Journal of Behavioral and Brain Science, 2016, 6, 184-198
Published Online April 2016 in SciRes. http://www.scirp.org/journal/jbbs
http://dx.doi.org/10.4236/jbbs.2016.64019

Contrasting the Amnesic Effects of Temporary Inactivation with Lesions of the Hippocampus on Context Memory

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Received 9 February 2016; accepted 12 April 2016; published 15 April 2016

Abstract

Lesions and temporary inactivation of the hippocampus (HPC) in rodents occasionally lead to discrepant amnesic effects. We directly compared and contrasted the retrograde amnesic effects that small HPC lesions (~50% damage), large HPC lesions (~80% damage), and combined dorsal and ventral HPC inactivation using the sodium channel blocker tetrodotoxin (TTX) had on contextual fear conditioning. Compared to control rats, large HPC lesions significantly reduced freezing during retention testing, a behaviour consistent with retrograde amnesia. In contrast, neither the small lesions nor the TTX inactivation significantly reduced freezing. The extent of damage was significantly and negatively correlated with retention performance ($r_{(9)} = -0.896$, $p < 0.001$), suggesting that 70% or more of the HPC needed to be damaged to observe deficits. Importantly, TTX inactivation disrupted spatial memory in the Morris Water Task, confirming that our inactivation procedure did impair one form of HPC-dependent memory. To assess the extent of the TTX inactivation, immediate early gene expression was quantified in the HPC following the Morris Water Task. However, despite the behavioural impairment, we did not find a significant reduction in expression. We conclude that temporary inactivation of the HPC may fail to impair context fear memory because this technique does not sufficiently disrupt the HPC.

Keywords

Hippocampus, Retrograde Amnesia, Lesion Size, Temporary Inactivation, Fear Conditioning Memory, Rat

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How to cite this paper: Scott, G.A., Saucier, D.M. and Lehmann, H. (2016) Contrasting the Amnesic Effects of Temporary Inactivation with Lesions of the Hippocampus on Context Memory. *Journal of Behavioral and Brain Science*, **6**, 184-198.
http://dx.doi.org/10.4236/jbbs.2016.64019

1. Introduction

Our understanding of the role of the hippocampus (HPC) in long-term memory comes, in part, from studies examining the amnesic effects of lesions or temporary inactivation of the HPC in non-human animals. Commonly, lesions involve destroying the neurons in the targeted region by either injecting a neurotoxin, passing current (electrolytic) or radiofrequency through the region, or by aspirating the tissue. In contrast, temporary inactivation involves reversibly "shutting off" neuronal activity in the target structure by infusing a compound that prevents action potentials, such as a sodium channel blocker (e.g., lidocaine) or a gabaergic agonist (e.g., muscimol). Disrupting the HPC using either method typically has equivalent retrograde amnesic effects on spatial memory tasks, such as the Morris Water Task [1]-[4].

When considering context fear memory, the effects of HPC inactivation do not always parallel the retrograde amnesic effects of HPC lesions. HPC lesions made shortly after learning consistently cause retrograde amnesia for contextual fear conditioning (see [5]). In contrast, there are many instances in which HPC inactivation failed to cause retrograde amnesia in this task [6]-[11], despite some studies reporting positive amnesic findings [11]-[15]. Logically, damaging HPC neurons or preventing their functioning should result in similar impairments in memory; the differential outcome between these two techniques in contextual fear conditioning is perplexing. However, these discrepancies may result from differences in conditioning protocols or differences in the extent of HPC disruption among studies.

Typically, studies that examine the effects of temporary inactivation bilaterally infuse a small amount of inactivating compound either at one site in the dorsal HPC [6] [7] [9] [10] or at most at one site in each of the dorsal and ventral HPC [8]. By contrast, permanent lesions of the HPC using a neurotoxin are typically conducted with many more injection sites per hemisphere (e.g., 10 sites) [16]-[21], presumably leading to more complete drug diffusion through the entire structure and greater disruption of neural functioning. Although a few studies have shown retrograde amnesia in contextual fear conditioning after small lesions of the dorsal HPC [22]-[24], most fail to find impairments when the damage is induced outside the window of cellular consolidation [25] [26]. Thus, the lack of retrograde amnesia following HPC inactivation reported in some contextual fear conditioning studies might result from insufficient disruption of the HPC. The current study assessed this possibility by directly contrasting lesions with inactivation of the HPC in rats that were trained with the same contextual fear conditioning parameters.

We directly compared the retrograde amnesic effects of HPC inactivation with neurotoxic HPC lesions of different sizes. We manipulated lesion size because the number of neurotoxin infusion sites needed to lesion the HPC is easily manipulated under stereotaxic surgery. In contrast, the extent of HPC inactivation cannot be easily manipulated because of the physical limits in the number of guide cannulae that can be intra-cranially implanted to perform the injections. We did, however, follow an inactivation protocol that to our knowledge matches or exceeds those of most, if not all, other HPC inactivation studies [8] [11], meaning that we induced what would be considered a large inactivation. Specifically, we compared the amnesic effects of small neurotoxic HPC lesions (~50% damage) with large neurotoxic HPC lesions (~80% damage) and with dorsal and ventral HPC inactivation created with the potent sodium blocker tetrodotoxin (TTX). Importantly, the small HPC lesions and temporary inactivation were made using the same coordinates and injection parameters, to allow a comparable extent of drug diffusion in the HPC. Contextual fear conditioning was performed using the same conditioning protocol for all rats. To confirm that our regimen of HPC inactivation disrupted HPC function, the rats that experienced HPC inactivation were also trained and tested in the hidden platform version of the Morris Water Task, a spatial memory task that is very sensitive to HPC interference [27]. Finally, we assessed immediate early gene expression in the HPC following inactivation in order to quantify the extent of neural suppression caused by the TTX injections.

