; Vanderwolf, 1987, 1988).

Thus, perhaps the observed sex differences in spatial learning ability may be due in large part to differences in neural organization and possibly neurotransmitter systems between males and females, due to early exposure to androgens during the critical period of

sexual differentiation in prenatal and early postnatal development. The effect of transplantation of opposite sex neural tissue would thus appear to be an importation of these neural and neurotransmitter differences into the brains of the host animal, which may then serve to affect behavior.

It must be noted, however, that the study of neural contributions to observed sex differences in behavior is still in its infancy and much more research into the area is necessary before any definite conclusions may be reached regarding the role of the CNS in the expression of sexually dimorphic behavior. At this time it can only be concluded that the transplantation of opposite sex brain tissue does appear to effect the expression of sexually dimorphic behavior in recipient animals, and that the available research supports the hypothesis that these effects may be mediated to a large extent by differences in the neural structure of the CNS between males and females.

In conclusion, we are encouraged by the trends that are apparent in the data collected in this experiment. We believe that, had a larger number of subjects been used in each condition, we

would have seen a facilitation of male spatial behavior in females implanted with neonatal male hippocampal tissue.

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Table 1.

Mean times-to-goal in multiple t-maze for both conditions across five days.

SHAM

	Day 1	Day 2	Day 3	Day 4	Day 5
Mean	185.72	193.07	199.37	203.27	191.30
SD	101.22	122.30	124.56	134.04	134.31
SE	41.32	49.93	50.85	54.72	54.83
Min	28.90	15.20	11.60	8.00	6.80
Max	280.40	300.00	300.00	300.00	300.00

TISSUE

	Day 1	Day 2	Day 3	Day 4	Day 5
Mean	160.57	127.50	133.30	116.00	116.50
SD	101.25	112.84	130.61	126.88	142.21
SE	41.33	46.07	53.32	51.80	58.06
Min	61.60	47.00	36.60	27.40	19.20
Max	300.00	300.00	300.00	300.00	300.00

Figure Captions

Figure 1. Overhead view of the multiple t-maze used in this experiment. (S) = Start box. (G) = Goal box. Diagram drawn to scale: 1 mm =1 cm.

Figure 2. Location of the submerged platform in the Morris water maze as well as the start position in which subjects were placed in the pool. Diagram drawn to scale: 1 mm = 1 cm.

Figure 3. Mean times for subjects to traverse the multiple t-maze to reach the goal box for both conditions across five consecutive days of testing.

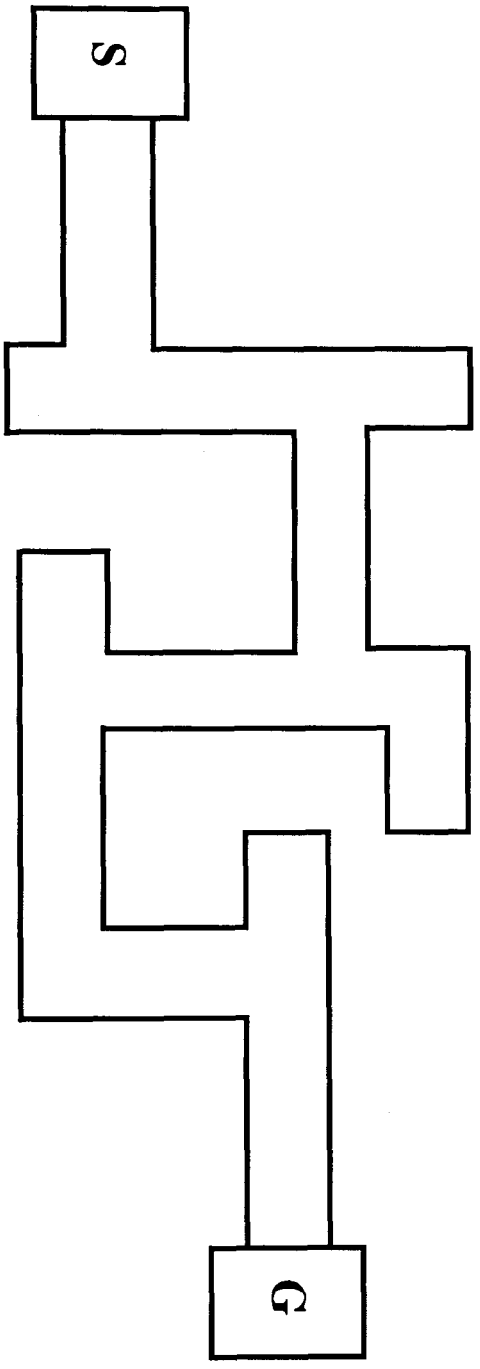
Figure 4. Mean number of errors made in traversing the multiple t-maze by subjects in both conditions over five consecutive days.

