ARC PROTEIN EXPRESSION WITHIN DISCRETE SUBFIELDS OF THE HIPPOCAMPUS FOLLOWING TRACE FEAR CONDITIONING

By

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ABSTRACT OF THE THESIS

Arc protein expression within discrete subfields of the hippocampus following trace fear conditioning

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Thesis Director:

Tim Otto

A growing body of evidence suggests not only that the dorsal and ventral subregions of the hippocampus contribute differentially to some forms of memory, but that the contribution of the discrete subfields (CA1, CA3) within the hippocampus may also be dissociable. In the present study, we examined the regional distribution of learning-related Arc (activity-related cytoskeletal protein) expression following training in hippocampal-dependent trace fear conditioning. . We have recently shown that trace fear conditioning enhances Arc protein levels, and that both trace fear conditioning and the associated learning-related enhancement of Arc can be blocked by infusing either Arc antisense oligodeoxynucleotides (ODNs) or the NMDA receptor antagonist APV into the dorsal or ventral hippocampus prior to training. Thus while NMDAr –dependent Arc expression in both dorsal and ventral hippocampus appears to be critically involved in the acquisition of trace fear conditioning, the extent to which Arc is differentially expressed within the discrete subfields of the hippocampus following learning has yet to be characterized. Different groups of subjects were either trained in our auditory trace

fear conditioning paradigm, a modified trace fear conditioning paradigm with pre-exposure to the conditioning context, received simple exposure the novel training context, or served as home-cage, handled control subjects. Consistent with our earlier findings, the present results suggest a substantial percent increase in Arc expression in both the dorsal and ventral hippocampus following either trace fear conditioning paradigm. With respect to the regional distribution of Arc, expression was greater in CA3 relative to CA1 in the dorsal hippocampus, whereas expression increased in both CA1 and CA3 in ventral hippocampus compared to home cage controls. Interestingly animals exposed to the novel training context also exhibited increased levels of Arc protein expression in patterns similar to those of animals trained in trace fear conditioning, though to a lesser extent. When considered together with our previous data regarding learning-related Arc expression and the known anatomical connections between the hippocampus and the amygdala, these results further support a dissociable role of NMDA mediated Arc expression, not just along the dorsal-ventral axis, but within the discrete subfields of both hippocampal subregions.

ARC PROTEIN EXPRESSION WITHIN DISCRETE SUBFIELDS OF THE HIPPOCAMPUS FOLLOWING TRACE FEAR CONDITIONING

Accumulating evidence suggests that the expression of Arc (activity-regulated cytoskeletal protein), an effector immediate early gene (IEG), is tightly coupled to both the maintenance of long term potentiation (LTP) and the acquisition and retention of several forms of memory (reviewed in Guzowski, 2002). For example, like many forms of memory, both LTP and Arc transcription are NMDA receptor dependent and are induced by similar patterns of neuronal activity (Guzowski, 2002; Czerniawski et al., 2011). Moreover, within the amygdala and hippocampus, two brain regions implicated in Pavlovian fear conditioning, inhibiting Arc translation via anti-sense oligodeoxynucleotides (ODNs) blocks both the maintenance of LTP and the acquisition of some forms of memory thought to be mediated via LTP-like mechanisms (Guzowski et al., 2000; Ploski et al., 2008; Czerniawski et al., 2011; Chia & Otto, 2013). Arc protein is also of particular interest due to its unique translation/transcription dynamics in which Arc mRNA is rapidly and robustly transported to activated synaptic zones where the protein product associates with cytoskeletal proteins (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998). Finally, Arc expression appears to be more closely linked to neuronal activity which induces synaptic plasticity as opposed to neuronal firing *per se* (Fletcher et al., 2006).

With respect to the hippocampus, mounting evidence suggests that this vigorously research brain area is not a uniform structure and can be dissociated both anatomically and functionally along both the septotemporal (dorsal-ventral) and transverse (DG-CA3-CA2-CA1) axes (reviewed in Amaral & Lavenex, 2007; Risold & Swanson, 1996). With respect to the septotemporal axis, the dorsal hippocampus receives its primary input from the entorhinal cortex which in turn receives projections from primary sensory cortical areas (reviewed in Moser & Moser, 1998; Pitkanen et al.,

2000), and has been implicated in spatial learning tasks (O'Keefe & Nadel, 1978; Eichenbaum et al., 1996). By contrast, the ventral hippocampus has strong monosynaptic and reciprocal connections with the amygdala (Pitkanen et al., 2000), and has been implicated in fear and emotion based learning tasks (Yoon & Otto, 2007; Rogers et al., 2006; Rudy & Matus-Amat, 2005; Maren & Holt, 2004). Consistent with these anatomical differences, a recent study from our laboratory has demonstrated a double dissociation of dorsal and ventral hippocampal function (Czerniawski et al., 2009). More specifically, we found that selective inactivation of dorsal, but not ventral hippocampus significantly impaired rats' performance of a spatial alternation task, while inactivation of ventral, but not dorsal hippocampus dramatically impaired acquisition of trace fear conditioning (Czerniawski et al., 2009).

