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Brain Damage and Associated Behavioral Deficits Following the Administration of L-Cysteine to Infant Rats[1]

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SHARPE, L. G., J. W. OLNEY, C. OHLENDORF, A. LYSS, M. ZIMMERMAN AND B. GALE. *Brain damage and associated behavioral deficits following the administration of L-cysteine to infant rats.* PHARMAC. BIOCHEM. BEHAV. 3(2) 291–298, 1975. – L-cysteine in doses of 1.2–1.3 mg/g, was administered to 4-day-old rats which were tested throughout development and as adults on a variety of behavioral tasks. Lesions of the type previously described from L-cysteine treatment were confirmed in several cortical and limbic structures. No neurodegenerative changes were observed in NaCl (10 mmoles/kg) controls. Surviving L-cysteine treated animals displayed no obvious impairments in motor ability or growth rate, but did show behavioral deficits when tested as adults on 3 behavioral tasks: spontaneous alternation; Lashley III maze; and pattern discrimination. Activity in the open field was significantly higher in the L-cysteine group at 20 days of age. Amphetamine, administered in doses of 1.5 to 3.0 mg/kg, had no differential effects on open-field activity in the two groups. The behavioral changes observed in L-cysteine treated animals is similar to that which has been reported in adult rats with extensive hippocampal damage.

L-cysteine Limbic lesions Behavioral deficits Learning Open-field activity Behavioral arousal Hippocampus

IT has been demonstrated that monosodium L-glutamate (MSG), when administered to infants of several species, produces acute necrosis of neurons in the inner layers of the retina [4, 29, 34, 44] as well as neurons of the arcuate nucleus of the hypothalamus [33, 37, 38, 40, 41, 50]. Further investigations have shown that this MSG-type of brain lesion can be reproduced by administering other acidic amino acids to infant mice [39]. From microelectrophoretic studies, it has been demonstrated that these acidic amino acids are neuroexcitatory in their action on CNS neurons [6]. However, unique observations were made with regard to L-cysteine. This amino acid, which is neither acidic nor excitatory, induces a more extensive degree and different pattern of brain damage involving the dorsal hippocampus, amygdala, cerebral cortex and thalamus [36,42]. The dose of L-cysteine (1.2 mg/g) required to induce this lesion pattern does not damage the retina or hypothalamus.

L-cysteine's tendency to damage the limbic system of infant animals prompted the present study. Our purpose was to investigate some of the long-term behavioral effects of the L-cysteine-type neurodegenerative syndrome by injecting infant rats with L-cysteine and testing them as adults on a variety of behavioral tasks.

METHOD

Animals

Seventeen litters of 145 male and female Sprague-Dawley rats were used. Using a split-litter technique, 100 pups were injected subcutaneously at 4 days of age with a single dose of 1.2–1.3 mg/g of L-cysteine (10 mmoles/kg, free base). L-cysteine was freshly prepared as a 10% (w/v) aqueous solution and neutralized to pH 6.9 ± 0.1 with NaOH. The control group consisted of the remaining animals in each litter which were injected with an equal

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molar dose (10 mmoles/kg) of a NaCl solution. When feasible, one male and one female per litter for both the experimental and control groups were sacrificed 24 hr after treatment by perfusion fixation so that the brains could be examined by combined light and electron microscopy as described elsewhere [35,37]. The selection of the treatment age (4 days), the L-cysteine dose (1.2–1.3 mg/g) and the posttreatment time for sacrifice (24 hr), was based on previous experiments in which over 500 infant rodents received various doses of L-cysteine and were sacrificed at different time intervals following treatment [42].

After treatment all pups were marked for individual identification and returned to their respective litters. At 21 days of age, all surviving animals were weaned and housed individually in cages 17.5 × 24 × 17.5 cm high. Unless stated otherwise, food (laboratory chow) and water were provided ad lib throughout experimentation.

Behavioral Methods

At various ages animals were run through a battery of 6 behavioral tests which were: (1) open-field activity; (2) post-amphetamine activity (1.5 mg/kg); (3) post-amphetamine activity (3.0 mg/kg); (4) spontaneous alternation; (5) Lashley III maze and (6) visual discrimination. The animals were run in a randomized sequence. A blind testing procedure was used in all instances; that is, during testing the experimenter did not know which animals belonged to the experimental or control group. Body weights were obtained at least every 5 days throughout experimentation.