2. Materials and Methods

This study was carried out in strict accordance with the guidelines of the Canadian Council on Animal Care and the research protocol was approved by the Trent University Animal Care Committee. All surgical procedures were performed under gas anesthesia (isoflurane), analgesics were administered during the one-week surgery recovery period, and all efforts were made to minimize suffering.

2.1. Subjects

Twenty-five male Long-Evans rats weighing 300 - 400 g (Charles River, St. Constant, Quebec) were used. Rats

were housed in pairs under a 12 h light/dark cycle with light onset at 7:00 am. They were provided with 25 - 30 g of rat chow daily and water was available *ad libitum*.

2.2. Materials

2.2.1. Contextual Fear Conditioning

Contextual Fear Conditioning was conducted in a 30 × 26 × 26 cm chamber, which was made of a Plexiglas front, back, and top and two metal sides. The floor of the chamber was comprised of 18 steel rods (2 mm diameter) spaced 1.5 cm apart. Foot shock (2 sec; 0.75 mA) was delivered through the rods with an SGS 003 shock generator/scrambler (BRS Foringer; Beltsville, MD). Rat behaviour was recorded using a webcam and laptop computer and analyzed using ANY-Maze tracking software (Stoelting Co., Wood Dale, IL).

2.2.2. Morris Water Task

A pool (140 cm in diameter and 60 cm deep) was filled with water 32 cm deep (21˚C ± 2˚C). The water in the pool was made opaque with skim milk powder. A Plexiglas platform (11 cm in diameter) provided escape from the water and was submerged 2 cm below the surface of the water. The room included a variety of extra-maze cues (e.g., wall posters, door, tables, etc.). The swim patterns of the rats were recorded using a webcam and laptop computer and analyzed using ANY-Maze tracking software (Stoelting Co., Wood Lane, IL).

2.3. Surgical and Drug Infusion Procedures

2.3.1. HPC Lesions

The lesions were performed under gas anaesthesia using isoflurane (Janssen, Toronto, Ontario) in oxygen at a rate of 0.8 l/min at 14.7 PSIA at 21˚C (Benson Medical Industries, Markham, Ontario). Once anesthetised, the rats were administered 0.02 mL of Metacam (5 mg/mL; Boehringer-Ingelheim, Burlington, Ontario) subcutaneously as an analgesic. The scalp was shaved and cleaned aseptically and they were placed in a stereotaxic frame (Kopf Instruments, Tajunga, CA). The scalp was then incised along the midline to expose the skull and small holes were drilled in the skull to enable the lowering of a 30-gauge injection needle into the HPC. At each injection site, N-methyl-D-aspartic acid (NMDA; 7.5 µg/µl; Sigma Chem., St Louis, MO) was infused at a flow rate of 0.4 µl/min (Large lesions) or 0.5 µl/min (Small lesions). The Large lesions involved injections at 10 sites bilaterally, whereas the Small lesions involved injections at 2 sites bilaterally (see **Table 1** for stereotaxic coordinates and injection volume per site). The injection needles were left in place for an additional 2 min after each

Table 1. Stereotaxic coordinates for NMDA injection sites for large and small HPC lesions (mm) relative to Bregma [28] as well as the volume injected at each site.

	Anteroposterior (AP)	Mediolateral (ML)	Dorsoventral (DV)	Volume injected (µL)
Large lesions				
	−3.1	±1.0	−3.6	0.3
	−3.1	±2.0	−3.6	0.3
	−4.1	±2.0	−4.0	0.3
	−4.1	±3.5	−4.0	0.3
	−5.0	±3.0	−4.1	0.3
	−5.0	±5.2	−7.3	0.3
	−5.0	±5.2	−5.0	0.3
	−5.8	±4.4	−4.4	0.3
	−5.8	±5.1	−7.5	0.4
	−5.8	±5.1	−6.2	0.4
Small lesions				
	−3.5	±2.0	−4.0	0.5
	−5.8	±5.0	−6.0	0.5

injection to maximize diffusion. Following all injections, the scalp was sutured and the rats were given 0.2 - 0.6 cc of diazepam (10 mg/ml; Hoffman-La Roche, Mississauga, Ontario) intraperitoneally as an anticonvulsant. The rats in the Sham control group received the same surgical procedures but did not have holes drilled in the skull, and thus had no damage to the brain. All the rats recovered from surgery for 10 days and were administered Metacam (Oral Suspension 0.1 ml; 1.5 mg/mL, p.o.; Boehringer-Ingelheim, Burlington, Ontario) as an analgesic daily during the first 7 days of recovery.

2.3.2. Cannulation for HPC Inactivation

The rats were anaesthetized and had their scalps incised in the same manner as described above for the HPC lesions. Burr holes were drilled in the skull to accommodate 23-gauge stainless-steel guide cannulae (PlasticsOne, Roanoke, VA). Cannulae, 10 mm in length, were lowered into the dorsal HPC bilaterally, whereas 13 mm long cannulae were lowered into the ventral HPC bilaterally (see **Table 2** for coordinates). Jeweller's screws were fixed to the skull and the guide cannulae were fixed to the skull and jeweller's screws using dental acrylic. Following surgery, each guide cannula was occluded with a stainless-steel obturator of equal length as the respective guide cannula. The rats recovered from surgery for 10 days and were administered oral Metacam (Oral Suspension 0.1 ml; 1.5 mg/mL, p.o.; Boehringer-Ingelheim, Burlington, Ontario) as an analgesic daily for the first 7 days.