Functional and anatomical dissociations along the transverse axis (DG-CA3-CA2-CA1) are less clear but emerging evidence suggests that the hippocampal subfields CA1 and CA3 may also play dissociable roles in memory (Gilbert & Kesner, 2004; Vago et al., 2007; Hoge & Kesner, 2007; Hunsaker & Kesner, 2008; Goodrich et al., 2008; Hunsaker et al., 2006; Lee et al., 2005). In this regard most of the research investigating functional and anatomical differences between the CA1 and CA3 subfields has focused on the dorsal hippocampus. This research identifies hippocampal subfield CA3 as a potentially critical component of an "autoassociative network" (Hoang & Kesner, 2008; Rolls & Kesner, 2006; McNaughton & Morris, 1987) in which CA3 maintains recurrent collateral connections as well as prominent projections to CA1 along the dorsal-ventral span of the hippocampus (Amaral & Lavenex, 2007). Lesions of this region affect performance on "pattern completion" tasks (reviewed in Kesner, 2007). The CA1 subregion, which receives input primarily from the entorhinal cortex and from other hippocampal regions, including CA3 (reviewed in Moser & Moser, 1998; Pitkanen et al.,

2000), has been implicated in tasks requiring “temporal processing,” (Hoge & Kesner, 2007), while both CA1 and CA3 seem to be necessary for contextual fear conditioning (Hunsaker & Kesner, 2008), temporal sequence of spatial locations (Hunsaker et al., 2008), and temporal pattern completion (Hoang & Kesner, 2008).

Within the transverse subfields of the ventral hippocampus, which have received far less attention, CA1 establishes dense and reciprocal connections to the amygdala region while CA3 receives afferent input only (Pitkanen et al., 2000). Consistent with these anatomical projections, recent evidence suggests that lesions of ventral CA3 produce impairments in tasks which involve input from the amygdala to the hippocampus (Hunsaker & Kesner, 2008).

Several previous studies have explored the extent to which the expression of Arc protein in the hippocampus can be modified by experience (Ramirez-Amaya et al., 2005; Monti et al., 2010; Li et al., 2009). While these studies have identified a number of temporal and experiential factors contributing to hippocampal Arc expression, until recently none have examined the relationship between Arc expression and forms of learning known to depend critically on hippocampal integrity. However, our laboratory has recently found that hippocampal-dependent trace fear conditioning dramatically enhances both Arc mRNA and protein within dorsal and ventral hippocampus, and that both trace fear conditioning and the associated learning-related enhancement of Arc can be blocked by infusing either Arc antisense ODNs or the NMDA receptor antagonist APV into the dorsal or ventral hippocampus prior to training (Czerniawski et al., 2011; Czerniawski et al., 2012). Evidence of potentially differential roles of the hippocampal subfields along the septotemporal axis is supported by the results of several immunohistochemical studies examining Arc protein expression following a variety of manipulations thought to engage hippocampal processing (Ramirez-Amaya et al., 2005;

Monti et al., 2010; Li et al., 2009). While immunohistochemical evidence suggests that novel context exposure (Rameriz-Amaya et al., 2005) and exposure to drug cues (Monti et al., 2010; Li et al., 2009) enhance Arc protein expression in the dorsal hippocampus, the extent to which these exposure paradigms specifically engage the hippocampus in a meaningfully critical manner is unknown.

While these studies clearly suggest that Arc expression within the hippocampus may play a critically important role in the acquisition and retention of hippocampal-dependent memory, the extent to which Arc is induced differentially across the CA3 and CA1 cell fields of dorsal and ventral hippocampus following learning has not been explored. When considered together with the emerging evidence suggesting that the hippocampal subfields along both the septotemporal and transverse axes of the hippocampus likely play dissociable roles in memory (Kesner et al., 2010; Fanselow & Dong, 2009; Hunsaker & Kesner, 2008; Yoon & Otto, 2007; Rogers et al., 2006; Moser & Moser, 1998), an important question remains to be explored: to what extent does the regional expression of Arc reflect the putative roles of each of these subregions in memory? Immunohistochemical procedures allow for the examination of the expression patterns of Arc protein across the various subfields of the hippocampus, thereby permitting a more precise identification of the distribution of neurons activated and likely undergoing plasticity during experience. Given the evidence from our laboratory and others suggesting functional and anatomical dissociations within the hippocampus, identifying the specific subfields within the hippocampus in which Arc is preferentially expressed following training in a hippocampal-dependent task will provide evidence addressing the potential role of Arc-dependent neuronal plasticity in these regions for uncompromised task performance.

The proposed experiments are the first to attempt to characterize the regional distribution of Arc protein expression induced by hippocampal-dependent learning. More specifically, the proposed studies focus on Arc expression induced by trace fear conditioning. Given the evidence suggesting differential contributions of dorsal and ventral hippocampus to the acquisition of trace fear conditioning, this task is ideally suited to investigate regional subfield (CA1, CA3) differences in Arc protein expression between dorsal and ventral hippocampus. Hence the primary goal of the present study was to better characterize the expression of Arc protein across both the septotemporal and transverse axes of the hippocampus following acquisition of a form of learning known to be hippocampal dependent, and provide more information on specific paradigmatic features of the trace fear conditioning task itself which lead to Arc expression.

