ABSTRACT

DORSAL HIPPOCAMPUS INFUSIONS OF CNQX INTO THE DENTATE GYRUS DISRUPT EXPRESSION OF TRACE FEAR CONDITIONING

by Jamie L Pierson

Few studies have specifically looked at dorsal hippocampus (DH) subregion (CA1, CA3, dentate gyrus) involvement in trace fear conditioning. In the present study, rats received a bilateral cannulation targeting one of the three DH subregions. Following surgery, animals were trace conditioned. Forty-eight hours following training, rats received an infusion of the CNQX or vehicle. Ten minutes following the start of the infusion, rats were placed in a novel context for the tone test. Rats that received CNQX into the dentate gyrus froze significantly less to the tone than control rats. Rats that received CNQX into the CA1 or CA3 subregion showed no difference in freezing to the tone compared to controls. These data support a role for the dentate gyrus in the expression of trace fear conditioning. This is different from the pattern observed for context fear conditioning, suggesting dissociable processes within the DH for trace and context fear.

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GYRUS DISRUPT EXPRESSION OF TRACE FEAR CONDITIONING

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Jamie L. Pierson
Miami University
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Advisor _____
Jennifer Quinn
Reader _____
Stephen Berry
Reader _____
Dragana Clafin

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Introduction

The hippocampus makes considerable contributions to the consolidation of explicit (also called declarative) long-term memories (e.g., Scoville and Milner 1957; O'Keefe & Nadel, 1978; Squire 1982; Zola-Morgan et al., 1986; Zola-Morgan and Squire 1990; Kim and Fanselow 1992; Kim et al. 1995; Quinn et al. 2008; Broadbent et al., 2010; Parsons and Otto 2010; Gaskin et al. 2011). Damage to the medial temporal lobe, including the hippocampus, diminishes a patient's ability to form new memories and recall some events from the past, while sparing the implicit (nondeclarative) memories, showing that not all memories are hippocampus-dependent (Scoville & Milner, 1957; O'Keefe & Nadel, 1978; Mishkin, 1978; Mahut, Zola-Morgan, & Moss, 1982; Squire 1982; Malamut, Saunders, & Mishkin, 1984; Zola-Morgan et al., 1986; Zola-Morgan & Squire, 1986; Zola-Morgan and Squire 1990). Additional research has shown that these hippocampal-dependent memories require the patient to be aware of the stimulus contingencies during training in order to acquire the memories (e.g., Clark and Squire, 1998; Manns et al., 2000; Clark et al., 2002). Patients with more select damage to the hippocampus alone had many of the same memory deficits as those who had more extensive damage to the entire temporal lobe (Zola-Morgan et al., 1986). Many of the memory deficits seen in human patients have been replicated in animals using lesions and inactivations of the hippocampus and/or surrounding structures. Such manipulations allow for more targeted disruption of the hippocampus and for precise quantification of the extent of damage.

Pavlovian fear conditioning is a widely used model to study the neural mechanisms of learning and memory and is a commonly used model for studying hippocampus-dependent memories (Ribot 1882; Kim and Fanselow, 1992; Squire 1992; Knowlton and Fanselow 1998). Discrete cue conditioning occurs when a conditional stimulus (CS), such as a tone, is paired with an unconditional stimulus (US), such as a footshock. Following conditioning, the CS elicits an unconditional response (UR), such as freezing. Delay conditioning involves the presentation of a CS that overlaps and co-terminates with a US. Trace conditioning is very similar, except that a stimulus-free trace interval separates the CS and US presentations. Trace conditioning depends upon an intact hippocampus, whereas delay conditioning typically does not (Selden et al., 1991; Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Quinn et al., 2002; but see Quinn et al., 2008; Maren, 2008). In addition to fear elicited by the discrete CS, the conditioning context itself acts as a CS and has the ability to elicit a fear response. Fear memories are a reliable way in

which we can study learning and memory because they are very stable, remaining intact for at least 16 months following conditioning (Gale et al., 2004, Quinn et al., 2008).