Open-field activity test. The open-field apparatus, painted a flat neutral grey, consisted of a 1.2 × 1.2 m plywood floor with 0.3 m high walls. The floor was sectioned with black lines into 36 equal squares, 20.3 × 20.3 cm. The only illumination during testing was a 40 W light suspended 0.5 m above the floor of the apparatus.

The testing procedure began by placing the rat into a barrier located in one corner of the apparatus. The barrier was removed 15 sec later allowing the animal to move freely about the entire floor of the apparatus. Motor activity was determined by recording the number of squares in which the animal's head and forepaws entered during a 3 min time period. Defecation was quantified by counting the number of boluses dropped during the 3 min interval as an index of emotional reactivity [8].

A total of 23 control (saline treated) and 12 experimental (L-cysteine treated) animals were tested every 3–5 days between the ages of 7 to 75 days. All animals were tested between 1400 and 1700 hr and the floor of the apparatus was wiped with a damp cloth between trials.

Amphetamine activity tests. The open-field apparatus as well as the testing procedure described above were used. Two amphetamine activity tests were run. Initially, a total of 23 control and 17 experimental animals, ranging from 100–150 days of age, were administered a control injection of physiological saline (1.0 ml/kg i.p.) and tested 1 hr later in the open-field apparatus for 3 min. This procedure was repeated the following day except that d-amphetamine sulfate (Sigma Chemical Co.) in a dose of 1.5 mg/kg was given. The second amphetamine test followed 110 days later and the procedure was identical in every respect to the first with the exception that the dose of amphetamine was 3.0 mg/kg. Several comparisons of activity scores, both within and between groups were made, such as: control vs experimental groups; saline vs amphetamine injections; male vs female; and low dose vs high dose.

Spontaneous alternation. The apparatus consisted of a plywood T-maze painted a flat neutral grey and measured 13 cm wide throughout with walls 13 cm high. The stem and cross piece measured 60 cm long including a 15 cm start box at the base of the stem and 2 goal boxes 22 cm long at each end of the cross piece. The start and goal boxes were equipped with plywood guillotine doors, and wire mesh covered the top of the maze. Illumination was provided by a 40 W light suspended 1.0 m above the center of the maze.

The testing procedure consisted of two trials which began by placing the rat in the start box for 10 sec, after which all 3 guillotine doors were raised allowing the rat to enter either goal box. Once the choice was made, the doors were lowered and the rat was confined to the goal box for 30 sec, with the second trial following immediately. The floor of the maze was wiped with a damp cloth between trials.

A total of 25 control and 20 experimental animals at ages 45–46 days of age were tested in the T-maze. None of the animals were food deprived and all were naive prior to testing. Spontaneous alternation was defined as an animal choosing on Trial 2 the goal box opposite from that chosen on Trial 1.

Lashley III maze. This maze, described in detail by Lashley [27] was constructed of plywood with the alleys measuring 10 cm throughout and the walls 10 cm high. A 30 cm long start box and a 38 cm long goal box were positioned at the opposite ends of the maze and were equipped with guillotine doors. In an errorless run, the animal had to travel a distance of 2 m from from start to finish through the alleys requiring alternate right and left turns every 50 cm. The maze was painted a flat neutral grey and was covered with wire mesh.

The procedure for a test trial consisted of placing the rat in the goal box for 10 sec, after which both guillotine doors were raised allowing the animal to traverse the maze to the goal box. Once inside the goal box, the door was lowered and the animal was confined until it ate two 45 mg Noyes pellets that were placed in a small food cup. The next trial began immediately.

A total of 25 control and 14 experimental animals completed the task in the Lashley III maze. All animals were accustomed to a 22 hr food deprivation schedule 3 days prior to testing. Immediately after testing food was provided in the home cages for 2 hr a day. Prior to testing animals were pretrained for 2 days which consisted of eating Noyes pellets in the goal box. All animals were run 10 trials a day until 60 trials were completed. The number of errors (incorrect turns) as well as the amount of time to complete all trials were recorded. The maze was wiped with a clean cloth between trials.