Consistent with the evidence reviewed above, a simplified diagram of relevant amygdala and hippocampal projects hypothesized to support trace fear conditioning is illustrated in Figure 1. Briefly, we suggest that trace fear conditioning is likely supported by amygdala projections to CA3 of the ventral hippocampus and reciprocal connections between ventral CA1and the amygdala, as well as reciprocal ventral CA1 connections to dorsal CA3. Based on this proposed circuit we expect trace fear conditioning to enhance Arc expression primarily in ventral hippocampal CA1 and CA3, and to a lesser extent dorsal CA3; we further hypothesize that this enhancement will be specific to animals learning the CS-US association relative to those in a variety of control conditions (see below). Moreover, based on the anatomical projections and functional dissociations described earlier, we expect that simple exposure to a novel context will enhance Arc expression in dorsal CA1 and CA3, with greater levels of Arc expression in dorsal CA3 for animals learning the CS-US association.

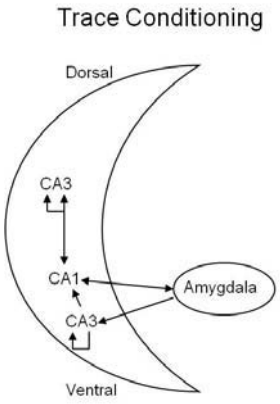

Figure 1. A simplified diagram depicting the hippocampal circuits proposed to support the acquisition of trace fear conditioning.

Materials and Methods

All procedures have been approved by Rutgers University's Institutional Animal Care and Use Committee (Protocol #96-033).

General Methods

The present experiment examined the expression patterns of Arc protein in the dorsal and ventral hippocampus in several different groups of animals, each of which are hypothesized to produce unique patterns of protein expression. Subsets of subjects were trained in trace fear conditioning or a variety of other conditions described more fully below. Animals were sacrificed one hour after training and their brains removed for immunohistochemical analysis of Arc expression across the transverse and septotemporal axes of the hippocampus. Levels of Arc protein expression between and within training conditions were compared. Data were analyzed using separate one- or two-way analyses of variance as well as non parametric Dunn's analyses, as appropriate. An α level of 0.05 was used for all statistical analyses. *Post hoc* comparisons, when necessary, were conducted using Student-Newman-Keul's (SNK) *post hoc* test.

Subjects

Twenty eight male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 225-250g, served as subjects. Animals were housed in clear plastic tub cages in an approved animal vivarium. Animals were under a 12 h light/dark cycle with all behavioral procedures occurring during the light cycle. Subjects had access to food *ad libitum*. All subjects were handled for 2 min daily for 5 days prior to training.

Apparatus

Fear conditioning and testing chambers. Auditory trace fear conditioning, context testing, and novel context exposure (described below) were conducted in 3 different behavioral chambers, each of which was located in a sound attenuating enclosure. A one-way glass window on the front door of the sound attenuating enclosure allowed an experimenter to observe and score the behavioral measure of freezing using a hand switch that was connected to the computer controlling all paradigmatic events. The training chamber was cleaned with a commercially available cage cleaner (Research Laboratories Inc.) between sessions. The testing session for trace fear conditioning took place in a novel chamber located in a different experimental room. The testing chamber had the same measurements and configuration as the training chambers but was differentiated from the training chamber in that the entire floor was covered with black Plexiglas and a black and white striped panel was attached to two of the opposing walls. The testing chamber was cleaned with alcohol between sessions.

Procedure

Auditory trace fear conditioning, tone testing, context testing and novel context exposure. All experimental groups were trained or exposed to the training context after the initial 5 days of handling. Auditory trace fear conditioning (TFC) (n=5) was conducted using procedures identical to those in previous experiments carried out in the laboratory (Czerniawski et al., 2012), and consisted of seven pairings of a tone (16 sec, 3.9 kHz, 80 dB) and footshock (2 sec, 0.6 mA), with a trace interval of 28 seconds between the offset of the tone and onset of the shock; individual trials were separated by a 2 minute intertrial interval (ITI). The behavioral response of freezing, defined as a rigid posture and lack of movement except that required for respiration, was recorded throughout the entire conditioning session by an observer blind to the experimental condition of the subject.

Another group of animals (exTFC) (n=5) received auditory trace fear conditioning identical to other subjects with the exception that these animals were exposed to the conditioning chamber for two hours immediately prior to trace fear conditioning.

Novel context exposure (NCE) (n=5) was conducted using the same training apparatus used for trace fear conditioning. Animals in this condition were exposed to the training chamber for a period of time yoked to the auditory trace fear conditioning protocol (19 min 22 s), but no tones or footshocks were delivered.

A final group of home cage subjects (n = 5) were sacrificed for immunohistochemical analysis without any training or novel context exposure after the same 5 days of handling for 2 minutes per day. Arc expression in these animals was subsequently used as the primary control against which Arc protein expression in other groups was compared.