2.3.3. Intracranial Drug Infusion for HPC Inactivation

Thirty minutes prior to retention testing in contextual fear conditioning and the Morris Water Task, the cannulated rats were infused with either saline (0.9%; Saline group) or TTX dissolved in saline (4 ng/μL; Sigma-Aldrich Canada, Oakville, Ontario; TTX group). The saline or TTX infusions (0.5 μL) were delivered through 30-gauge injection needles connected to 10 μL Hamilton syringes via polyethylene tubing (PE-50). The syringes were located in a microinfusion pump, which enabled controlled delivery of the drugs over 60 sec. The tips of the injection needles protruded into the tissue 0.5 mm past the intracranial ends of each guide cannula. The injection needles remained in the guide cannulae for 60 sec following completion of the infusions to maximize drug diffusion. The infusions were completed by injecting in the dorsal HPC in one hemisphere while simultaneously infusing in the ventral of the other hemisphere and then vice versa. Overall, the infusion procedure for each rat took approximately 6 min to complete. Immediately following the injections, the rats were returned to their home cage for 30 min until behavioural testing commenced.

2.4. Behavioural Procedures

2.4.1. Contextual Fear Conditioning

HPC Lesions: The rats were transported from their home cage to the testing room where they were placed in the conditioning chamber. During the conditioning session, they were allowed to explore the chamber for 3 min before receiving 5 foot-shocks with a1 min interval between each. The rats remained in the chamber for an additional minute after the onset of the final shock. Three days later, they either received Large lesions, Small lesions, or Sham control surgery. The 3-d training-to-surgery interval was selected as it is argued to be sufficiently long to avoid possible peripheral cellular consolidation disruption that can be associated with neurotoxic lesions [25] [26]. Following the post-surgery recovery period, the rats were returned to their conditioning chamber for a 5-min retention test. Retention was operationally defined as the percentage of time freezing (complete lack of movement except for breathing), which was quantified for each rat. Prior to conditioning and testing of each rat, the chamber was cleaned with 70% ethanol.

HPC Inactivation: Conditioning and testing was conducted in the same manner as described above. The retention test, however, was given 3 days following conditioning and the rats received intra-hippocampal infusions of either saline (Saline; control group) or TTX (TTX; HPC inactivation group) 30 min prior to the retention test.

Table 2. Stereotaxic coordinates of cannulae implantations relative to Bregma (mm) [28].

Anteroposterior (AP)	Mediolateral (ML)	Dorsoventral (DV)
−3.5	±2.0	−3.5
−5.8	±5.0	−5.5

2.4.2. Morris Water Task

A minimum of one week following retention testing for contextual fear conditioning, the cannulated rats were trained in the hidden platform version of the Morris Water Task. This task required the rats to learn and remember the location of an escape platform submerged below the surface of the water using distal spatial cues. For each swim trial, the rats were placed in the pool either at the SE, SW, or NE quadrant in a pseudorandom order, with the platform always located in the NW quadrant. The rats had to find the platform within 60 sec to escape the water. If a rat did not find the platform within this period, then it was guided to the platform by hand. The rats remained on the platform for 15 sec. The rats were given a 2 min rest between trials. The rats were given 8 swim trials per day for 2 consecutive days for a total of 16 trials. On the second day, the rats received intra-HPC injections of saline or TTX 30 min prior to the behavioural testing. The distance swam to reach the platform was used as index of spatial memory performance.

2.5. Perfusions, Histology, and Immunohistochemistry

2.5.1. Perfusions and Histology

All the rats were anaesthetized with intraperitoneal injections of 0.3 - 0.4 ml of sodium pentobarbital (320 mg/mL; Schering Inc., Montreal, Quebec) and perfused intracardially with 200 ml of phosphate-buffered saline followed by 200 ml of 4% paraformaldehyde. The brains were removed from the skulls and stored in 4% paraformaldehyde for 24 h before being transferred to 0.1% sodium azide/30% sucrose solution for cryoprotection. Once the brains were no longer buoyant in the solution, they were frozen with dry ice and sectioned on a freezing microtome (American Optical Corporation, Buffalo, NY) at a thickness of 40 µm. Each section through the HPC was collected and the sections were divided into 12 tissue series (section sampling fraction $1/12^{th}$). Hence, one series consisted of every 12^{th} section throughout the entire HPC. Each series was stored in 0.1% sodium azide. The sections from one series were mounted on to gelatine-coated glass slides and stained with cresyl violet to enable lesion assessment or cannula placements.

2.5.2. Immunohistochemistry

Using the same procedures as above, the Saline control or TTX HPC inactivation rats were anesthetised and perfused 50 - 60 min after the end of the final trial in the Morris Water Task. Specifically, for these rats, a second series of sections was used to label and assess Zif268 expression, a marker of neuronal activity [29]. Importantly, the test-to-euthanization interval has been shown to be adequate to detect behaviourally-induced expression of Zif268 protein [30] [31]. We predicted that HPC inactivation would reduce Zif268 expression because TTX suppresses neural activity. Thus, comparison of the quantification of Zif268expression in the TTX HPC inactivation rats with the Saline control rats aimed to assess the extent of the HPC inactivation.

Once sectioned, the tissue was rinsed in a 0.1% sodium azide solution for 8 - 10 min and then incubated for 24 h at room temperature on a rotator in a 1:1000 primary rabbit anti-*Erg*-1 (Zif268) antibody (Santa Cruz Biotechnology, Dallas, TX) and 0.1% Triton X/PBS. The sections were then rinsed 3 times for 8 - 10 min in phosphate buffered saline, and transferred for a second incubation period (24 h, room temperature on a rotator) with a secondary antibody solution containing 1:1000 secondary antibody (donkey anti-rabbit Cy3; red); Jackson Immuno Research Lab, West Grove, PA) and 1:2000 fluorescent Nissl stain (Neurotrace® green fluorescent Nissl stain; Invitrogen, Eugene, OR). Sections were then mounted in phosphate buffered saline on glass slides and cover slipped immediately using Invitrogen Slow Fade™ Gold (Life Technologies, Burlington, Ontario).