Visual discrimination. The two-choice visual discrimination apparatus (painted a flat neutral grey) consisted of a 30 cm long and 12.5 cm wide plywood runway with 18 cm high walls. At the goal end of the runway were two clear plastic doors (each 5.5 cm^2) 1 cm apart. The doors were hinged at the top so that when pushed at the bottom the rat could obtain 2 Noyes pellets placed in a foodwell immediately behind the door. Either door could be blocked preventing access to food. The discriminating visual stimuli consisted of horizontal and vertical striped patterns (5.5 cm^2) made of poster board and were placed behind the

clear plastic doors so that they could be viewed from the end of the runway. Each black and white stripe measured 3 mm wide so that the overall brightness of both patterns were equal. In the runway an opaque guillotine door was placed next to the doors containing the stimulus patterns and another guillotine door made of clear plastic was placed 10 cm from the stimulus patterns.

At the beginning of the trial the rat was confined to the rear of the runway. The opaque door was raised permitting the animal to view the horizontal and vertical patterns from behind the clear plastic guillotine door. Ten sec later the clear plastic door was raised allowing the rat to make only 1 response (noncorrection technique) to either the positive or negative stimulus pattern. The rat responded correctly if it pushed the unblocked door containing the designated positive stimulus pattern and received 2 Noyes pellets. The rat responded incorrectly if it pushed the blocked door containing the designated negative stimulus and received no food. Immediately after a response was made, the trial was terminated by lowering first the opaque door and then the clear plastic guillotine door which forced the animal (without handling) to the rear of the runway to await the next trial (about 15 sec later).

A total of 10 animals from each group was randomly selected for the visual discrimination task. One animal from the control group and 2 from the experimental group died resulting in N's of 9 and 8, respectively. The ages varied from 160 to 230 days in each group. All animals were accustomed to a 22 hr food deprivation schedule as described above and were pretrained in the apparatus for 2–3 days to obtain food from both foodwells without the presence of the visual stimulus patterns. The horizontal striped pattern was designated the positive stimulus for half the animals in each group and the vertical striped pattern was the positive stimulus for the other half in each group. A total of 10 trials were run each day until a criterion of 9 out of 10 correct responses were made. The left–right position of the positive and negative stimulus pattern was predetermined from trial to trial according to a Gellermann schedule [11]. On each trial, both foodwells contained food pellets in order to eliminate the significance of odor cues in the discrimination task.

RESULTS

Mortality

Out of 100 experimental animals that received L-cysteine (1.2–1.3 mg/g) at 4 days of age, 13 were sacrificed for histological examination. Out of the remaining 87 animals, 57 died within 5 days following the injection. Eight more animals died within the next 12 days resulting in a 75 per cent mortality rate for the experimental group during the preweaning period (1–21 days of age). This is compared to a preweaning mortality rate of 30 per cent for the control group which received saline injections at 4 days of age. Very few deaths occurred during the postweaning period in either group.

The general physical condition of the surviving animals treated with L-cysteine appeared normal in every respect when compared to the saline treated controls. There were no obvious differences between the two groups in body hair appearance, locomotor ability or food and water intake. The apparent good health of the L-cysteine treated animals is reflected in the fact that increases in body weight did not differ from the control group at any age (Fig. 1).

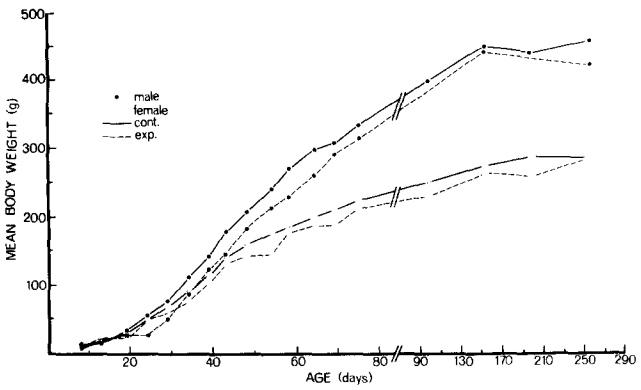

FIG. 1. A comparison of the growth rate of rats treated with 1.2–1.3 mg/g of L-cysteine at 4 days of age (exp.) with rats treated with an equal molar dose of NaCl (10 mmoles/kg) at 4 days of age (cont.). No statistical significance was found between experimental (exp.) and control (cont.) groups of the same sex. N = 4, exp. males; N = 8, exp. females; N = 10, cont. males; N = 13, cont. females.