The role of the hippocampus in the expression of fear memories is time-limited (Ribot 1882; Squire 1992; Knowlton and Fanselow, 1998, Quinn et al., 2008). Kim and Fanselow (1992) showed that rats receiving electrolytic dorsal hippocampal lesions 1 day following delay fear conditioning did not display freezing to the context in which they were conditioned, while those rats receiving hippocampal lesion 7, 14, or 28 days following conditioning did display context-specific freezing behavior. Those rats that received a lesion 28 days following training showed freezing similar to that of control animals. On the other hand, hippocampal lesions had no effect on freezing to the tone regardless of the duration of the training-to-lesion interval. This provides support for hippocampus involvement in some, but not all, types of fear memories.

The hippocampus has often been studied as a unitary structure, but there has been reason to look at its different subregions based on anatomical, physiological, and behavioral evidence (Treves and Rolls, 1994; McClelland and Goddard, 1996; Wiebe et al., 1997). Anatomically, the hippocampus can be distinguished in several ways. Studies have shown that there are dorsal and ventral differences in anatomy of inputs and outputs as well as behavioral functioning (Swanson and Cowan, 1977; Moser and Moser, 1998; Fanselow and Dong, 2010). These distinctly different inputs, which include two distinct perforant paths from the entorhinal cortex, and outputs may lead to the possible differences in functionality (Swanson and Cowan, 1977) where the dorsal hippocampus is most associated with memory and cognition while the ventral hippocampus has been associated with emotion (Kim and Fanselow, 1992; Henke, 1990; Squire et al., 2001; Dedovic et al., 2009). In addition to dorsal/ventral distinctions in the hippocampus, there are distinctions among the three subregions; CA1, CA3, and the dentate gyrus. These anatomical differences have been studied since Ramón y Cajal (1901) and Lorente de No' (1934) looking at the morphological differences in cell types. In addition to the fact that the hippocampus can be divided into these subregions, distinctions have been made based on whether they are located dorsally or ventrally using gene expression research in the C57Bl/6 mouse (Thompson et. al., 2008; Dong et al., 2009; Fanselow and Dong 2010). This gene expression supports the anatomical and behavioral differences that have been seen in previous studies, and serves to clarify boundaries and inconsistencies in previous literature in defining the regions of the hippocampus (Fanselow and Dong, 2010).

Theoretical computational modeling based on anatomical connections in the hippocampus among subregions and connections to neighboring structures have been used to model possible functions of each distinct subregion during the formation and retrieval of memories (Treves and Rolls, 1994; McClelland and Goddard, 1996; Wiebe et al., 1997). Many of the computational models, examining the role of the hippocampus in context conditioning, identify the CA3 region as the autoassociation recurrent network that will form rapid and arbitrary associations among hippocampal afferent inputs and circulate the information within its own network (Lee and Kesner, 2004; Treves and Rolls, 1994; McClelland and Goddard, 1996; Wiebe et al., 1997). This rapid association would allow the CA3 region to form an initial representation of spatial cues during acquisition, and store it for a short time before sending the information on for further processing. The dentate gyrus seems to function as a pattern separator that can parse input patterns that need to be further differentiated from each other before they reach CA3 (Lee and Kesner, 2004; Treves and Rolls, 1994). Together CA3 and the dentate gyrus may play a critical role in the acquisition of a context-specific memory, which has been supported in behavioral research (Lee and Kesner, 2004; Hernandez-Rabaza et al., 2008). Traditionally, CA1 has been thought of as an output region, but more recent findings have shown that it may do additional computational processes prior to outputting information, including modulating additional feedback inputs to the hippocampus due to the bidirectional pathway between the entorhinal cortex and CA1 (McClelland and Goddard, 1996; Hasselmo et al., 2000; Lee and Kesner, 2004).