2.5.3. Stereology

HPC Lesion Quantification: In rats that received a neurotoxic lesion, the extent of HPC damage was quantified using unbiased/assumption-free stereology principles and the Cavalieri point-counting method (Mouton, 2002). Using a Nikon Eclipse 80i microscope with a 1600 × 1200 megapixel digital colour camera sending a live video feed to a Dell Precision computer (T3500), the cresyl violet-stained sections containing HPC cell fields (CA1-3, hilus, fasciolarum cinereum, and dentate gyrus; 10 - 12 sections per brain) were examined at a magnification of 2X and with Stereologer 2000 software (Stereology Resource Center Inc., MD). A sampling grid with an area per point of 0.05 mm^2 was randomly superimposed on each section. Grid points intersecting healthy tissue on any of the HPC cell fields were counted for each section. The total number of points counted for each brain was divided by the average count from 5 Sham control rats (Mean = 631, SD = 46.93) and multiplied by 100 to pro-

duce an estimate of the percent of remaining HPC tissue, the complement of which corresponded to the lesion size.

Quantification of Zif268: The estimate for Zif268-positive cells in the HPC of each rat in the inactivation condition (Saline and TTX) was obtained according to unbiased/assumption-free stereology practices using the disector principle [32]. A grid of disectors with a spacing of 500 µm was superimposed on images of each section labelled with Zif268 containing HPC cell fields (10 - 12 sections per brain) at 4x magnification. At each disector that contacted HPC cell fields, magnification was increased to 100×, and Zif268-positive cells were counted within a 7921 μm^2 optical fractionator. Despite the section thickness averaging 35.45µm (SD = 1.45) at the time of quantification, only cells within the middle 15 µm of the tissue were counted. This provided a guard height greater than 5 µm in all sections to avoid quantifying near the cut surfaces of the sections where cells may be cleaved/removed by the blade of the microtome. Additionally, only the tops of cells that came into focus within the middle 15 µm of tissue were counted. These parameters were used to assure that approximately 200 Zif268-positive cells were counted in the HPC, which has been shown to be an ideal number of counted objects to obtain accurate and reliable estimates within a reference space [33]. Finally, the number of cells counted was multiplied by the inverse of 1) the respective section sampling fraction, 2) the area sampling fraction, and 3) the thickness sampling fraction to obtain the estimate of the total number of Zif268-positive cells in the HPC.

2.6. Statistical Analysis

Parametric statistical analyses were used to analyse all group differences. Specifically, independent t-tests were conducted when the experimental instances only involved two groups, whereas ANOVAs followed by LSD post hoc comparisons were conducted when the analyses involved more than two groups or repeated measures. The relationship between behavioural performance and lesion extent was analysed using a Pearson correlation.An alpha level of 0.05 was used as a critical factor for significance in all instances.

3. Results

3.1. Histology and Immunohistochemistry

3.1.1. HPC Lesion Quantification

Photomicrographs depicting representative HPC damage in the Small and Large lesion groups are shown in **Figure 1**, whereas the descriptive data for the lesion size estimates are presented in **Table 3**. One rat in the

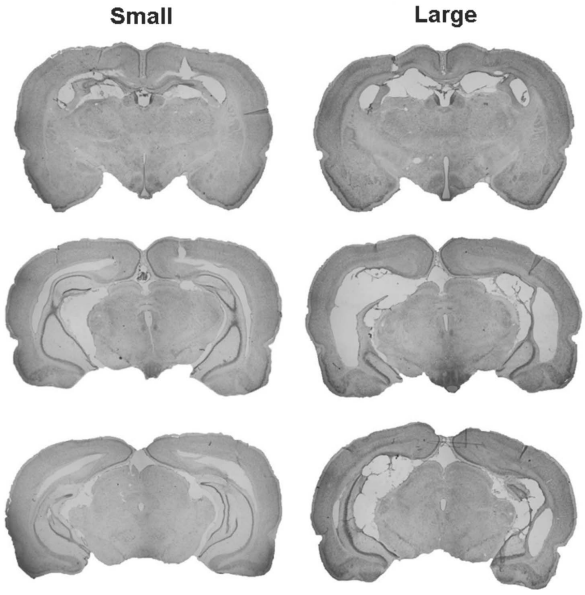

Figure 1. Photomicrographs (2×) of coronal brain sections stained with cresyl violet from a representative Small and Large HPC lesion. The small lesions resulted in damage proximal to the injection sites whereas the large lesions resulted in extensive damage to the entire HPC, leaving only small amounts of spared tissue in the most posterior portion of the HPC.

Table 3. Unbiased stereological estimates of the amount of HPC damage in each lesion group.

Group	n	Mean	SEM	Smallest	Largest
Large lesions	5	79.93%	3.44	68.30%	89.70%
Small lesions	4	50.28%	4.94	38.83%	60.70%

Small group had no noticeable damage to the HPC and was excluded from all analyses. Thus, the final n for the Sham, Small, and Large lesion groups was 6, 4, and 5 respectively. The cell damage in the Large lesion group was extensive and encompassed all HPC cell fields, averaging a lesion size of 79.9% of the HPC. The cell damage in the Small lesion group was largely confined to the areas immediately surrounding the injection sites in the HPC, averaging a lesion size of 50.3% of the HPC.

