Patterns of hippocampal cell loss based on subregional lesions of the hippocampus

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Abstract

It is widely accepted that the hippocampus plays an essential role in memory. Furthermore, studies have suggested that subregions within the hippocampus contribute differentially to specific behavioral components of memory. These studies typically rely on lesions produced by localized injections of neurotoxins (e.g., ibotenic acid or colchicine) into targeted subregions of the hippocampus. In the present study, the specificity of ibotenic acid lesions into areas CA1 and CA3 and colchicine lesions into the dorsal dentate gyrus (DG) was tested. Specifically, the effects of lesions within the dorsal hippocampus, the ventral hippocampus, and areas outside the hippocampus (e.g., lateral septum and entorhinal cortex) were evaluated using Fluoro-Jade, a histofluorescent stain for degenerating neurons. The results show that cell loss is relatively uniform after ibotenic acid injections into areas CA1 and CA3 and variable after colchicine injections into DG. CA1 and CA3 lesions appeared mostly localized to those relative subregions, and DG lesions appeared highly localized to the DG. Using these lesion procedures, little cell loss was apparent in the ventral hippocampus, and no cell loss was apparent in the entorhinal cortex. It is suggested that the lesion procedures described in this study produce relatively selective lesions of neurons within specific subregions of the hippocampus and should be useful for studies examining possible differential contributions of hippocampal subregions to memory processes.

1. Introduction

In recent years, studies have suggested that specific subregions of the hippocampus support unique behavioral functions [1]. For example, Gilbert et al. [4] showed a double dissociation between the dentate gyrus (DG) and CA1, where damage to the DG resulted in a deficit of spatial processing, while damage to CA1 resulted in a deficit of temporal processing. Lee and Kesner [12] demonstrated time-dependent contributions of hippocampal subregions in memory formation, with CA3 mediating short-term memory and CA1 mediating intermediate-term memory. In understanding and supporting this idea of specificity of function, it is valuable to know the nature and extent of damage caused by the neurotoxic lesions employed in these studies. Based on analysis of lesion volumes, intrahippocampal injections of neurotoxins (i.e., ibotenic acid and colchicines) appear to produce extensive cell damage in the target areas (i.e., CA1, CA3, DG) but minimal damage to subregions of the hippocampus in non-target areas [4,10,12,13]. However, it is possible that there is more damage than what has been estimated by volumetric analysis.

The present study used the histofluorescent stain, Fluoro-Jade, as an alternative method of assessing the degree of
neuronal damage produced by ibotenic acid and colchicine lesions. Fluoro-Jade is a sensitive and reliable histofluorescent marker for degenerating neurons and their processes, although the exact mechanism by which Fluoro-Jade marks degenerating neurons is not well known [21]. Hallam et al. [7] used Fluoro-Jade staining to observe degenerating neurons in the cortex, thalamus, hippocampus, caudate–putamen, brainstem, and cerebellum after the induction of brain injury via lateral fluid percussion and weight drop impact–acceleration. Degenerating neurons can be clearly seen with Fluoro-Jade because, unlike with silver staining methods, it causes degenerating neurons to appear bright green against a dark background [21]. This allows for clearer visualization of the extent of damage to the interconnected subregions of the hippocampus. The importance of more clearly visualizing damage produced by lesions to the hippocampus has been pointed out by Jarrard [9], who suggested that such lesions by ibotenic acid might cause undetected damage to the entorhinal cortex.

The purpose of this study is to produce neurotoxic insults with intrahippocampal injections of ibotenic acid into dorsal CA1 and dorsal CA3 and with colchicine injections into dorsal DG in order to observe the specificity of neuronal damage by staining with Fluoro-Jade. Cell loss was evaluated within subregions of the dorsal hippocampus, in the ventral hippocampus, and outside the hippocampus, especially in the entorhinal cortex. Animals were perfused 2 days, 4 days, or 7 days after receiving neurotoxic insult.

2. Materials and methods

2.1. Subjects

Eighteen adult male Long–Evans rats (mean body weight: 300 g) were used and maintained with free access to food and water.