Cysteine-induced Lesions

A total of 13 L-cysteine treated and 6 saline treated infants were sacrificed 24 hr posttreatment for histological examination. Figure 2 represents a reconstruction of a moderately severe lesion spread (shaded area) in a 5 day old animal 24 hr following a 1.2 mg/g subcutaneous dose of L-cysteine. Our experience has indicated that animals subjected to lesions of any greater extent would not have survived more than a few days after L-cysteine treatment. The lesions could be detected readily by light microscopy as bilateral, symmetrical rarefied zones in various regions of gray matter. White matter and bundle systems appeared to be uninvolved except for secondary degeneration of axons originating from acutely necrotic neuronal cell bodies in affected gray matter. The cytopathology in rarefied areas was characterized primarily by somatic swelling and pyknosis of the nuclei of several cell groups. Figure 3b depicts such a lesion affecting CA3 neurons in the hippocampus of a 5 day old animal that was mildly affected by L-cysteine treatment. The cytopathology induced in the hippocampus by L-cysteine resembles that produced in the retina or hypothalamus by monosodium glutamate (MSG) [33, 34, 35, 38], although neither the retina or hypothalamus of cysteine treated animals had lesions. The acute degenerative process was maximally evident 24 hr following L-cysteine treatment [42]. Except for a decreased number of neurons, examination of the brain within a few days after the acute stage disclosed little or no traces of the tissue reaction since destroyed neurons are phagocytized and degeneration products are efficiently eliminated [35].

Structures of the limbic system were the most vulnerable to L-cysteine since the order of sensitivity of structures involved in the L-cysteine-type lesion were: (1) hippocampus; (2) amygdala; (3) cortex and (4) thalamus. This ranking was based on the extent of lesion spread and the frequency that a given structure was involved among all animals examined. All of the 13 treated infants had some degree of degeneration within the dorsal and ventral hippocampus which included the subiculum and pyramidal cells of CA1 and CA3 of Ammon's horn. Portions of the fimbria,

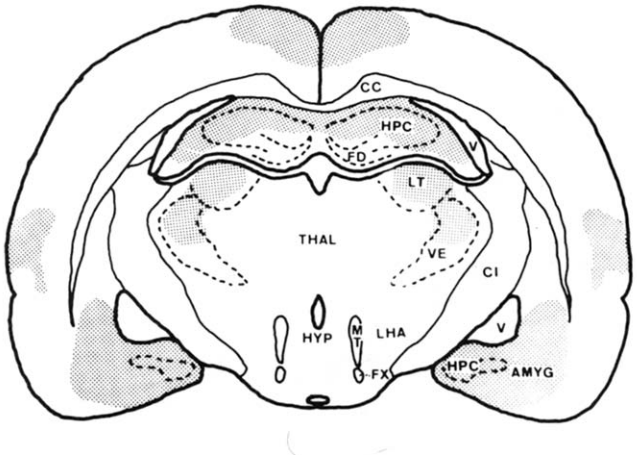

FIG. 2. Coronal section representing a moderately severe lesion spread (shaded areas) induced by a 1.2 mg/g dose of L-cysteine 24 hr following a subcutaneous administration to a 4 day old rat. Redrawn from Pellegrino and Cushman –1.6 mm from bregma [43]. AMYG = amygdala; CC = corpus callosum; CI = internal capsule; FD = denate gyrus; FX = fornix; HPC = hippocampus; HYP = hypothalamus; LHA = lateral hypothalamic area; LT = lateral nucleus of thalamus; MT = mamillothalamic tract; THAL = thalamus; V = ventricle; VE = ventral nucleus of thalamus.