In the Large lesion group, minor amounts of each of the CA fields were spared in the posterior portion of the HPC, with some dorsal HPC sparing in two rats. The dentate gyrus also tended to have small amounts of sparing in its most anterior and posterior portions. The dorsal subiculum sustained some damage in all the Large lesion rats, with some damage to the ventral subiculum in one rat. In this group, one rat had thinning of the neocortex and some damage extending into the parietal cortex (seemingly following the injection tracks), subiculum, presubiculum, and parasubiculum. In the Small lesion group, the damage was primarily found in the centre region of the HPC, with large portions of all principle cell fields spared. Two rats in the Small group exhibited some damage to the dorsal subiculum, The fornix was spared in all rats in the Large and Small lesion groups.

3.1.2. Injection Sites for HPC Inactivation

The locations of the injection sites for the rats in the Saline control and TTX HPC inactivation groups were within the HPC with the exception of one rat in the TTX group. The data from this rat was excluded from the analyses because one cannula was located outside the HPC, resulting with 3 rats in the Saline group and 4 rats in the TTX group.

3.1.3. Zif268 Cell Counts

A Photomicrograph of Zif268-positive cells in the HPC of a rat from the TTX HPC inactivation group is shown in **Figure 2**. Quantification of Zif268-positive cells throughout the HPC yielded an average of 651.16×10^3 positive cells in the Saline group (SD = 119.82×10^3) and 828.45×10^3 positive cells in the TTX HPC inactivation group (SD = 122.77×10^3). A coefficient of error (CE) was also computed for each rat. The mean CE for the Saline control group was 0.05 (SD = 0.01) and 0.05 (SD = 0.003) for the TTX HPC inactivation group. An independent t-test revealed no significant difference in the number of Zif268-positive cells between the two groups $t_{(5)} = -1.91$, $p = 0.12$, suggesting that HPC inactivation did not suppress Zif268 expression.

3.2. Behaviour

3.2.1. Contextual Fear Conditioning

HPC Lesions: The percent freezing observed during the retention test for each group (Sham, Small, and Large lesion) is shown in **Figure 3**. A one-way between-subjects ANOVA revealed a significant effect of Lesion size ($F_{(2,12)} = 22.63$, $p < 0.001$). Least Significant Difference (LSD) post hoc comparisons revealed that the Large lesion group froze for significantly less time than either the Sham control group ($p < 0.001$) or the Small lesion group ($p < 0.001$). There was no significant difference between the Small lesion group and the Sham control group ($p = 0.87$).

A scatter plot displaying the relation between lesion extent and retention performance in contextual fear conditioning is shown in **Figure 4**. For rats that had lesions, percent freezing was significantly and negatively correlated with percent damage to the HPC (Pearson $r_{(9)} = -0.896$, $p < 0.001$, 1-tailed), suggesting that retrograde amnesia for contextual fear conditioning became more severe as the extent of the HPC damage increased. Indeed, performance appeared to be most affected when the lesion reached or exceeded ~70% of the HPC, suggesting a potential threshold for the degree of damage before memory in this task is impaired.

HPC Inactivation: For conditioned fear, the percent freezing observed during retention testing for the Saline control and TTX HPC inactivation groups are shown in **Figure 5**. An independent t-test failed to reveal a significant difference between the two groups ($t_{(5)} = 0.341$, $p = 0.75$).

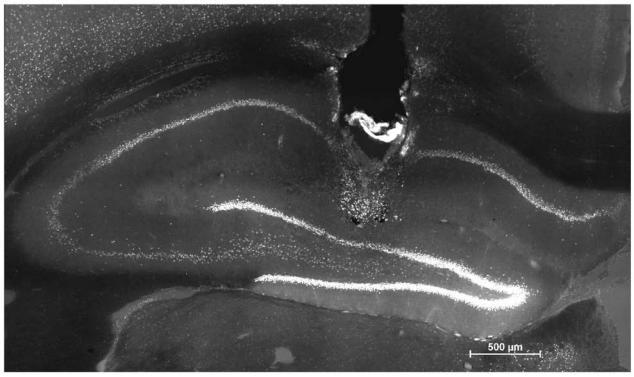

Figure 2. Photomicrograph (10×) of a coronal section of the dorsal HPC showing Zif268 expression surrounding the injection site following TTX inactivation of the HPC. The TTX infusions did not cause any suppression of Zif268 expression.

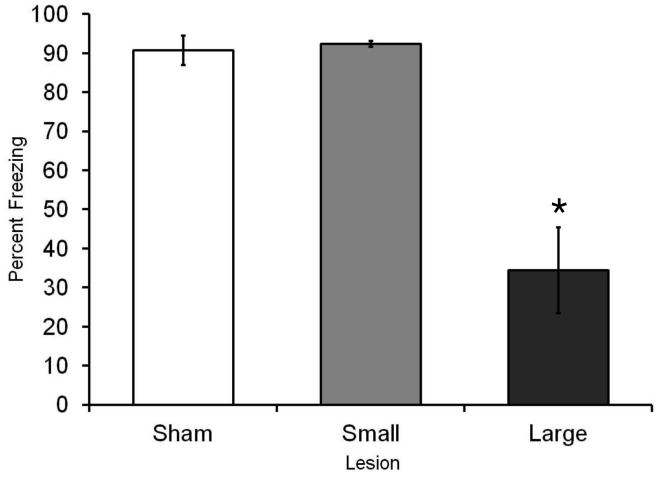

Figure 3. Mean (±*SEM*) percent freezing for the Sham, Small, and Large lesion groups during retention testing for contextual fear conditioning. Only the Large lesion group froze significantly less than the Sham group ($p < 0.05$; *), suggesting that large but not the small HPC lesions caused retrograde amnesia.