Electrophysiological data have also been used to look at the differential involvement of hippocampal subregions during both acquisition and retrieval. Single neurons in the dentate gyrus have been found to display a progressive increase in their firing rates over days of training to CS and US during trace fear conditioning, while CA1 neural firing rates increase at the beginning of training but, as days elapse, develop a learning related decrease in firing rate to both CS and US. Correlation analyses show that the dentate gyrus and CA1 have an inverse pattern of firing to the CS during trace fear conditioning, where the dentate gyrus firing rate increases and CA1 firing rate decreases. This pattern is maintained during retrieval tests (Gilmartin and McEchron, 2005). CA1 neurons have also been implicated in the encoding of the trace interval itself. Rabbits who were conditioned with a trace interval of 10 or 20 second trace periods in a

tone shock paradigm had CA1 firing during the tone test that matched the duration of the trace interval that was not seen in pseudoconditioned animals (McEchron et al., 2003)

In addition to previously mentioned studies, researchers have examined direct manipulations of the hippocampal subregions that shed light onto their possible functions during specific types and time points of memory formation and retrieval. These hippocampal subregional differences have been studied in context conditioning experiments (Lee and Kesner, 2004; Hernandez-Rabaza et al., 2008). Lee and Kesner (2004) showed that pretraining lesions of CA1, CA3 or the dentate gyrus all individually caused a deficit in acquisition of context fear, with CA1 and dentate gyrus lesioned animals sustaining the impairment over repeated acquisition sessions, and CA3 only showing the deficit during the first acquisition session. Eventually all lesion groups did acquire to similar levels. Twenty-four hours following training, context tests showed that animals with CA1 and dentate gyrus lesions showed a freezing deficit while CA3 lesions did not. In addition, pretraining colchicine lesions, which specifically target the dentate gyrus, produce deficits in acquisition of context fear conditioning (Goldschmidt and Steward, 1980; Hernandez-Rabaza et al., 2008).

Temporary inactivation and lesion studies have also looked at these hippocampal subregion differences, both dorsal versus ventral and CA1, CA3, and dentate gyrus using pretraining infusions and lesions. These studies have shown that the dorsal hippocampus is critical for the acquisition, consolidation, and expression of trace fear conditioned memories, but not other types of conditioning such as delay (McEchron et al., 1998; Quinn et al., 2002). Both the acquisition and expression of trace fear conditioning specifically relies on the contribution of hippocampus NMDA receptors and Arc gene activation, which is not the case for all types of Pavlovian conditioning (Quinn et al., 2005; Czerniawski et al., 2011). Yoon and Otto (2007) compared pretraining lesions of the dorsal or ventral hippocampus and showed that lesions of the dorsal hippocampus (DH) had no effect on freezing to the tone in trace fear conditioned rats, while lesions of the ventral hippocampus (VH) did. Upon closer inspection of the lesions, the DH lesions were very extensive, while the VH lesions were less so. The smaller of the VH lesions targeted the area of the dentate gyrus, sparing CA1. In post training lesions, both DH and VH lesions impaired expression of fear to the tone. In another study, researchers used infusions of mucimol, a GABA-A agonist, to temporarily inactivate either the DH or the VH prior to training or tone testing in trace conditioned rats. Pretraining inactivation of VH, but not DH,

produced a deficit in acquisition of trace fear conditioning. Similarly, pretest inactivation of VH, but not DH, disrupted the expression of fear trace fear memories (Czerniawski et al., 2009). Upon inspection of this histology, the DH infusions appeared to target CA1, and the VH infusions often targeted the dentate gyrus. Czerniawski and colleagues have also found that NMDA receptor agonist (APV) infused in either the DH or VH pre-training disrupted acquisition of both trace and contextual fear, but infusions pre-test only disrupted retrieval of trace and contextual fear when infused into the VH, not DH (Czerniawski et al., 2012). Since there were differences in the subregions targeted in both the lesion and the infusion studies, we hypothesized that it may not be DH and VH differences, but rather subregional differences. To determine if that could indeed be the case, we inactivated each of the three hippocampal subregions prior to retrieval of a trace fear conditioned memory. We hypothesized that the inactivation of the dentate gyrus would have an effect on expression, but inactivation of CA1 or CA3 would not. These predictions are tested in the present study, as all three subregions have yet to be examined in trace fear conditioning in the scope of a single experiment.