Importantly, a subset of animals were trained using procedures identical to the TFC (n=4) and exTFC (n=4) groups, but were not sacrificed for immunohistochemical analyses. These animals were tested 24hr later in a novel context for fear conditioned to the CS; a second testing session conducted in the original training chamber 24hr later (48hr after training) examined levels of contextually-elicited fear. The testing session for trace fear conditioning consisted of one session comprised of three trials (8 min 18 s). The timing of stimulus delivery and duration of both the CS and ITI was identical to that used during training except that footshock was not presented and the number of trials was decreased to 3 to reduce the potential effect of extinction during testing. As during conditioning, the behavioral measure of freezing was recorded throughout the entire testing session. The first two minutes in the testing chamber was used as a baseline measure of freezing behavior. These raw data were subsequently transformed into the

percentage of time spent freezing during the first ITI (ITI-1), and the remaining ITIs, CS, and trace intervals of the testing session.

Conditioned fear to the training context was assessed by placing each subject into the chamber in which training occurred for the same 3 trial period as used during CS testing, but no tones were presented. Freezing was recorded continuously during each testing session by an observer blind to the subjects' condition. These raw data were subsequently transformed into the percentage of time spent freezing during periods consistent with those during which the ITI, CS, and trace interval were presented during initial training.

Immunohistochemistry. One hour after the end of training or novel context exposure, subjects were administered a sub-lethal dose of sodium pentobarbital (100mg/kg i.p.) and perfused transcardially with phosphate buffered saline (PBS, pH 7.4) and 4.0% paraformaldehyde. Brains were then removed and post-fixed in 4% paraformaldehyde solution for approximately 18 h at 4°C before being transferred to 30% sucrose PBS solution for at least 48 hours at 4°C, or until the brains sank to the bottom of the jar. Brains were then frozen and sliced into free-floating coronal sections of 40-μm thickness using a cryostat. Starting at the most rostral portion of the hippocampus, every third section was taken and stored in PBS until immunohistochemical processing approximately 24 h later. Twelve slices per subject were chosen on the basis of uniformity between subjects and consisted of 6 dorsal hippocampal sections (-2.76 to -4.36 from bregma) and 6 ventral hippocampal sections (-4.86 to -6.00) for each subject.

Day one of immunohistochemistry consisted of washing slices in PBS for 3 x 10 min, blocking in PBS containing 1% bovine serum albumin (BSA), 0.1% Triton-X for 1 h,

and incubating 18-24 h at 4°C in anti-Arc antibody (Cruz Arc (C-7) sc-17839 mouse mono-IgG2A 1:500) in 1%BSA in PBS with 0.1% Triton-X. Day two began with another set of 3 x 10 min washes in PBS followed by incubation in secondary antibody (Vector labs (PK-6102) Vectastain ABC peroxidase kit (Mouse IgG) elite series) in 1%BSA in PBS with 0.1% Triton-X for 1 hr at room temperature. Slices were again washed in PBS for 3 x 10 min, followed by incubation in AB solution for 1 hr and a final 3 x 10 min washes in PBS. Slices were then developed in DAB peroxidase substrate for 3 min. Slices receive a final 3 x 5 min wash in diH_20 before sections were mounted on glass slides and coverslipped.

Histology. Slices were imaged with a Nikon Eclipse E400 light microscope and captured using ImageJ (NIH) at 4x and 10x magnification levels and saved as jpeg image files. Regions of interest (dorsal CA1 & CA3, ventral CA1 & CA3) were then outlined using the drawing tool in ImageJ, and were based on the demarcation of these areas derived from Paxinos & Watson (2007); the area of the region on the image was then quantified in mm^2. Arc-positive cells within the outlined area were marked and quantified using ImageJ by experimenters blind to the experimental condition of the subject. Individual cell counts per mm^2 were then totaled for the 6 dorsal hippocampal sections and the 6 ventral hippocampal sections from each rat, averaged across rats within a group, and then averaged across the three counters.

Results

Behavioral Training and Testing

As described previously, a subset of animals from the two groups of subjects trained in auditory trace fear conditioning (TFC: n=4; exTFC: n=4) were tested for freezing during training, during a testing session examining fear conditioned to the tone, and subsequently to fear conditioned to the original training context. The mean (±SEM) percentage of freezing exhibited by TFC and exTFC during training, CS testing, and context testing are presented in Figure 2.

Training. The data for Trial 1, prior to the delivery of the first US, were used to reflect a ‘‘baseline’’ measure of freezing, and are separated from those for Trials 2–7. Because freezing for each subject was stable across Trials 2–7 during conditioning, the data for each subject after the first US presentation were averaged into a single value (Trials 2–7). In order to determine if the two hours of pre-exposure to the context in the exTFC group resulted in significantly different levels of freezing during Trial 1 and Trials 2-7 of training relative to that in the TFC group, separate two-way repeated measures ANOVAs were conducted with training condition (TFC vs. exTFC) as the between subjects factor and trial period (ITI, CS, Trace) as the within subjects factor. For Trial 1 (Figure 2a) statistical analyses revealed there was no main effect of condition ($F_{(1,12)} = 0.264$, $p=0.626$), no significant main effect of trial period ($F_{(2,12)} = 1.762$, $p=0.213$), with no significant interaction between condition and trial period ($F_{(2,12)} = 0.238$, $p=0.792$).