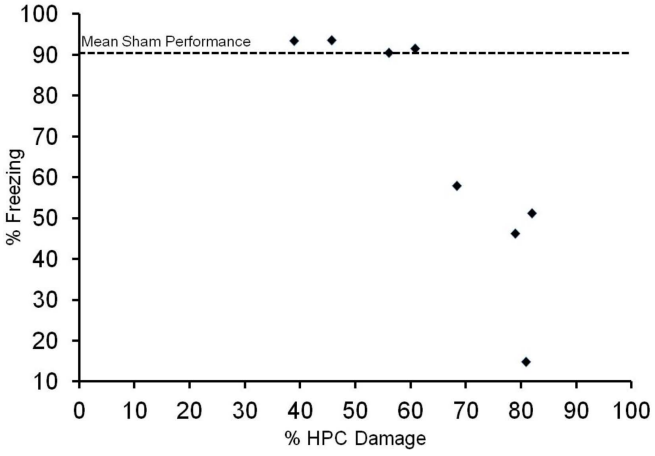

Figure 4. A scatter plot showing the relationship between the amount of HPC damage and freezing behaviour in contextual fear conditioning. The significant correlation ($r_{(9)} = -0.896$, $p < 0.001$, 1-tailed) indicates that as the amount of HPC damage increases, freezing behaviour decreases. In addition, the freezing impairment appears (deviation from mean sham performance; dashed line) when the damage starts exceeding ~70%, suggesting this to be the HPC damage threshold for amnesic effects in this task.

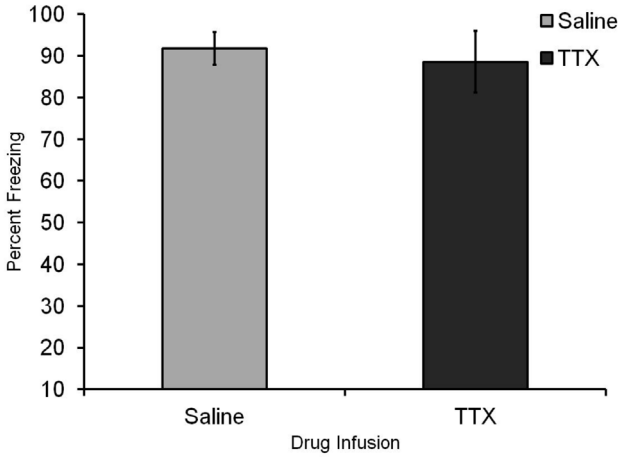

Figure 5. Mean (±*SEM*) percent freezing for the Saline control and TTX HPC inactivation groups during retention testing for contextual fear conditioning. There was no significant difference in the amount of freezing between groups ($p > 0.05$), suggesting that HPC inactivation did not cause retrograde amnesia.

3.2.2. Morris Water Task

For the Morris Water Task, the mean swim distance for the Saline control and TTX HPC inactivation groups was collapsed into 4 blocks (4 trials/block; **Figure 6**). The data for Day 1 were analysed separately from Day 2, using a 2 × 2 mixed design ANOVA with Block as a within-subjects measure and Group (Saline and TTX) as a between subjects measure. The ANOVAs were conducted separately for each day of testing as rats in the HPC Inactivation group only received TTX infusions on the second day. For Day 1, a significant effect of Block was found ($F_{(1,5)} = 33.94$, $p < 0.01$), consistent with the rats in both groups learning the task and using a shorter path to find the hidden platform. For Day 1, neither a significant effect of group ($F_{(1,5)} = 0.18$, $p = 0.69$), nor a significant Block x Group interaction ($F_{(1,5)} = 0.05$, $p = 0.83$) was observed. However, on Day 2 when the HPC was inactivated with TTX, a significant main effect of Group was found ($F_{(1,5)} = 218.7$, $p < 0.001$), with rats in the TTX HPC inactivation group swimming greater distances than those in the Saline control group. For Day 2, neither a significant effect of Block ($F_{(1,5)} = 0.02$, $p = 0.89$) nor a significant interaction between Block and Group ($F_{(1,5)} = 0.59$, $p = 0.48$) was observed.

3.3.3. Comparison of Lesions with HPC Inactivation for Contextual Fear

In order to directly compare the amnesic effects of the HPC inactivation to the lesions on the percent freezing observed during the retention test for each group, all groups (Large, Small, TTX, Control, and Saline) were compared using a one-way ANOVA, which revealed a significant group difference($F_{(4,17)} = 14.45$, $p < 0.001$). LSD post hoc comparisons revealed that the Large Lesion group froze for significantly less time than all the other groups (all p values < 0.001). No other significant differences were observed (all p values > 0.05).

4. Discussion

The purpose of the present study was to directly compare the retrograde amnesic effects of HPC inactivation to HPC lesions in contextual fear conditioning. Large lesions of the HPC (~80%) caused retrograde amnesia, whereas small lesions of the HPC (~50%) and HPC inactivation failed to cause a memory deficit. Importantly, all groups were trained in contextual fear conditioning using the same parameters. Thus, our data suggest that for contextual fear conditioning, HPC inactivation was more similar to the effects of small than large lesions of the HPC. Interestingly, lesion extent and retention performance were strongly correlated and only when the damage began to exceed ~70% did evidence of retrograde amnesia emerge. We therefore argue that the HPC inactivation disrupted less than 70% of the HPC, and that this was insufficient to cause retrograde amnesia for contextual fear under the present conditioning protocol.