Materials and Methods

Subjects

One hundred six (thirty-seven following histology) experimentally naive male Long-Evans rats (250-325g; Harlan Laboratories) were pair-housed in standard colony caging on 12:12 light:dark cycle, with food and water available *ad libitum*. All animals were handled for approximately 60 seconds per day the week prior to surgery and prior to the start of behavioral testing. All behavioral procedures were conducted during the cycle and were approved by Miami University IACUC.

Surgery

Animals were anesthetized in an induction chamber with 5% isoflurane (Vedco, St. Joseph, MO) in an induction chamber. Following induction, rats were placed on a standard stereotaxic apparatus (Kopf Instruments) with a nose cone for continuous 2-3% isoflurane maintenance. The scalp was shaved, incised, and retracted, The skull leveled in the horizontal plane by equating bregma and lambda prior to holes drilled into the skull above the desired cannula placement site. Dorsal hippocampus coordinates for bilateral guide cannula placement

were as follows: CA1 (AP: -3.5; ML: ± 2.5 ; DV: -3.2), CA3 (AP: -3.5 ML: ± 4.1 ; DV: -3.4), dentate gyrus (AP: -3.5; ML: ± 2.1 ; DV: -3.7) relative to bregma. Four skull screws were used to anchor dental acrylic used to stabilize the cannula. Obturators were placed into the guide cannulae to prevent debris from entering. All animals were also administered Rimadyl (5mg/ml in 0.9% saline, 1 ml/kg s.c.) as an analgesic and saline (3ml of 0.9%) for rehydration. Rats were then placed on a heating pad until they woke up, at which point they were returned to their home cage.

Apparatus

Eight observation chambers (32.4L x 21.6H X 25.4D cm; MED-Associates, Inc., Georgia, VT) were used for all phases of the experiment. Animals were fear conditioned and context tested in one of four identical chambers are located in the Context A room.. In each of these chambers the front door and ceiling are made of a clear Plexiglas, the sidewalls are aluminum, and the back wall is white plastic. The floor consisted of 19 equally spaced stainless steel rods wired to a shock generator and scrambler (MED-Associates, Inc.) A stainless steel pan beneath the grid floor was coated with a diluted vanilla flavor (50% in water, Meijer) to provide a distinct odor, and each chamber was wiped down with a 5% NaOH solution. The lighting in the chamber consisted of a white house light (125 lux), and a fan provided background noise (65 dB).

Animals were tested for freezing to tone in context B. These chambers (32.4 x 25.4 x 21.6 cm; MED-Associates, Inc.) were located in a different experimental room and were distinct from Context A. They consisted of a white Plexiglas floor, a black Plexiglas triangular insert forming the side walls with a peak in the center. The context was cleaned and scented with a 5% glacial acetic acid solution. The light box above the training chamber provided near infrared lighting (0 lux) for the purpose of the cameras.

During all training and testing rats were continuously monitored by a progressive scan video camera with a visible light filter (VID-CAM-MONO-2A; Med-Associates, Inc., St. Albans, VT) connected to a Windows computer in the experimental room running Video-Freeze software (Med-Associates) designed for automated assessment of defensive freezing and motion (Anagnostaras et al., 2010).

Drugs and Infusions

Injectors (33gauge) were connected to 10 μ l Hamilton syringes using clear polyethylene tubing (PE20). The injectors were inserted into the cannulae so that they extended 0.5 mm below the guide for CA1 and 1.1 mm below the guide for CA3 and the dentate gyrus. All infusions were delivered via an infusion pump (KD Scientific, Inc., Holliston, MA) at a rate of 0.5 μ l/min for 2 min. Rats were placed in plastic bins with approximately 3 cm standard bedding during infusions, and were left for 2 min following infusion to allow for diffusion. Animals were in one of two treatment groups: the AMPA/kainate receptor antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione (1% CNQX dissolved in 10% DMSO in aCSF, Tocris, Minneapolis, MN), or vehicle (10% DMSO in aCSF).

Procedure

Rats were trained in Context A using a trace fear conditioning program that consists of a 180 s acclimation period to the chamber followed by 10 pairings of tone (16 s; 2 kHz) and footshock (2 s; 0.9 mA) using a 28 s trace interval between tone offset and shock onset. The inter-trial interval was 256 s from tone onset to tone onset. Rats remained in the training context for 210 s after the final shock before being removed and returned to their home cages.