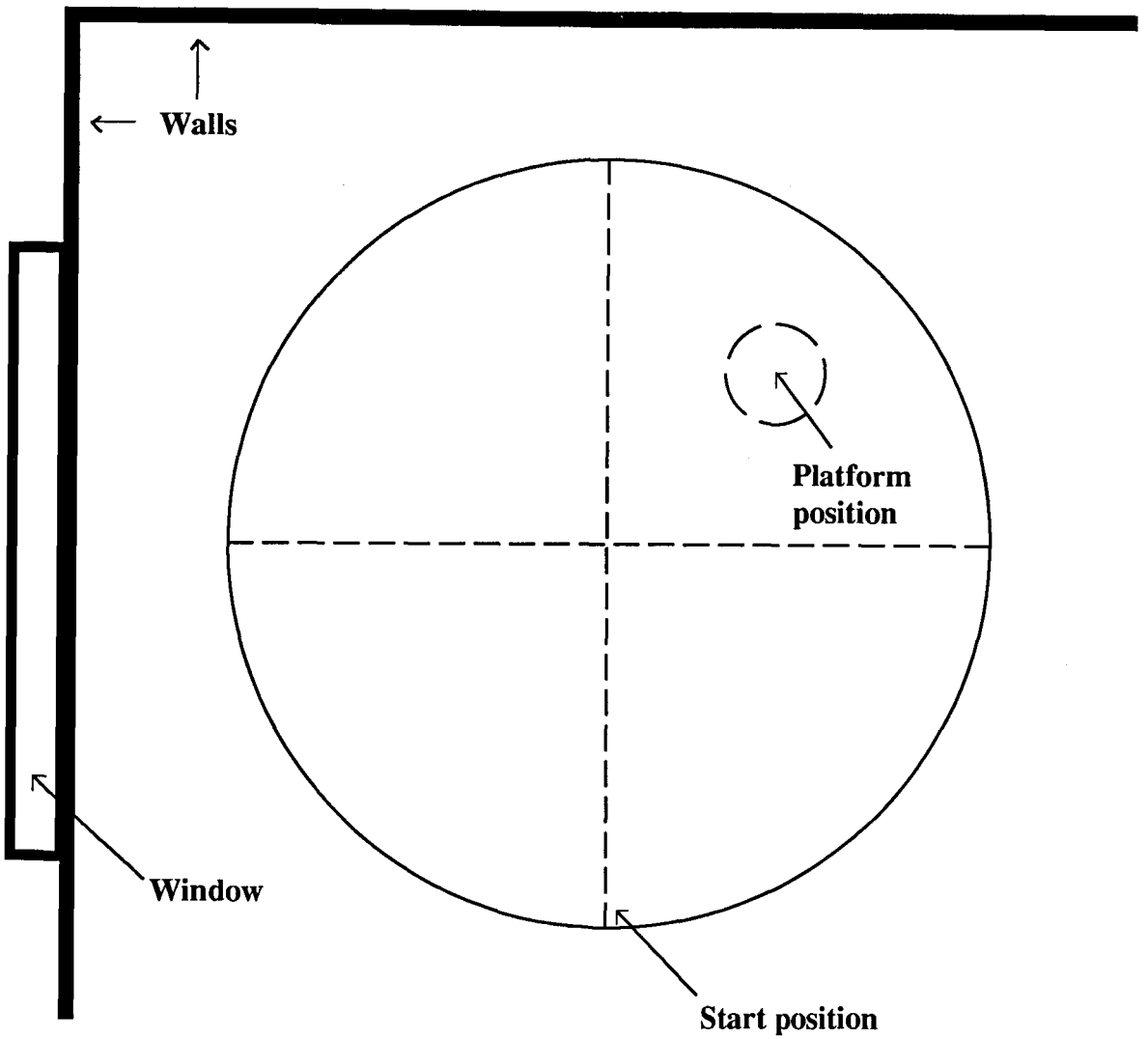
Figure 5. Mean times for subjects to reach the submerged platform in the Morris water maze for both conditions across five consecutive days of testing.