The lesion findings are consistent with other studies demonstrating that extensive HPC damage is more likely to cause retrograde amnesia or more severe retrograde amnesia than small lesions [16] [34]-[36]. Although these studies relied on partial lesions of similar size to the ones in the Small lesion group in the current study (~50%), the aforementioned studies limited the lesion to either the dorsal or ventral region of the HPC leaving the other

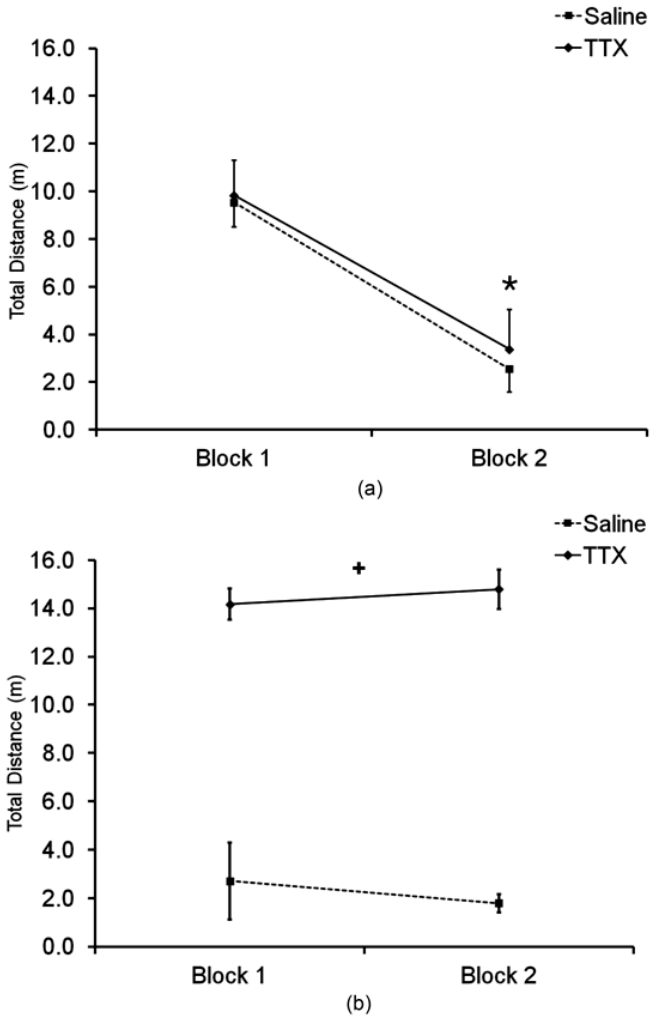

Figure 6. (a) Mean (±*SEM*) swim distance to locate the hidden platform for the Saline and TTX groups collapsed into two blocks (four trials per block) on the first day of training. Note that the HPC was intact on this day as the rats had yet to receive the intra-HPC drug infusions. Both groups showed similar evidence of learning as the distance swam decreased over the trials ($p < 0.05$; *) and these curves did not differ from one another ($p > 0.05$). (b) Mean (±*SEM*) swim distances for the Saline and TTX groups collapsed into two blocks (four trials per block) on the second day of training 30-min after drug infusions. The TTX-infused rats swam significantly greater distances than Saline rats ($p < 0.05$; $^{+}$), suggesting inactivating the HPC impaired spatial memory.

region intact. The remaining region may have provided sufficient HPC functionality to support memory in these studies. Perhaps partial damage that was more equally dispersed across the entire septo-temporal HPC axis would have caused retrograde amnesia comparable to complete HPC lesions. The current findings, however, suggest that this is not the case. We injected small amounts of neurotoxin in both the dorsal and ventral HPC, which lead to partial damage across the HPC axis and still found intact context fear memory.

The observed absence of retrograde amnesia for contextual fear conditioning following inactivation of the dorsal and ventral HPC with TTX, a potent sodium channel blocker that targets cell bodies and fibers of passage, replicates several other reported null results following temporary HPC inactivation in this task [6]-[11]. A likely explanation for these failures to impair memory is that an insufficient amount of HPC tissue was inactivated by the injections to cause amnesia. Contrasting the present freezing results of the HPC inactivation group with the Small lesion group substantiates this argument. Both the Small lesion group and TTX inactivation group received drug infusions that followed the same parameters, meaning the number of injection sites, injection coordinates, drug volume, and injection flow rate were identical, and both showed comparable levels of freezing and

did not significantly differ from the control groups. Also, because these injection parameters only caused 50% cell damage in the Small lesion condition, we speculate that approximately 50% of the HPC was disrupted by the TTX inactivations. Although it is possible that the TTX injections disrupted more than 50% of the HPC, we are confident that it is less than 70% because the correlation between lesion extent and performance revealed that ~70% of the HPC cell fields needed to be damaged in the permanent lesion experiment to reliably cause amnesia in this task. Hence, failures to find behavioural changes following inactivation of the HPC must take these estimates into consideration. This issue is even greater for studies that only examined the effects of dorsal or ventral HPC injections. In these instances, possibly 35% or less of the HPC would have been disrupted-half the maximum estimation of our combined injections.

A number of studies have quantified temporary inactivation extent by assessing the spread of an injected dye through the target structure [37]-[39] or autoradiographic estimates of the spread of radiolabeled drugs [40] [41]. These techniques appear to show that drug infusions of a similar volume to ours diffuse no further than 1 - 2 mm from the injection site [37], meaning that a substantial portion of the HPC is not directly affected by the injections. These estimates are also consistent with our estimate using behavioural change comparisons between HPC inactivation and lesions.

Instead of measuring drug diffusion to quantify the extent of the inactivation, a few studies have quantified changes in immediate early gene expression following HPC inactivation [11] [42]-[45]. The advantage of this technique is that it provides an indirect assessment of neural activity across the HPC, meaning including and beyond the area directly affected by the spread of the injected drug. In these studies, substantial decreases in immediate early gene expression are reported, including one study suggesting that the disruption is greater than the estimate from the drug diffusion studies cited above (up to 80% HPC disruption with combined dorsal and ventral HPC inactivation; [44]). We similarly examined the effects of the TTX injections on immediate early gene expression to estimate the extent of the HPC disruption. Specifically, we labeled for the protein Zif268 in rats that were sacrificed 90 min after the TTX injections and 60 min after swimming in the Morris Water Task, a behavioural test known to cause increases of immediate early gene expression in the HPC [46]. However, we found no decrease in Zif268 expression following the TTX injections across the HPC or in proximity of the injection location. This is surprising given the fact that TTX HPC inactivation markedly affected spatial memory in the Morris Water Task, demonstrating the efficacy of the TTX injections in disrupting HPC function. We have additional pilot work suggesting that neither altering the time course between TTX injections and perfusion/euthanization (10 min, 30 min, or 60 min) or that labeling for a different protein, such as c-Fos, lead to more success with this method in our laboratory.