Twenty-four hours following conditioning animals were returned to the training context (Context A) and the freezing was measured for 8 mins. Forty-eight hours following training, animals received the infusion through an injector placed into the guide cannula. Ten minutes following the start of the infusion, rats were placed into the novel tone test chamber (Context B). Following a 180 s baseline period, the rats were presented with 3 tones (same 16 s tone presented in the training session). Each tone was followed by an inter-trial interval of 256 s from tone offset to tone onset. Rats remained in the box for an additional 240 s following the offset of the final tone. Freezing was measured during the 3 tone and post tone periods.

Histology

Following all behavioral testing, animals were anesthetized with 0.2 ml Euthasol i.p. (Virbac Animal Health, Inc., Fort Worth, TX; 390 mg pentobarbital sodium + 50 mg phenytoin sodium per ml). and to visualize cannula placements, a 10% Cresyl violet acetate (10% in distilled water; Sigma-Aldrich, Inc.) was infused at each infusion site and the same rate and duration as the drug infusions. The rats were then perfused with 0.9% saline followed by 10% formalin. The brains were removed and stored in a 10% formalin/30% sucrose solution for at least 2 days. Brains were frozen and sliced into 50 μ m coronal sections on a cryostat ($-18 \pm 1^\circ\text{C}$),

mounted on glass slides, and stained using 0.5% thionin (Sigma-Aldrich, Inc.), coverslipped, and infusion locations were verified using a light microscope (Fisher Scientific Steromaster Microscope, MicronUSB)

Behavioral analysis

Throughout conditioning, context testing, and tone testing, defensive freezing was observed and recorded. Freezing can be defined as the absence of movement (except for respiration) with significant muscle tone, and is a reliable measure of conditional fear (e.g., Fanselow & Bolles, 1979). Freezing data were analyzed using one-way or repeated measures ANOVAs with infusion site or drug used as between-subjects factors. *A priori* planned comparisons were made using Fisher's PLSD. An alpha value of 0.05 was used for all analyses.

Results

Histology: Figure 1 shows a histological representation of drug infusion placements, as well as a photograph of a representative sample of a coronal section that was deemed for each placement. There were a total of 61 misplaced infusions, 14 dentate gyrus, 15 CA1, and 32 CA3. Animals in which the infusion was misplaced were excluded from statistical analyses.

Context test: The percentages of time spent freezing over the 8-minute context test are presented in Figure 2. Rats were matched based on their context freezing to drug condition creating no significant difference based on the context freezing levels between future drug groups [$F(5,31)=0.075$, $p=0.996$].

Tone Test: During the first three minutes of the tone test baseline freezing was assessed. An ANOVA revealed no significant difference in drug infusion or location during the baseline period, [$F(5,31)=0.579$, $p=0.716$]. *A priori* planned comparisons between the average freezing during the three tones were conducted and there was a significant difference in freezing between the vehicle and CNQX condition at the dentate gyrus infusion location, with CNQX animals freezing significantly less [$t(17)=2.118$, $p=0.049$], but not CA1 [$t(10)=-0.083$, $p=0.935$] or CA3 [$t(4)=-0.873$, $p=0.432$], these data are represented in Figure 3. The average post-tone period was broken up into 16-second bins for analysis, and are presented in Figure 4. Repeated measures revealed that infusion of CNQX into the dentate gyrus reduced post-tone freezing compared to vehicle infused controls [$F(1,17)=4.739$, $p=0.044$]. There is also a time by drug interaction with the CNQX infused animals freezing significantly more than vehicle controls at the beginning of the post-tone period (bins 1-7) and leveling off to the same amount of freezing as vehicle

controls by the end of the post-tone period [$F(14,238)=2.854$, $p=0.001$]. There is also a significant effect of time for the CA1 region in the during the post-tone period [$F(14,140)=5.906$, $p<0.005$], with freezing decreasing over time in both vehicle and CNQX groups, but there was no significant differences in drug groups [$F(1,10)=1.651$, $p=0.228$ }, and no interaction [$F(14,140)=0.594$, $p=0.866$]. There is also a significant effect of time for the CA3 region during the post-tone period [$F(14,56)=2.644$, $p=0.005$], with freezing decreasing over time. There were no significant differences in drug [$F(1,4)=0.328$, $p=0.597$ }, and no interaction [$F(14,56)=1.065$, $p=0.407$].