the stratum oriens and stratum radiatum which convey or receive processes from these cells also exhibited degenerative changes. Interestingly, the tissues within the hippocampal region that were least affected by L-cysteine were the pyramidal cells of CA2 and CA4 of Ammon's horn and the dentate gyrus. The second most affected structure, the amygdala, had neurodegenerative changes in all nuclear groups in moderately severe cases as shown in Fig. 2. In less severe cases the lesions were confined primarily to medial aspects of the basal and cortical amygdaloid nucleus surrounding and including the ventral aspect of the hippocampus. The piriform and entorhinal cortices were rarely involved in the lesion process. Ranked third was the neocortex with spread of the lesion varying widely among animals but most frequently being confined to the inner layer of cortical tissue above the rhinal fissure and the midline area as shown in Fig. 2. The thalamic regions mostly involved in L-cysteine-induced lesions were the lateral nucleus (LT) and the dorsal part of the ventral nucleus (VE). In instances with lesions more severe than depicted in Fig. 2, the midline thalamus also became involved but the midline periventricular structures such as the habenula, stria terminalis and the paraventricular nucleus remained completely intact [42]. No neuropathology was evident in the brains of saline treated infants.

Open-field Activity and Amphetamine Tests

Since males and females did not differ in locomotor activity during the 3 min period in the open-field, their data were combined into experimental and control groups, as shown in Fig. 4. In both groups, spontaneous motor activity increased sharply during the second and third week of life which was followed by a decline in activity lasting until 30 days of age. This brief period of behavioral excitation during the development of altricial mammals has been described by Campbell and Mabry [3]. The peak activity of 20 days of age in the L-cysteine treated animals was significantly higher than that of the saline controls at the same age ($p<0.02$, t test). The L-cysteine group was also, on the average, slightly more active than controls after 45 days of age, but these differences were not statistically significant. The number of boluses dropped during the open-field testing was no different between the two groups at any age, reflecting no difference in emotional reactivity.

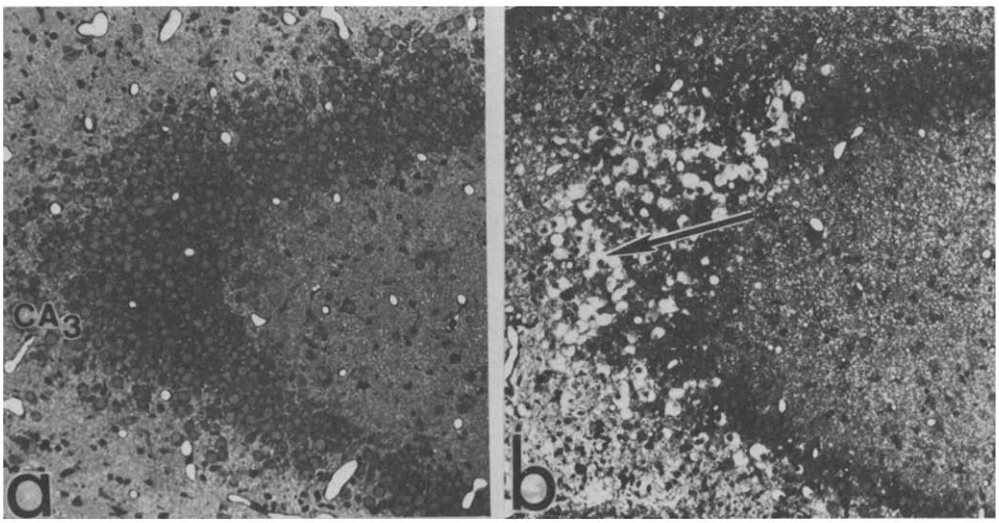

FIG. 3. Light micrographic illustrations of 1 μ sections through the hippocampus of 5-day-old infant rats 24 hr after being treated with 10 mmoles/kg NaCl (a) and 1.2 mg/g of L-cysteine (b). The pyramidal cells in field CA3 of Ammon's horn appeared uneffected in the NaCl treated animal (a), whereas a large number of the pyramidal cells in the L-cysteine treated animal have swollen soma and pyknosis of the nuclei (arrow). The rarefied appearance of tissue lateral to field CA3 (lower left in b) probably represents L-cysteine-produced cytopathology and may involve the basal dendrites of pyramidal cells as well as proximal parts of the axons (X 120).

The administration of amphetamine in 2 doses (1.5 and 3.0 mg/kg) at different ages produced no differences between the two groups when tested in the open field for 3 min. While amphetamine produced a slight mean increase in activity over saline control injections, other comparisons such as control vs experimental groups, males vs females and low dose (1.5 mg/kg) vs high dose (3.0 mg/kg) were not statistically significant.