2.2. Neurotoxin surgery

The rats were assigned to receive a lesion to a specific subregion of the hippocampus: either CA1 (N = 6), CA3 (N = 6), or the dentate gyrus (N = 6). Unilateral lesions were made so that the contralateral unlesioned side could serve as a control. Each rat was first given an intraperitoneal injection of atropine sulfate (0.2 mg/kg) followed 10 min later by an intraperitoneal injection of sodium pentobarbital (68 mg/kg). The animal was placed in a stereotaxic device (Kopf Instruments, Tujunga, CA), with its skull parallel to the stage. Small holes were drilled into the skull at measured locations. The injections were made using a 10 μl Hamilton syringe with a microinjection pump (Cole Palmer Instrument Company, Vernon Hill, IL).

Colchicine (2.5 mg/ml; Sigma-Aldrich, St. Louis, MO) was used for the lesions to the DG because it destroys the granule cells [6,16] in this region. Injections of 0.60 μl/site (1.5 × 10⁻³ mg/site) at 20.00 μl/h were made at the following stereotaxic coordinates: (a) 2.8 mm posterior to bregma, 1.4 mm lateral to midline, and 3.4 mm ventral from the dura; and (b) 4.3 mm posterior to bregma, 2.3 mm lateral to midline, and 3.0 mm ventral from the dura. Ibotenic acid (6.0 mg/ml; Sigma-Aldrich, St. Louis, MO) was used to lesion CA1 and CA3. All injections with ibotenic acid were made at 4.00 μl/h. The volumes and coordinates for the three CA3 injections were: (a) 0.10 μl (6 × 10⁻⁴ mg) injected at 3.6 mm posterior to bregma, 1.0 mm lateral to midline, and 1.9 mm ventral from the dura; (b) 0.10 μl (6 × 10⁻⁴ mg) injected at 3.6 mm posterior to bregma, 2.0 mm lateral to midline, and 1.9 mm ventral from the dura; and (c) 0.15 μl (9 × 10⁻⁴ mg) injected at 3.6 mm posterior to bregma, 3.0 mm lateral to midline, and 1.9 mm ventral from the dura. The volumes and coordinates for the three CA1 injections were: (a) 0.05 μl (3 × 10⁻⁴ mg) injected at 2.8 mm posterior to bregma, 3.0 mm lateral to midline, and 3.2 mm ventral from the dura, (b) 0.08 (4.8 × 10⁻⁴ mg) μl injected at 3.3 mm posterior to bregma, 3.4 mm lateral to midline, and 3.2 mm ventral from the dura, and (c) 0.15 μl (9 × 10⁻⁴ mg) injected at 4.1 mm posterior to bregma, 4.2 mm lateral to midline, and 3.3 mm ventral from the dura. The doses of colchicine and ibotenic acid injected were the same as doses used for previous behavioral research, so the lesion effects could be compared on histological and behavioral levels.

2.3. Histology

Either 2 days, 4 days, or 7 days after surgery (N = 6 for 2 days, N = 6 for 4 days, and N = 6 for 7 days), the animals were given an intraperitoneal injection of sodium pentobarbital (100–200 mg/kg). After breathing had ceased, the rats were perfused intracardially with normal saline for 5 min followed by 10% formalin in phosphate-buffered solution for 5 min. The brain was removed from the skull and stored at 4 °C in a solution of 10% formalin/30%. A tissue block was cut perpendicularly from each brain. The block was frozen to about −18 °C, then 32 μm sections were cut on a Minotome cryostat (International Equipment Co., Needham Heights, MA), and these sections were placed on gelatinized slides. These slides were immersed sequentially in the following solutions: 100% ethanol for 3 min, 70% ethanol for 1 min, dH₂O for 1 min, 0.06% potassium permanganate for 15 min with gentle shaking, dH₂O for 1 min, 0.001% Fluoro-Jade staining solution for 30 min with gently shaking, and 3 times in dH₂O for 1 min each. The Fluoro-Jade staining solution comprised of 20 ml 0.01% Fluoro-Jade stock solution (50 mg Fluoro-Jade powder and 500 ml of dH₂O) and 180 ml 0.1% acetic acid (180 μl acetic acid and 180 ml dH₂O). The slides were then dried at room temperature for 2 h, immersed in xylene 3 times for 2 min each, coverslipped with Permount mounting medium (Fisher Scientific, Fairlawn, NJ 07410), and dried overnight.
Descriptions of neuronal injury are based on observation of Fluoro-Jade-stained brain sections using a Nikon Eclipse E600 microscope equipped for epifluorescence (Nikon, Inc. Garden City, NY 11530) with a FITC fluorescence filter cube (Nikon B-2A, Tokyo, Japan). Photomicrographs were taken at 40× and 100×. Sustained Fluoro-Jade staining was defined 70% to 100% of the cells fluorescing within a region, while intermittent staining was 30% to 70%. Fig. 1 shows photomicrographs of sustained (Fig. 1a) and intermittent (Fig. 1b) cell loss to provide an example of our definition of these two conditions, where the degeneration is indicated by arrows.