Motion was scored automatically during the first three minutes (baseline) of the tone test (see Figure 5). Overall, there was no difference in freezing between the three subregions [$F(2,31)=.257$, $p=.775$]. Repeated measures ANOVA showed that there was also no significant difference in motion during the three minute baseline period between CNQX and vehicle-infused animals for any DH subregion [dentate: $F(1,17)=.127$, $p=.726$; CA1: $F(1,10)=1.638$, $p=.229$; CA3: $F(1,4)=1.534$, $p=.238$]. All units for motion index are arbitrary units (Anagnostaras et al., 2010).

Discussion

In the present study, we examined the effects of inactivation on three DH subregions in expression of trace fear conditioned memories. We observed that infusions of CNQX into the dentate gyrus region of the DH disrupts expression of trace fear conditioned memories, but similar infusions into CA1 or CA3 do not. Infusion of CNQX into the dentate gyrus also disrupts freezing during the post-tone period, while rats infused with CNQX into CA1 or CA3 show similar levels of freezing as vehicle controls. To our knowledge, this is the first study to demonstrate the dentate gyrus' critical role in expression of trace fear memories as well as post-tone freezing. These data indicate that the DH does not function as a unitary structure as it has often been treated in the past, but each subregion plays a unique role in expression. Previous studies have also shown that DH manipulations can cause hyperactivity, so we examined the motion index of the first three minutes of the tone test to determine if the differences in freezing could be explained by hyperactivity, rather than fear memory (Blanchard, Blanchard, Lee, & Fukunaga, 1977; Douglas & Isaacson, 1964; Maren et al., 1997; Nadel, 1968; Teitelbaum & Milner, 1963). We show that there is no difference in motion in any subregion regardless of drug

condition.

The hippocampus can be divided into subregions based on function and anatomy. These regions include the DH and VH, with the DH being more closely associated with cognitive functions and the VH with stress and emotion (Kim and Fanselow, 1992; Fanselow, 2000; Gray and McNaughton, 2000; Dedovic et al., 2009; Fanselow and Dong, 2010). Our data are consistent with previous studies showing that the DH is necessary for the acquisition and expression of trace fear conditioning (McEchron et al., 1998; Quinn et al., 2002; Quinn et al., 2005; Czerniawski et al., 2011). Additional investigations suggest further delineation of function within the dentate gyrus, CA1, and CA3 cell populations of the hippocampus. Cells in the dentate gyrus have a learning related increase in firing to both the CS and US during trace fear conditioning. Cells in CA1 actually decrease their firing rate over days of acquisition trials to both the CS and US compared to their initial firing rates, and these patterns are maintained during expression (Gilmartin and McEchron, 2006).

Additional, prior research involving context only conditioning (i.e., unsignaled) shows a slightly different pattern from our trace tone data. Lee and Kesner (2004) found that pretraining lesions of either CA1 or the dentate gyrus effect expression, but lesions of CA3 do not. Lesioned rats were slow to acquire context conditioning but eventually reached the levels of controls. Further, rats with lesions to the CA3 acquired more quickly than those who had lesions to either CA1 or the dentate gyrus (Lee and Kesner, 2004). The CA3 of the DH has been shown to play a role in the early stages of contextual fear acquisition, but not in later recall (Lee and Kesner 2002, 2004).

To further support our data, Liu et al. (2012) used optogenetics in mice to label cells in the dentate gyrus that were activated during acquisition of fear conditioning and then later optically reactivated these same cells in a different environment. Mice showed an increase in freezing upon light stimulation alone, which was not seen in fear conditioned mice that were exposed to a non-fear conditioned context or in non-fear-conditioned mice. This optogenetic manipulation was sufficient to activate downstream areas such as CA3 and amygdala. This light-induced freezing also upregulated c-Fos expression in the amygdala and this immediate early gene expression has been shown to correlate highly with freezing levels (Ramirez et al., 2012). Both optogenetic studies support a crucial role for the dentate gyrus in the expression of fear conditioned memories in an intact hippocampus.