For Trials 2-7 (Figure 2b) statistical analyses revealed there was no main effect of condition ($F_{(1,12)} = 1.423$, $p=0.278$), a significant main effect of trial period ($F_{(2,12)} = 20.109$, $p<0.001$), and no significant interaction between condition and trial period ($F_{(2,12)} = 1.481$, $p=0.266$). Given that the main goal of these analyses was to identify

differences in freezing for TFC and exTFC subjects, significant differences among trial period were not of interest so *post hoc* analyses are not reported here.

Tone Testing. The data from ITI-1 during tone testing are interpreted as a "baseline" level of freezing to the novel context and are separated from the other ITI freezing data (Figure 2c). In order to determine if the two hours of pre-exposure to the context in the exTFC group resulted in significantly different levels of freezing to the tone CS during testing relative to that in the TFC group, a two-way repeated measures ANOVA was conducted with training condition (TFC vs. exTFC) as the between subjects factor and trial period (ITI-1 vs. ITI, CS, Trace) as the within subjects factor. Statistical analyses revealed there was no significant main effect of condition ($F_{(1,18)}$ = 0.201, p=0.669), a significant main effect of trial period ($F_{(3,18)}$ = 1555.42, p<0.001), with no significant interaction between condition and trial period ($F_{(3,18)}$ = 1.54, p=0.238). Again, significant differences among trial period were not the focus of this analysis so *post hoc* analyses are not reported here.

Context Testing. A separate two-way repeated measure ANOVA was conducted on context test data with training condition (TFC vs. exTFC) as the between subjects factor and trial period (ITI vs. CS, Trace) as the within subjects factor. Data for one subject in the exTFC group for the context text was lost due to a computer recording error resulting in only 7 (TFC n=4; exTFC n=3) subjects being represented within this set of analyses (Figure 2d). Statistical analyses revealed there was no main effect of condition ($F_{(1,10)}$ = 1.405, p=0.289), no main effect of trial period, ($F_{(2,10)}$ =0.1, p<0.906), with no significant interaction between condition and trial period, ($F_{(2,10)}$ = 0.504, p=0.619).

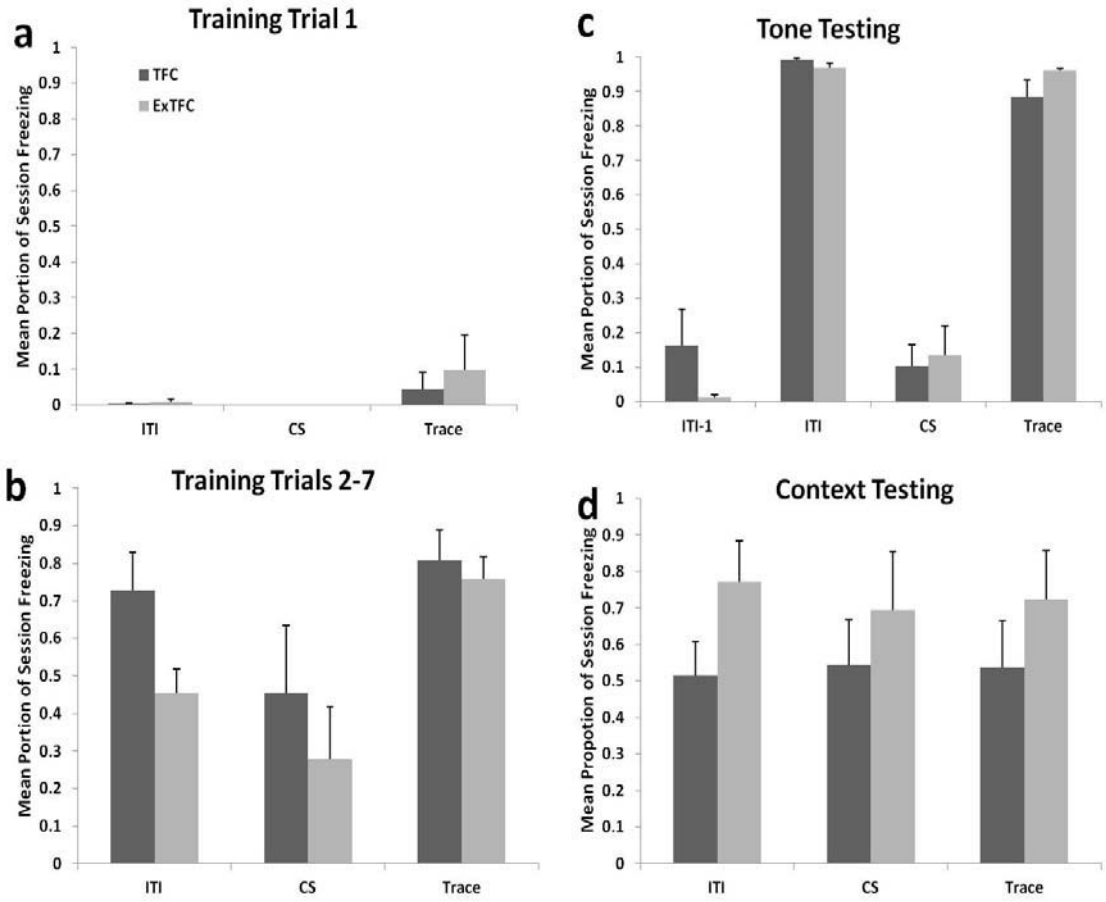

Figure 2. Mean +/- SEM period of session freezing during (a) training trial 1, (b) training trials 2-7, (c) tone testing, and (d) context testing.