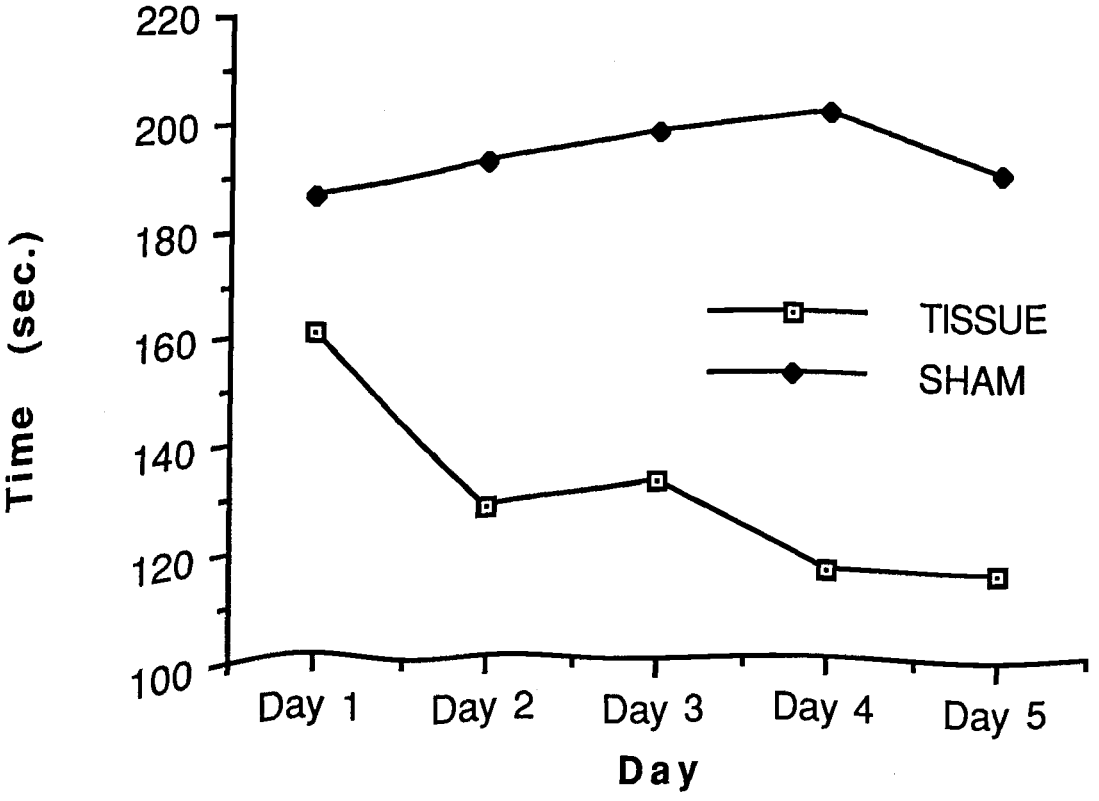
Figure 6. Mean times for subjects to find the food reward in the open field box for both conditions across five consecutive days of testing.