Our data appears to differ from other data on DH involvement in the expression of fear memories. Previous findings have shown that inactivations or lesions of the DH had no effect on trace or contextual fear conditioning (Rogers et al., 2006; Yoon and Otto, 2007; Czerniawski et al., 2009). Judging from the histology representations in their publications, manipulations targeting the DH were primarily targeting CA1, while manipulations of the VH were primarily targeting the dentate gyrus. With the histological representations taken into consideration, the data is consistent with ours, as targeting the CA1 region of the DH has no effect on the expression of trace fear conditioning. It remains to be determined whether a similar subregion difference exists in VH.

The dentate gyrus plays a functional role in orthogonalizing (i.e., making more different) the input from the entorhinal cortex (Andersen et al., 2007). Each dentate gyrus granule cell communicates with only about 15 CA3 pyramidal cells, but each pyramidal cell will receive input from about 70 granule cells (Amaral et al., 2007). Neural networking studies suggest that the dentate gyrus is taking the information received from the entorhinal cortex and is generating a “sparse code” that it transmits to CA3. This ability for the dentate gyrus to take input coming from the entorhinal cortex and turn it into a sparse code is what has given the dentate the functional distinction of a “pattern separator”. As mentioned previously, the CA3 is considered as a powerful autoassociating recurrent network that can form rapid arbitrary associations among hippocampal afferent inputs and circulate the information within its own network. Because of the proposed role of CA3 in associating arbitrary inputs and holding the association for a short period of time, the CA3 is ideally suited for the formation and temporary maintenance of configural representations, as has been shown in contextual fear conditioning (Hess et al., 1995; Lee and Kesner, 2004). However, recent work has shown that the dentate gyrus may be involved in both pattern separation and pattern completion, depending on the age of the granule cell. Mice who have older granule cell output inhibited, but leaving the majority of the younger adult granule cell output intact showed a normal or increased ability to distinguish between two similar contexts (pattern separation), but a deficit in pattern completion. This notable ability for pattern separation was lost when the younger adult granule cells were ablated, suggesting that these cells are involved in pattern separation (Nakashiba et al., 2012). This would implicate dentate gyrus involvement more broadly and may explain the very detrimental effects of glutamatergic receptor antagonism on the expression of trace fear conditioning.

The projections from the dentate gyrus to CA3, followed by CA3 projections to CA1 constitutes the “trisynaptic loop”, the primary source of neurotransmission within the hippocampus. In addition to the recurrent collaterals within CA3 and the output to CA1, CA3 also projects to the contralateral CA3 region as well as the lateral septal nucleus, some of these connections are glutamatergic, and interestingly the lateral septal nucleus is involved in the acquisition of delay tone fear conditioning. Infusions of glutamic acid into the lateral septal nucleus promotes conditioning to the tone, while the antagonist, kynurenate, disrupts conditioning (Calandreau et al., 2007; 2010).

The projections from the dentate gyrus to the CA3 region of the DH are not uniform throughout the hippocampus. CA3 pyramidal neurons closest to the dentate gyrus granule cells receive mossy fiber inputs onto both the apical and basal dendritic trees, whereas those pyramidal cells located more distally in CA3 only have mossy fiber inputs onto the proximal portion of the apical dendritic tree (Blackstad et al., 1970, Andersen et al., 2007). The mossy fiber inputs targeting these distal CA3 pyramidal cells are originating from only the infrapyramidal blade of the dentate gyrus, rather than both the suprapyramidal and infrapyramidal blades (Andersen et al., 2007). This distinction may help to clarify the lack of effect following CNQX infusions into CA3 in our study. By targeting the most lateral portion of CA3, it is possible that we have inadvertently preserved the area of CA3 that is most critical in expression of trace fear conditioned memories.