Immunohistochemical examination of the regional patterns of Arc expression

Hippocampal neuronal cells positive for Arc protein expression were quantified across the septotemporal (Figure 3a) and transverse (Figure 3b) axes of both dorsal and ventral hippocampus.

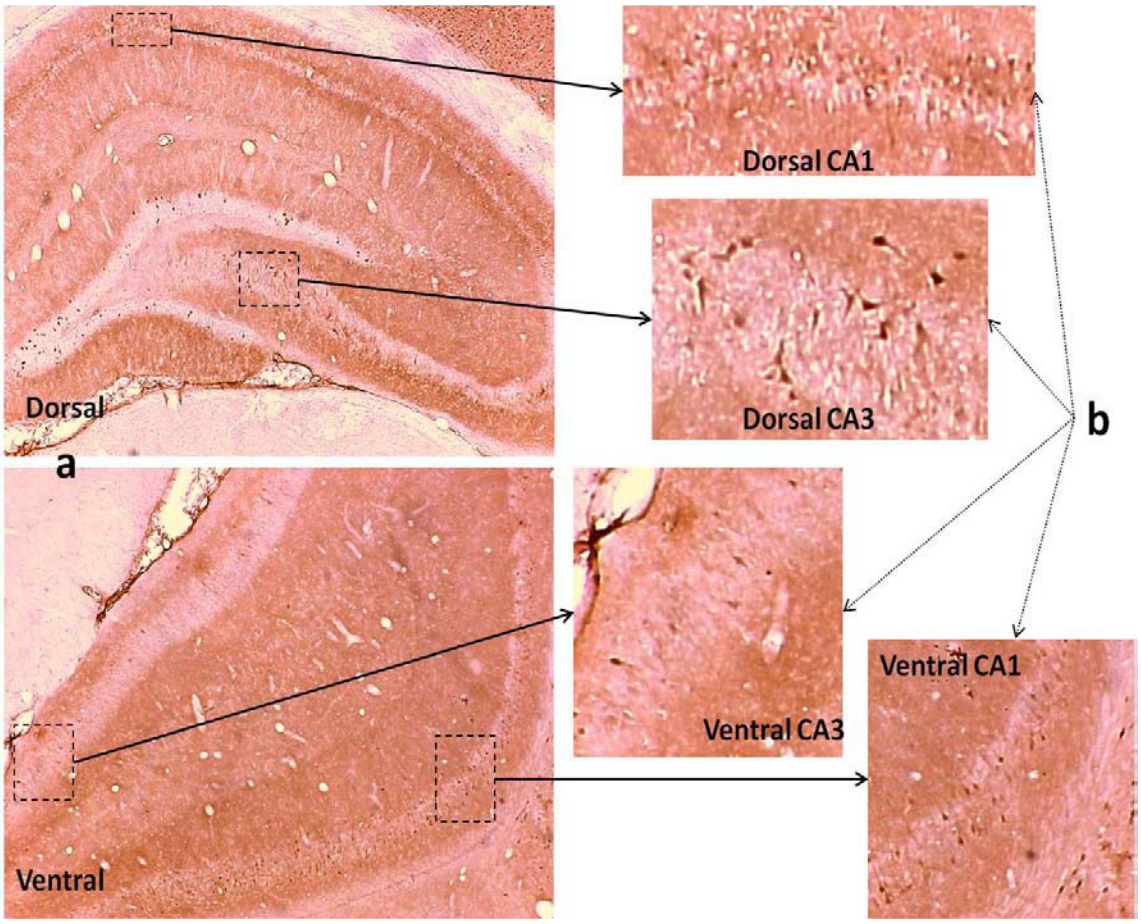

Figure 3. Representative Arc positive protein expression in (a) dorsal (top left) and ventral (bottom left) hippocampus (septotemporal axis) and within (b) CA1 and CA3 subfields (transverse axis) for a subject trained in trace fear conditioning.

Transformation of raw cell count data. Direct statistical comparisons of absolute cell counts between hippocampal subfields are restricted due to cytoarchitectural differences between those subfields. Specifically, anatomical data indicate that the pyramidal cells within the CA1 subfield of the hippocampus are smaller and more densely packed than pyramidal cells in either CA3 or the dentate gyrus (Pyapali et al., 1998). This higher density of CA1 pyramidal cells thus could lead to a greater number of cells available to express Arc protein compared to CA3 and dentate gyrus, thus making direct comparisons between CA1 and the other subfields potentially misleading. Additionally, there are also differences in the actual size of the subfields within which Arc positive

cells were quantified. For example, the size of the ventral CA1 hippocampal subfield is, at some points, twice as large as the dorsal CA1 subfield (Paxinos & Watson, 2007). To account for this issue, Arc positive cell counts were converted into a count per unit area. This conversion is easily made using ImageJ (NIH) by scaling the image and outlining the quantifiable region for a given subfield to determine the size of the area being assessed for the presence of Arc positive cells. Thus with respect to the data presented here, raw Arc positive cell counts obtained using immunohistochemistry were normalized to the size (mm^2) of the area over which Arc expression was quantified.