3. Results

For each subregion lesion (CA1, CA3, or DG), perfusions were performed 2, 4, or 7 days after surgery (animals are designated as day 2, day 4, or day 7). With each condition, two animals were used, and their average cell loss was described in the anterior (% bregma = -1.8 mm to % bregma = -2.8 mm), middle (% bregma = -3.0 mm to % bregma = -4.3 mm), and posterior (% bregma = -4.5 mm to % bregma = -6.8 mm) regions of the hippocampus. In the figures, degeneration is indicated in CA1 by white arrows, in DG by black arrows, and in CA3 by black arrowheads.

3.1. CA1 lesions

At day 2, cell injury after ibotenic acid injection extended from bregma = -2.8 to bregma = -5.8 (Fig. 2a and Table 1) and was sustained through the entire CA1 region, sustained on the dorsal blade of the DG posteriorly, and intermittent through CA3 in the anterior and middle hippocampus (CA3 damage not shown in Fig. 2a). One rat showed intermittent cell loss in the lateral septum at bregma, bordering the lateral ventricle. Degeneration at day 4 extended from bregma = -1.8 to bregma = -5.3 (Fig. 2d and Table 1). In CA1, cell loss was sustained through the entire dorsal hippocampus, and, in DG, it was sustained on the dorsal blade through the anterior and middle hippocampus. Intermittent damage appeared in CA3 from bregma = -1.8 to bregma = -3.5 (CA3 and DG damage not shown in Fig. 2d). At day 7, degeneration was from bregma = -1.8 to bregma = -5.0 (Fig. 2g and Table 1). For one of the brains, it was sustained through the medial two-thirds of CA1 and on the dorsal blade of the DG. For the other brain, cell damage was the same except it showed sustained damage in both blades of the DG in the posterior hippocampus (not shown in Fig. 2g). Intermittent cell loss appeared in CA3 through the anterior and middle hippocampus (not shown in Fig. 2g).

3.2. CA3 lesions

Lesions in CA3 appeared localized to that subregion anteriorly, with some cell loss in other regions posteriorly. At day 2 (Fig. 2b and Table 1), from bregma = -1.8 to bregma = -5.8, cell loss appeared sustained through all of CA3 and CA2 and on the lateral half of CA1. Sustained cell loss also occurred on the lateral tips of the DG blades. In one of these rats, the lateral septum showed intermittent degeneration at bregma, bordering the lateral ventricle. One of the day-4 lesions, shown in Fig. 2e and Table 1, indicated cell loss from bregma = -2.3 to bregma = -6.8 and was sustained through CA3 and the lateral 0.5 mm of the ventral blade of the DG. Cell damage was intermittent through CA2 and the lateral third of CA1. The other day-4 animal (not depicted in Fig. 2e) showed sustained cell loss through CA3, CA1, and the DG. Fig. 2h and Table 1 show the cell loss at day 7, which appears from bregma = -2.3 to bregma = -6.8. It was sustained through all of CA3 and CA2 and the lateral third of CA1 (Fig. 2h shows cell damage on about the lateral 0.5 mm of CA1). In the DG, cell loss is apparent on the lateral tips of its blades, appearing intermittent in the middle hippocampus and sustained in the posterior hippocampus.