Spontaneous Alternation

Normal animals have been reported to show a significant tendency to avoid the arm of the T-maze entered on the previous trial [7]. Figure 5 shows that about 70 per cent of the saline treated control (CONT.) animals alternated on the second trial whereas only 30 per cent of the L-cysteine treated animals (EXP.) alternated ($p<0.05$, Chi-square).

Lashley III Maze

Figure 6 shows that the animals treated with L-cysteine (○ - - - - ○) made significantly more mean errors per trial than the controls (● ——— ●) during all but the last 10 trial block ($p<0.01$ for the Trial Blocks 1–4 and $p<0.05$ for Trial Block 5, t test). However, Fig. 7 shows that only during the first 10 trials did the L-cysteine treated animals take more time to run the maze than controls ($p<0.05$, t test).

Visual Discrimination

The ability of L-cysteine treated and saline treated animals to discriminate between horizontal and vertical striped patterns is presented in Fig. 8. More saline treated animals than L-cysteine treated animals learned the discrimination task with criterion scores at or less than the median criterion score of 162 ($p = 0.04$, median test, [49]).

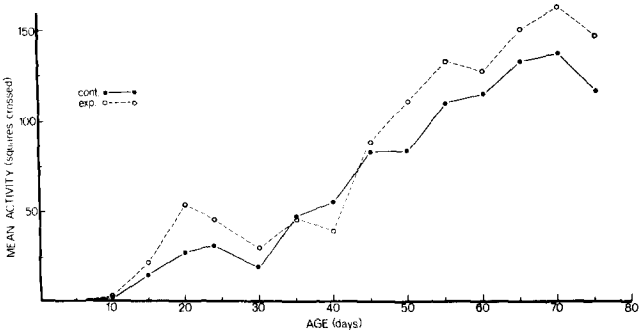

FIG. 4. Mean open-field activity scores (3 min test) of L-cysteine treated (exp., N = 12) and NaCl treated (cont., N = 23) rats tested from 7–75 days of age. Significant differences between experimental and control groups occurred only at 20 days of age ($p<0.02$, t test).

DISCUSSION

These experiments confirm prior reports [36,42] that the administration of L-cysteine to infant rats produces a pattern of brain damage prominently involving limbic forebrain structures and demonstrate that animals thus treated have measurable behavioral deficits as adults. The preweaning mortality rate of animals treated with L-cysteine (1.2–1.3 mg/g) was high (75%) which suggests that a slightly lower dose, perhaps 1.1 mg/g might be a better regimen to follow. Surviving animals appeared healthy as was reflected in their body weights (Fig. 1) and open-field activity (Fig. 4).

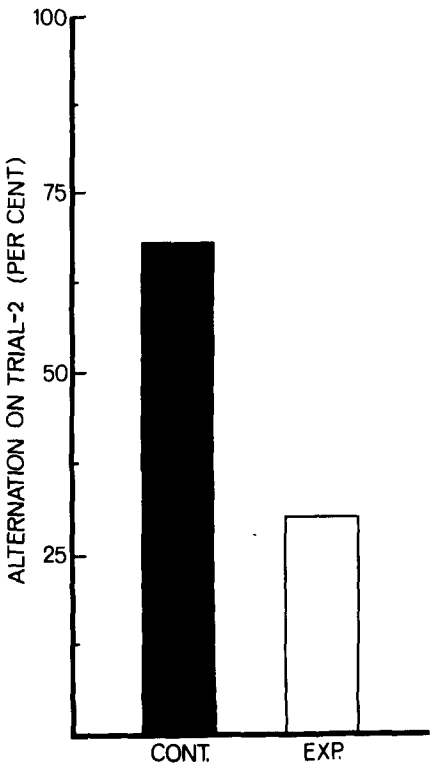

FIG. 5. Percentage of NaCl treated animals (CONT. N = 25) and L-cysteine treated animals (EXP., N = 20) that displayed spontaneous alternation in a T-maze. Statistical significance between groups = $p<0.05$, Chi-square test.