Another important transformation of these data was normalization of each group to the density of Arc positive cells in home control cage subjects. Normalization to home cage levels of Arc expression allows for a more systematic comparison of the specific contributions of Arc protein expression between different behavioral groups (TFC, exTFC, NCE). While basal Arc expression is typically quite low (Vazdarjonova & Guzowski 2004), the distribution of baseline Arc protein expression across subregions may not be uniform and our immunohistochemical results support this notion (Figure 4). As such the percent increase in Arc expression relative to that in home cage control subjects provides additional information regarding how a particular experience changes Arc expression while controlling for potential differences in baseline expression.

Separately, a transformation of the raw data was made due to our specific theoretical interests in the CA1 and CA3 subfields and their involvement in trace fear conditioning, as well as previous data from our laboratory in which Arc expression was quantified by Western Blot analyses. This transformation involved summing the cell count per mm^2 across CA3 and CA1 in order to better compare immunohistochemical data to previous Western Blot data.

For one home cage animal the perfusion procedure resulted in hippocampal slices which were not appropriately stained with DAB peroxidase. This animal was excluded from the analyses. Two other animals demonstrated levels of Arc expression in ventral CA1 which were determined to be outliers by Dixon's outlier test: one home cage subject ($Z_{(3)}$ =1.48, p<0.05), and one exTFC subject ($Z_{(4)}$ = 1.715, p<0.05). These two animals were also excluded from statistical analyses; final sample sizes for were TFC: n=5; exTFC: n=4, NCE: n=5; HC: n=3.

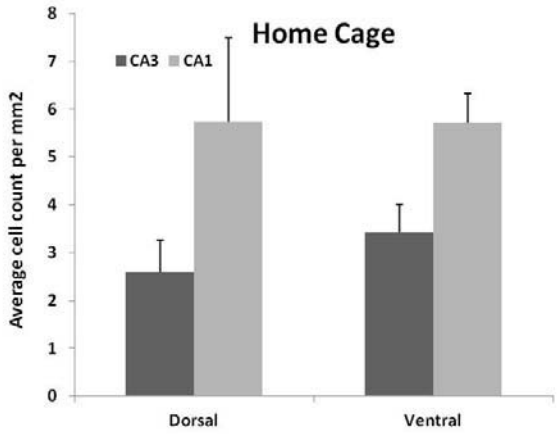

Figure 4. Mean ± SEM Arc positive cells per mm^2 in dorsal and ventral hippocampus in home cage subjects (n=3).

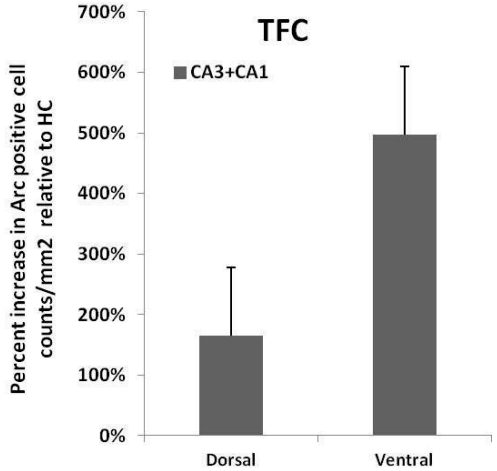

Figure 5. Mean ± SEM percent increase in Arc positive cells per mm^2 over home cage subjects in the dorsal and ventral hippocampus for animals trained in trace fear conditioning.

Arc positive cell counts per mm^2 combined across CA1&CA3 subfields in dorsal and ventral hippocampus

Animals trained in trace fear conditioning (TFC) exhibited a substantial change in Mean percent increase in Arc positive cells per mm^2 relative to home cage subjects in both the dorsal and ventral hippocampus (Figure 5). Within the dorsal hippocampus specifically, Arc positive cell counts are presented as Mean ± SEM (Figure 6a), but due to a violation of normality (p<0.05), for statistical analysis, data are also presented as Medians with 1st and 3rd quartile range as error bars (Figure 6b). Multiple comparisons using Dunn's nonparametric statistical analysis, using home cage animals as the control group, demonstrated a statistically significant difference between the exTFC and home cage animals ($H_{(3)}$ = 2.83, p<0.05). TFC, exTFC and Novel Context Exposure subjects were not significantly different than home cage subjects (Figure 6b).