3.3. Dentate gyrus lesions

DG lesions were mostly localized to the DG, and cell loss was less severe with colchicine (2.5 mg/ml) used as the neurotoxin. Table 1 and Figs. 2c, f, and i show the
Fig. 2. 40× photomicrographs of neurotoxic lesions stained with Fluoro-Jade. (a) Two days after CA1 lesion; note the sustained degeneration through CA1 (white arrows) and the dorsal blade of the DG (black arrows). (d) Four days after CA1 lesion; sustained cell loss through CA1 (white arrows) is apparent. It is possible that the lack of apparent cell loss in CA3 is because all CA3 cells had already died when stained with Fluoro-Jade. However, this is not likely because significant cell loss can be observed in other lesions at days 4 and 7, suggesting that significant cell loss continues through at least 7 days after neurotoxic insult. (g) Seven days after CA1 lesion; sustained cell loss appears through most of CA1 (white arrows) and in the medial part of the dorsal blade of the DG (black arrows). (b) Two days after CA3 lesion; sustained degeneration appears through CA3 (black arrowheads), CA2, and the lateral half of CA1 (white arrows). As well, the lateral edge of the dorsal tip of the DG (black arrow) shows sustained cell loss. (e) Four days after CA3 lesion; sustained degeneration through CA3 (black arrowheads) and the lateral tip of the ventral blade of the DG (black arrows). Furthermore, CA2 and the lateral 0.5 mm of CA1 (white arrows) show intermittent cell damage. (h) Seven days after CA3 lesion. CA3 (black arrowheads), CA2, the lateral edge of CA1 (white arrow), and the lateral tip of the ventral blade of the DG (black arrow) show sustained cell loss. (c) Two days after DG lesion; very intermittent cell loss appears through both blades of the DG (black arrows), with very intermittent cell loss also appearing in lateral CA1 (white arrows). (f) Four days after DG lesion; slightly more cell loss than day 2, though degeneration still appears intermittent through both blades of the DG (black arrows), and very intermittent cell loss appears in the lateral 0.5 mm of CA1 (white arrows). (i) Seven days after DG lesion; cell damage visible only in the dorsal blade of DG. (j–l) Two days after CA1, CA3, and DG lesions, respectively. No cell loss apparent in the entorhinal cortex.
3.4. Other cell loss

The ventral hippocampus, entorhinal cortex, and lateral septum were evaluated. In two CA3 day-4 animals and one DG day-7 animal, intermittent cell loss appeared in the dorsal half of the ventral hippocampus. The entorhinal cortex showed no cell loss for any of the lesions. Figs. 2j–l show all cells of the entorhinal cortex intact after CA1, CA3, and DG lesions, respectively. Each of these slides represent day-2 animals. Some intermittent cell loss appeared in the lateral septum, which is indicated above. The fluorescence in the cells of the lateral septum was not obvious, but it is mentioned as the only other potential degeneration.

4. Discussion

The original findings in this study were that the lesion procedures employed herein produce relatively specific damage to targeted subregions of the hippocampus. Damage to the lateral septum appeared in one CA3-lesioned animal and one CA1-lesioned animal. This could be due to anterograde degeneration as some researchers [1,2,18] have described a projection from CA3 to the lateral and medial nuclei. The CA1 lesion which damaged the lateral septum also damaged CA3, so it may be that CA3-lesioned cells projecting to the lateral septum caused the anterograde degeneration in the lateral septum. Sections from approximately bregma 0.0 to bregma −7.0 mm were examined, and, within that vicinity, a lesion of a subregion of the dorsal hippocampus did not produce any retrograde degeneration in the entorhinal cortex, contrary to other observations [9]. The area most strongly affected by neurotoxic insult was the hippocampal subregion targeted.