Noteworthy, two of the studies reporting immediate early gene suppression following HPC inactivation, including the 80% HPC disruption, found it following seizure induction (electroconvulsive shock or kainic acid injection) to promote extensive c-Fos expression [44] [45] and not following behaviourally-induced immediate early gene expression as in this study. Although Broadbent *et al.* [43] reported that HPC inactivation successfully suppressed immediate early gene expression within the region of the injection following behavioural testing, their inactivations were chronic (7 days) rather than acute. Our findings contrast most, however, with those of Kubik *et al.*'s [42] study in which the same behavioural task and inactivating compound were used. They report decreased Arc mRNA expression in the HPC around the TTX injection site after testing in the MWT. Perhaps the assessment of mRNA in their study vs. protein in this study accounts for the discrepancy. Indeed, immediate early gene protein levels are influenced by more factors than mRNA levels and mRNA assessment may provide a more accurate measure of changes in neural activity than protein assessment because of a lower signal-to-noise ratio [29]. The protrusion of the injection cannula beyond the guide cannulae in the present study may also have mitigated the chances of finding TTX-induced suppression in Zif268 because Gulbrandsen & Sutherland [44] were only successful in detecting decreases in immediate early gene expression when the injector remained flush with the chronically implanted guide cannulae. Regardless of the specific factor preventing us from finding a reduction in immediate early gene expression following the TTX injections, we emphasize that the method cannot easily be used to assess the extent of the inactivation. Moreover, our comparison between the behavioural effects of the TTX injections to permanent lesions suggests less than 70% HPC disruption with combined dorsal and ventral inactivation.

Although several laboratories and we have failed to find retrograde amnesia for contextual fear conditioning following HPC inactivation [6]-[11], others do report impairments [11]-[15]. These discrepant findings may be accounted for by differences in the fear conditioning protocols used across studies that lead to memories of

varying strength, and thus varying vulnerability to HPC disruption. For instance, Gulbrandsen *et al*. [11] found that HPC inactivation failed to cause retrograde amnesia in rats that had received six context-shock pairings, but successfully caused amnesia in rats that had only received three. Therefore, it is not that inactivation of HPC cannot cause retrograde amnesia for context fear memory, but it seems to be under a narrow range of conditioning parameters and weaker context fear memories.

In the current study, there was a minor procedural difference between the permanent lesion experiment and the temporary inactivation experiment. The conditioning-to-test interval was seven days longer in the lesion experiment. This difference occurred because we matched the conditioning-to-HPC disruption (TTX injections or NMDA injections) in both experiments and the neurotoxic lesion rats required a post-surgery recovery period following the disruption. Although this one-week difference could slightly alter retention performance, we believe it is a negligible factor and that the conditioning-to-test interval was a much more critical variable to match when designing the study.

Despite the TTX HPC inactivation failing to cause retrograde amnesia for context fear memory, it caused pronounced retrograde amnesia on the hidden platform version of the Morris Water Task. During the first day of training on the spatial memory task in the pool, all rats showed a marked decrease in the distance required to find the platform, suggesting learning of the platform location. This was expected because the HPC was intact during this learning session and also demonstrates that the prior fear conditioning experiment did not prevent learning in this task. However, on the second day when the HPC was inactivated, the TTX group showed impaired memory as they swam significantly greater distances than the control group to find the platform. Moreover, the performance of the inactivation group closely resembled that of their initial learning trials on the first day of training when they were completely naïve to the platform location. Thus, the dissociable effects of HPC inactivation on context fear and spatial memory in this study suggest that spatial memory is more sensitive to HPC disruption than context memory. The underlying account for this difference is unclear because both tasks require the acquisition, retention, and use of configural information [47]. The difference, however, may simply lie in the task demands. The Morris Water Task requires recognition, triangulation of a location, and location updating using extra-maze cues during navigation [48] [49], whereas contextual fear condition may simply involve recognition processes. Thus, context memory would require less cognitive processing and would be less susceptible to HPC interference than spatial memory requiring navigation.

5. Conclusion

In summary, the present study found that combined bilateral infusions of the sodium channel blocker TTX into the dorsal and ventral HPC spared memory for contextual fear conditioning. We argue that this null finding likely comes as a result of too little HPC tissue being disrupted by the inactivation. Specifically, we estimate the disruption to be less than 70% of the HPC, based on the relationship we found between permanent lesion size and performance, in which damage below this threshold did not lead to significant impairments. Temporary inactivations confer several important advantages and make possible a range of research designs not available when using permanent lesions (see [50] for review). However, there are limitations with respect to the effectiveness or extent of the inactivation that can lead to insufficient amnesic effects or behavioural effects. Thus, inactivation findings at odds with permanent lesion findings should be interpreted with caution. Moreover, we conclude that inactivation of the HPC, whether targeting the dorsal HPC, ventral HPC, or both combined, is more comparable to small rather than large HPC lesions.

Acknowledgements

We thank the Natural Sciences and Engineering Research Council of Canada (NSERC; 355934; http://www.nserc-crsng.gc.ca) and the Canada Foundation for Innovation (CFI; 29438; http://www.innovation.ca) for funding this research.The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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