The pattern of performance deficits manifested by animals treated neonatally with L-cysteine resembles that described in adult rats which have sustained massive hippocampal damage. Several studies have shown that, unlike normal animals, hippocampectomized animals do not show spontaneous alternation in a T-maze [26, 28, 47, 52]. They also make more errors than controls in the acquisition of a variety of complex tasks [1,20] including the Lashley III maze [2, 18, 51] and complex two choice discrimination tests [16,22]. When performing these tasks, hippocampal animals have frequently been described as having a much greater tendency than controls to repeat previous responses in mazes, to form position habits in discrimination tasks and to be inattentive to appropriate environmental cues in novel situations. Such terms as, lack of habituation [7], reduced stimulus satiation [12], reduced attentiveness [1], response perseveration or fixation [16], and deficits in response inhibition [23] have been used to describe the behavior. Several neuronal mechanisms have been proposed to explain these behavioral deficits [1, 9, 15, 16, 20, 23, 24]. There appears to be general agreement that one of the main functions of the hippocampus is to inhibit neural systems concerned with nonspecific levels of activation or arousal. It is possible that treatment of neonatal rats with L-cysteine prevented the normal maturation of the hippo-

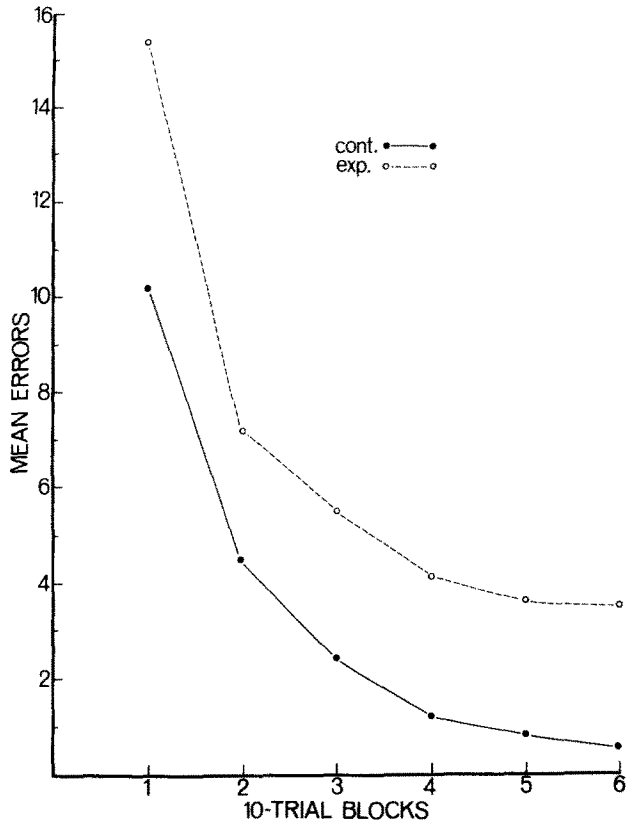

FIG. 6. Mean number of errors per trial over 60 trials in Lashley III maze for NaCl treated (cont., N = 25) and L-cysteine treated (exp., N = 14) animals. Groups were statistically different in mean error scores on all 10-trial blocks except for the last ($p<0.01$ for Trial Blocks 1–4 and $p<0.05$ for Trial Block 5, t test).

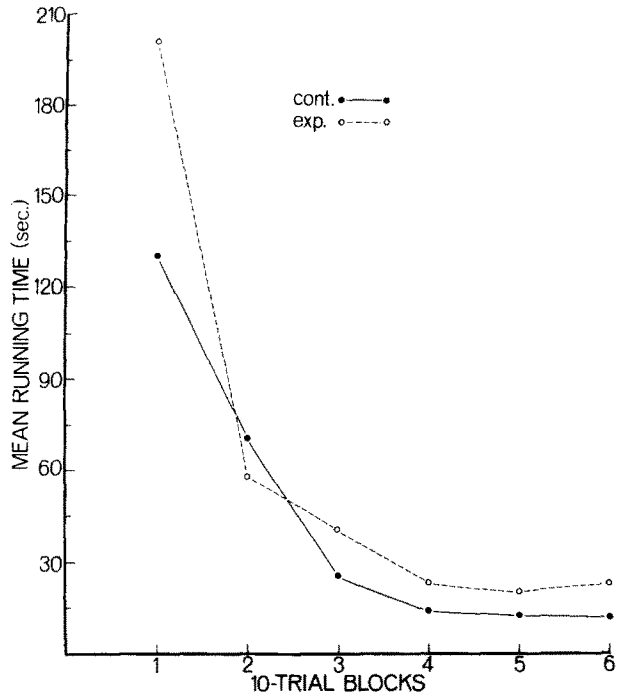

FIG. 7. Mean running time per trial in the Lashley III maze for NaCl treated (cont., N = 25) and L-cysteine treated (exp., N = 14) animals. Statistical significance was obtained between groups only for the 1st 10-trial block ($p<0.05$, t test).