The dissociations between hippocampal subregions during the acquisition and expression of fear memories may be, in part, due to differences in inputs to the distinct hippocampal subregions coming from the entorhinal cortex. Entorhinal projections to dentate gyrus and CA3 both originate from the perforant path which is composed of outputs from layer II of the entorhinal cortex, implying that the information coming from the entorhinal to dentate gyrus and CA3 is similar. This differs from the entorhinal input to CA1, which originates from layer III, rather than layer II (Andersen et al., 2007). Both layer II projections may be necessary for the expression of trace conditioned memories, whereas layer III projections may be necessary for expression of context memories based on context conditioning data (Lee and Kesner, 2004). As far as layer III’s role in the expression of trace fear conditioning, Shu et al. (2011) found that transgenic mice who had the layer III input from the entorhinal cortex specifically inhibited during the acquisition of trace fear conditioning froze significantly less than controls during

acquisition and expression of the tone memory. On the other hand, when entorhinal layer III was functioning properly during acquisition, and only inhibited during the tone test, there was no difference in freezing between mutants and controls. While our data for CA3 is limited currently, this may explain why we see a possible deficit in expression across both CA3-infusion groups (if this region was damaged structurally by the cannula placement). It is also possible that we do not see the deficit in CA1 because our infusions are unable to antagonize enough of CA1. In addition to not disrupting enough of CA1 or CA3 to produce a deficit, it is also possible that the infusion into the dentate gyrus is inactivating the crucial portion of CA3 or flowing back up the cannula tract to CA1. It could be that the inactivation of the dentate gyrus plus dysfunction to either CA1 and/or CA3 disruption in the expression of trace fear memories.

Going forward, the present data provides a framework to study the complexities of the hippocampus in trace fear conditioning. If we are able to determine if the same region of the DH (dentate gyrus) is critical during the acquisition of trace fear conditioned memories, or if CA1 and/or CA3 also play a pivotal role, we would be deciphering a critical piece of a very complex puzzle, a piece that would allow us to build on it and reveal other mysteries that have long eluded us. With the difference in subregion function found in the DH, it can be hypothesized that there are also subregional differences in the VH in expression and acquisition. These data would be important to clarifying the functional distinctions between DH and VH seen in the previous literature. In addition to manipulations of the hippocampus directly, it would also be crucial to look at the input and output structures that support hippocampal function. Both the entorhinal cortex and the septum play key roles in hippocampal functioning, and each structure has distinct inputs and outputs that would be more likely to serve as crucial components for supporting memory acquisition and expression.

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Figures and Figure Captions

Figure 1. Histological representation of infusion locations for the dentate gyrus, CA1, and CA3 regions. Photomicrographs of representative samples of each infusion location.

Figure 2. The percent of time freezing during the context test. Rats were matched based on context freezing data to future drug groups; therefore there are no significant differences between future vehicle and CNQX groups. Error bars $\pm$ 1 SEM.

Figure 3. Tone test results. The percent of time freezing during the baseline (first 3 minutes) of tone test show no significant differences in drug or location. The amount of time spent freezing to the tone expressed as an average percentage of freezing over the three tone presentations. Rats with an infusion of CNQX into the dentate gyrus showed a significant decrease in freezing compared to vehicle controls. Rats with an infusion of CNQX into either CA1 or CA3 showed no significant difference in freezing to their respective vehicle controls. Error bars $\pm$ 1 SEM.

Figure 4. Average freezing during the post-tone period. Each post tone period was broken into 15 16-second bins. An average was taken across each of these 15 bins for all three post-tone periods. Rats with an infusion of CNQX into the dentate gyrus froze significantly less overall during the post-tone period than control animals, and most specifically during bins 1 through 7, and by the end of the post-tone period, both control and CNQX infused animals were freezing the same amount. All three groups froze less during the post-tone period as time went on. Error bars $\pm$ 1 SEM.

Figure 5. Average motion during the baseline period of the tone test. Three minute baseline was broken into 1-minute bins. There is no significant difference across subregions in motion during the baseline period. There is also no significant difference in motion in rats with an infusion of CNQX versus their vehicle-infused controls into any subregion of the DH. au, Arbitrary Units.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.