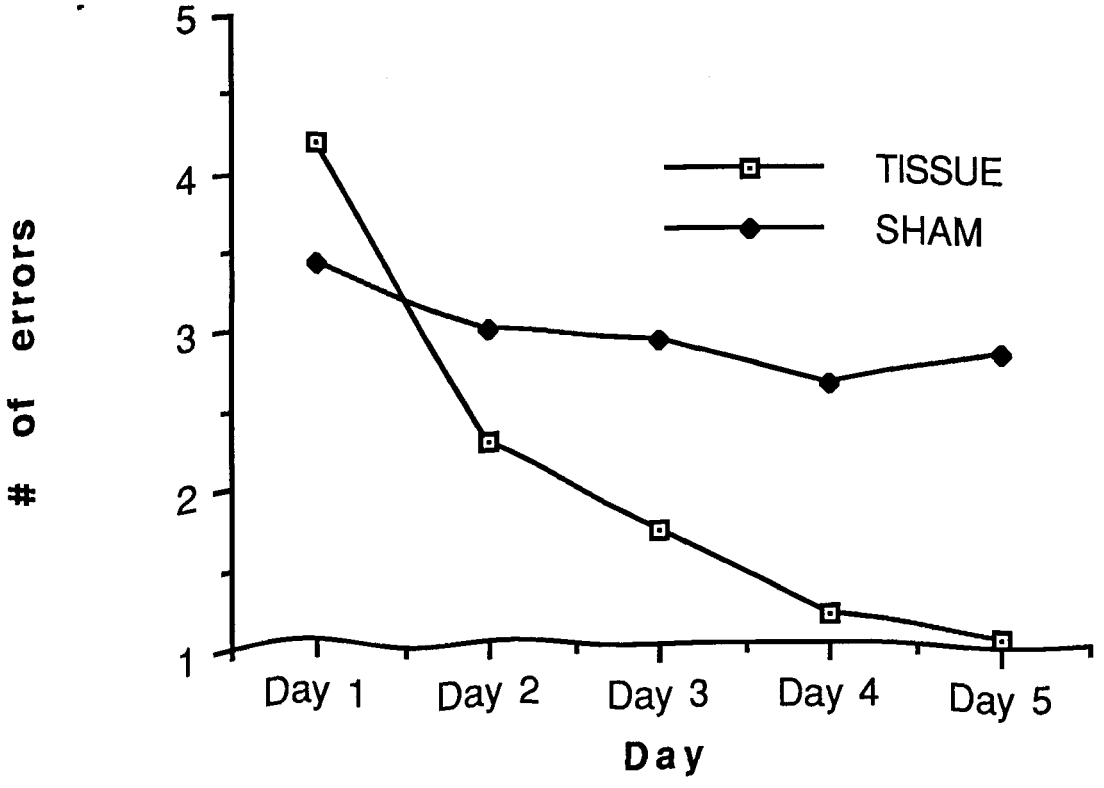
Figure 7. Mean activity levels (squares entered/minute) of subjects

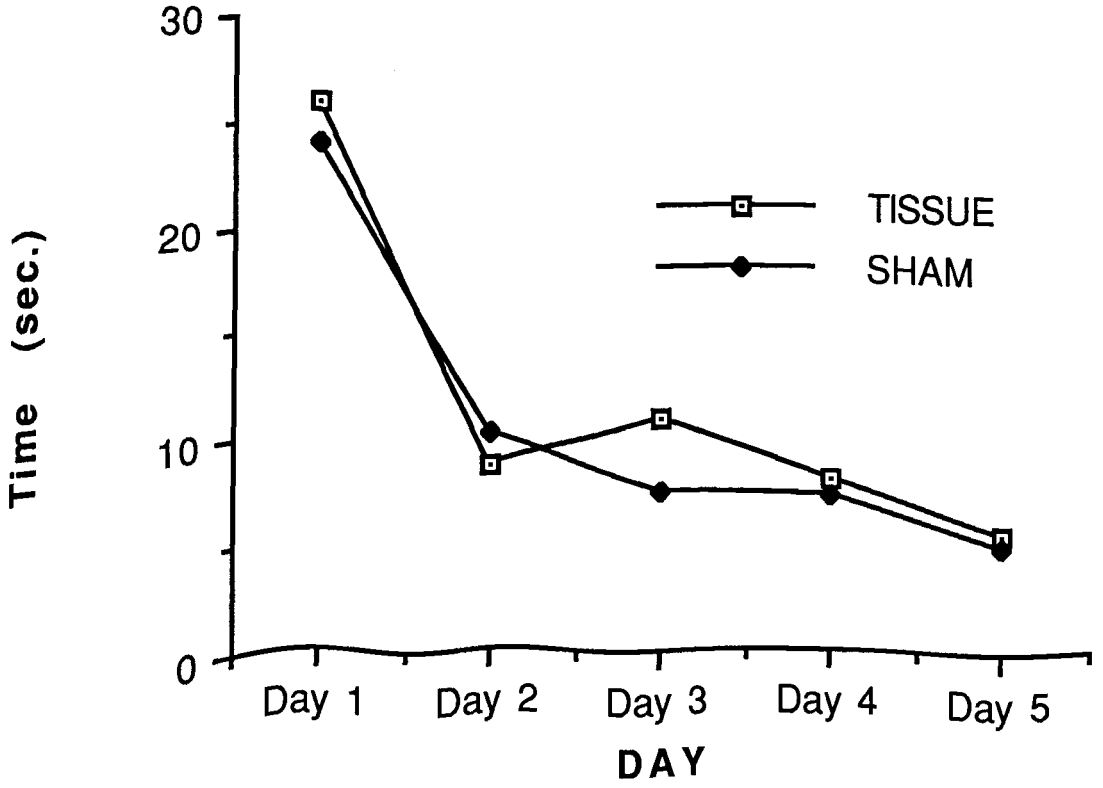
in both conditions across five days of testing in the open field box.