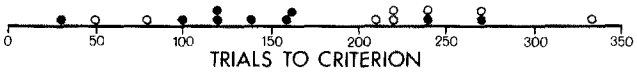

FIG. 8. Number of trials required for animals to learn the visual pattern discrimination problem. Criterion trials have been excluded. Each L-cysteine treated animal is indicated by an open circle and each NaCl treated animal is indicated by a closed circle. The two groups were significantly different in criterion scores (p = 0.04, median test [49]).

campal response-inhibitory system by damaging limbic structures such as the hippocampus. For example, it has been suggested that damage to the hippocampal field CA3 (as depicted in Fig. 3b), which sends axons through the fimbria to the septal area [45,46], interferes with response inhibition and timing behavior [20,30]. Although damage to mouse retina from a high dose (3 mg/g) of L-cysteine has been reported [37], the lower doses (1.2–1.3 mg/g) employed here induced no change in either the retina or optic tract of animals examined histologically. Thus, damage to the visual receptive system would be an unlikely explanation for the observed deficits in visual pattern discrimination of our experimental animals.

A sustained increase in the general drive state or motivation level has also been postulated to explain impaired performance of animals with hippocampal lesions [20]. Hippocampectomized rats have been reported to be more active than controls in the open-field [19, 22, 31, 53] and in situations that evoke goaldirected behavior [13]. Studies using stabilimeters [53] and activity wheels [10,21] have not produced clear results of such an increase in general activity. However, in the neonatal rat, damage to the hippocampus or the frontal cortex was reported to produce an increase over controls in hunger-induced activity as measured by the stabilimeter, but only after 15 to 20 days of age, indicating that these limbic forebrain structures begin to develop inhibitory control over behavioral arousal at this time [32]. In the present study, the L-cysteine treated animals had a significantly higher spontaneous activity score than the controls at an age (20 days) when a decrease in locomotor activity usually occurs in the rat. There is also some indication that giving L-cysteine to neonatal rats produced a slight but sustained increase in general activity in the open-field, especially after 45 days of age (Fig. 4). Increased activity is further indicated by the fact that, except for the first 10 trials, running time in the Lashley III maze was not statistically different between the experimental and control groups (Fig. 7) even though the experimentals made significantly more errors (Fig. 6). That is, L-cysteine treated animals could have wasted more time making errors (incorrect turns) and still equalled the running time of controls if, due to a higher activity level, they ran faster.

Other tests, such as passive avoidance and differential reinforcement learning (DRL), which have been shown to differentiate the performances of hippocampectomized

from normal rats [5, 14, 17, 22, 25, 48] might also be explored. It should be mentioned that lesions in other brain regions, such as the septum and entorhinal cortex, which have exclusive connections to the hippocampus [45,46], also produce deficits similar to the hippocampal syndrome on many but not all of these tests [1]. In addition, the hippocampus probably does not serve a single function, but rather different hippocampal regions may be concerned with separate functional processes [20,52].

Since the L-cysteine pattern of brain damage is not confined exclusively to the hippocampus nor even to the limbic system, it is not possible to conclude unequivocally that behavioral deficits accompanying the syndrome reflect damage localized to either the hippocampus or other limbic forebrain structures. That damage to several cortical regions might have contributed to the performance deficits in at least some of the animals must be born in mind. However, the tests reported upon here were chosen because of their demonstrated usefulness in identifying a hippocampal or limbic component to a behavioral deficit syndrome and the brain regions most consistently and severely damaged in our experimental animals were the hippocampus and amygdala. It, therefore, seems reasonable to view the behavioral manifestations of L-cysteine-induced brain damage as predominantly a limbic dysfunction syndrome.

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