Among CA1 lesions, all animals showed sustained loss in CA1, with some loss evident elsewhere in the dorsal hippocampus. A possible reason for damage to DG with CA1 lesions is from the downward spread of ibotenic acid when it is injected into CA1, even though the slow rate of 4.00 μl/h was employed, which is a likely possibility because the most severe damage in DG often appeared ventral to the injection site for CA1. Other studies have reported apparent spread of drug during injection with a radioactive marker [22], so some degree of spread may be an inevitable consequence of neurotoxic injections into CA1. If the damage in the DG was due to degeneration over time through intrahippocampal connections rather than immediate drug effect, we would have seen more cell loss in CA3 because CA3 connects DG with CA1. However, only CA1 and DG were affected in these lesions, so DG damage was not due to a direct connection. Thus, ibotenic acid injections into CA1 had their most pronounced effects on the targeted subregion. These findings are applicable to studies which use similar lesion procedures and parameters and which have suggested functions specific to CA1. Such findings have shown CA1 to be important with temporal pattern association [8], transitive inference [20], and intermediate-term memory [12,13], and these functions are not highly associated with other subregions of the hippocampus.

CA3 lesions, also produced by ibotenic acid injections, showed a fairly localized effect in the anterior hippocampus, with some spread posteriorly. In most of the lesions, the lateral portions of CA1 and the DG were also affected, possibly through connections via the longitudinal fibers of CA3. Furthermore, it is likely that lateral CA1 was affected by the vertical penetration of the injection needle because ibotenic acid always spreads up the needle during injection, and it is the lateral CA1 that was always affected. It seems more difficult to achieve localized lesions in CA3, but these parameters for surgery have been used in other studies.
where a double dissociation has been shown between CA1 and CA3 and between DG and CA3. Other reports suggesting that CA3 has a unique function in memory include those evaluating spatial pattern association 
[3,19], spatial pattern completion 
[5,14,15,17], short-term memory processing 
[11,13], and novelty detection 
[12,13]. Although CA3 lesions with ibotenic acid appear to be less localized than other lesions in this study, the parameters used herein have been shown to produce specific behavioral effects in other studies.

Previous studies using direct injection of colchicine into the DG described highly localized neuronal degeneration of dentate granule cells 
[6,16]. The present results are consistent with these earlier studies. It has been reported that lesions in the DG produced by colchicine also damage neurons in CA3 
[9]. However, the present results do not support this observation. There was small amount of cell loss in CA1 after DG lesions, which was probably due to mechanical damage from the injection needle, as the affected area in CA1 was minimal (as shown in Figs. 2i and j), and colchicine does not disrupt pyramidal cells 
[6,16]. Evidence that colchicine produces relatively specific lesions to the DG supports behavioral data which show a double dissociation between the DG and other subregions, where the DG appears involved in the orthogonalization of sensory inputs into a spatial (metric) domain 
[4].

The pattern of chronological degeneration with colchicine lesions shows cell loss 2 days after surgery that is less severe than 4 days after surgery. This can be explained by the action of colchicine via inhibition of protein synthesis, which results in cell death that is not immediate. Ibotenic acid produced severe cell loss on day 2 as it causes more immediate excitotoxic effects. Chronological cell loss pattern after neurotoxic insult by ibotenic acid resulted in small differences across days. It should be noted that, with these lesions, it is difficult to determine whether the increases or decreases in degeneration that occur across time are meaningful because the lesions made at days 2, 4, and 7 may not have been identical. Because of the nature of the procedure, it was not possible to do a within-subjects analysis, so there is a possibility that the small differences we observed with ibotenic acid lesions were due to variability in the exact size and location of the lesions.

Based on previous research using similar lesion parameters and employing Nissl and cresyl violet stains 
[4,12,13], hippocampal subregional lesions have appeared localized, with the boundary of affected cells clearly defined. In this study, different degrees of degeneration (sustained and intermittent) were evaluated with Fluoro-Jade staining, which revealed a more specific pattern of cell loss. While some regions (i.e., CA3, CA1, DG) showed obvious sustained or intermittent damage, fluorescence in the lateral septum was less clear. Despite this limitation, it was clear that entorhinal cortex showed no cell loss. In summary, lesions to subregions of the hippocampus were highly localized to the hippocampus and mostly localized to the targeted subregion.

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**References**