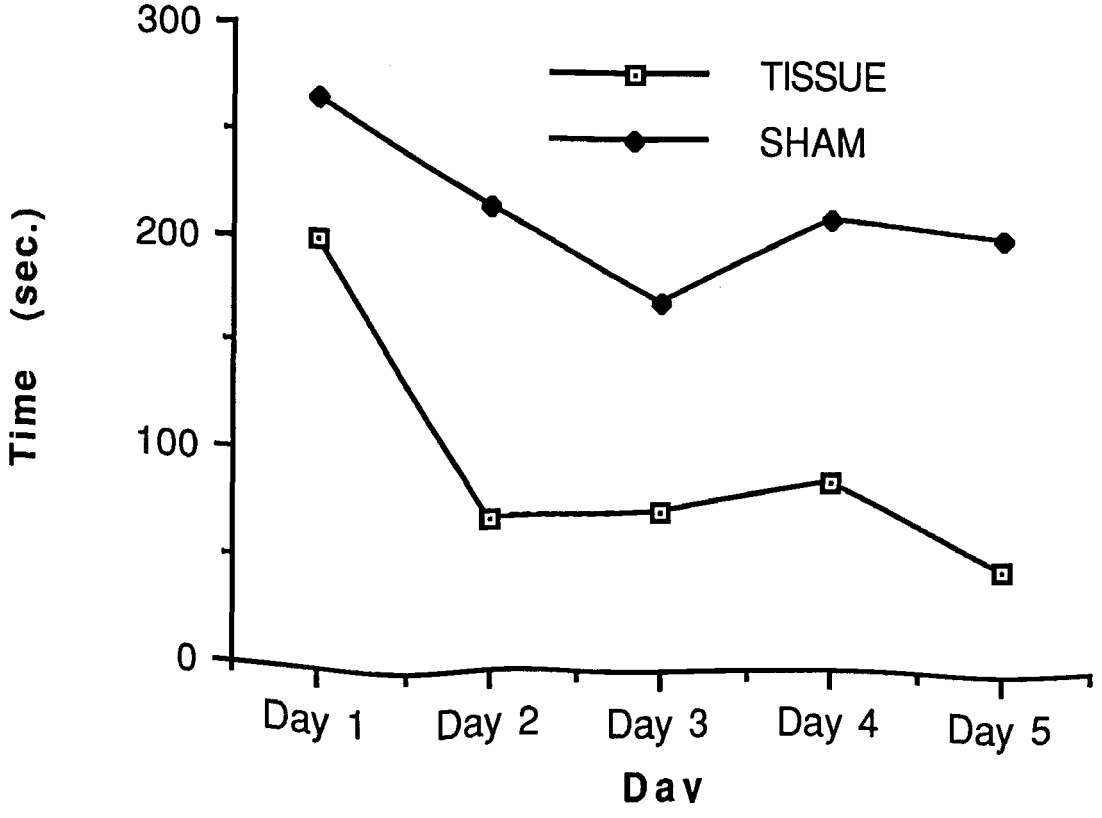

S
G

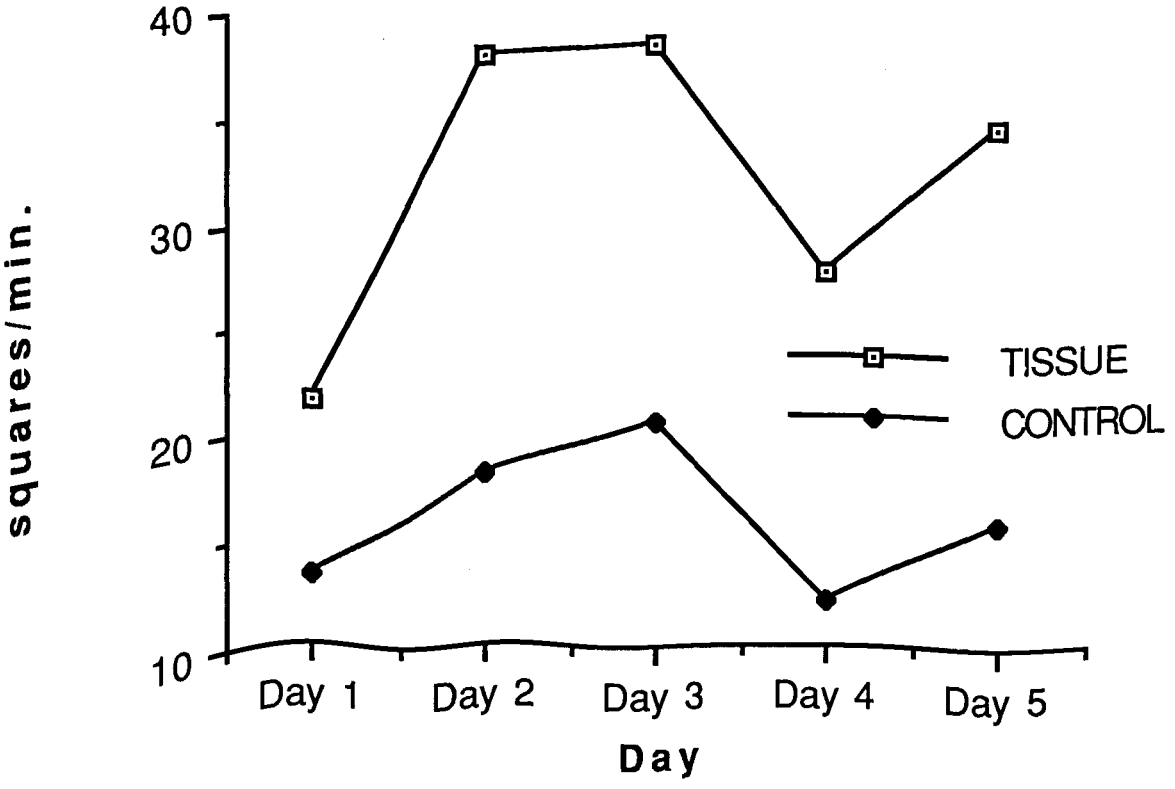

Walls
Platform position
Window
Start position


220
200
180
160
140
120
100
Time (sec.)
TISSUE
SHAM
Day 1
Day 2
Day 3
Day 4
Day 5
Day


5
4
3
2
1
# of errors
TISSUE
SHAM
Day 1
Day 2
Day 3
Day 4
Day 5
Day


30
20
10
0
Time (sec.)
TISSUE
SHAM
Day 1
Day 2
Day 3
Day 4
Day 5
DAY


300
200
100
0
Time (sec.)
TISSUE
SHAM
Day 1
Day 2
Day 3
Day 4
Day 5
Day


40
30
20
10
squares/min.
TISSUE
CONTROL
Day 1
Day 2
Day 3
Day 4
Day 5
Day