Within the ventral hippocampus, a one-way ANOVA comparing mean Arc positive cell counts per mm^2 totaled across CA1 &CA3 revealed a significant effect of training condition ($F_{(3)}$ = 4.337, p=0.025). Subsequent SNK *post hoc* tests determined that Arc-positive cell counts for TFC subjects were significantly different than home cage subjects (p=0.015). Other experimental groups approached significant differences relative to home cage subjects, but were just above our statistical cut off of α = 0.05. TFC, exTFC and Novel Context Exposure subjects were not significantly different from one another (Figure 6c).

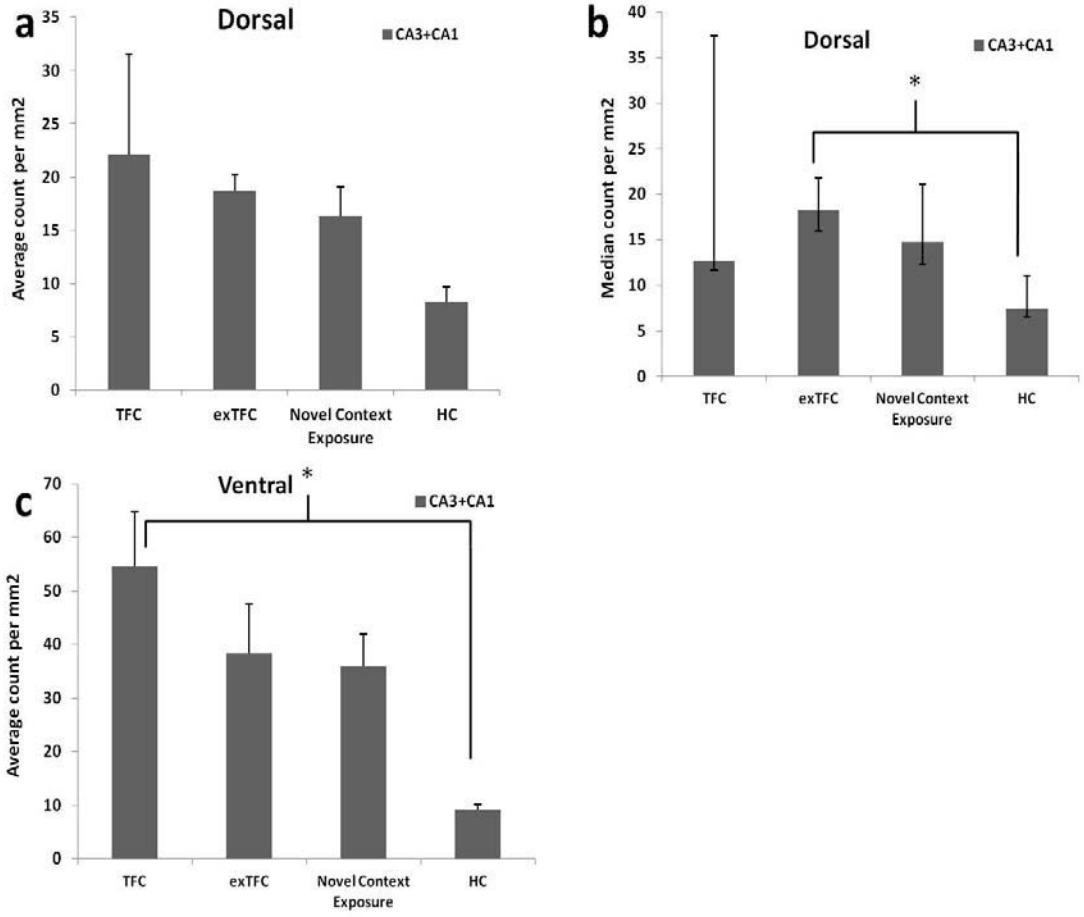

Figure 6. Arc positive cells counts in CA3+CA1 subfields in dorsal and ventral hippocampus. Significant differences noted with asterisk. (a) Mean ± SEM Arc positive cells per mm^2 in dorsal hippocampus (normality assumption violated). (b) Median, 1st and 3rd quartile range of Arc positive cells per mm^2 in dorsal hippocampus, Dunn's non parametric multiple comparison with home cage subjects as control. (c) Mean ± SEM Arc positive cells per mm^2 in ventral hippocampus ($p<0.05$).

Percent increase in Arc positive cells per mm^2 in dorsal and ventral hippocampal subfields CA1 & CA3 relative to HC control subjects

In dorsal hippocampus (Figure 7a) a two-way repeated measures ANOVA with training condition (TFC, exTFC, NCE) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor revealed no significant main effect of condition ($F_{(2,11)} = 0.437$, $p=0.656$), a significant main effect of hippocampal subfield

($F_{(1,11)}$ = 6.187, p=0.03), and no significant interaction between training condition and hippocampal subfield ($F_{(2,11)}$ = 1.717, p=0.224).

In ventral hippocampus (Figure 7b) a two-way repeated measures ANOVA with training condition (TFC, exTFC, NCE) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor demonstrated no significant main effect of condition ($F_{(2,11)}$ = 1.623, p=0.241), no significant main effect of hippocampal subfield ($F_{(1,11)}$ = 2.972, p=0.113), and no significant interaction between training condition and hippocampal subfield ($F_{(2,11)}$, = 0.247, p=0.785).

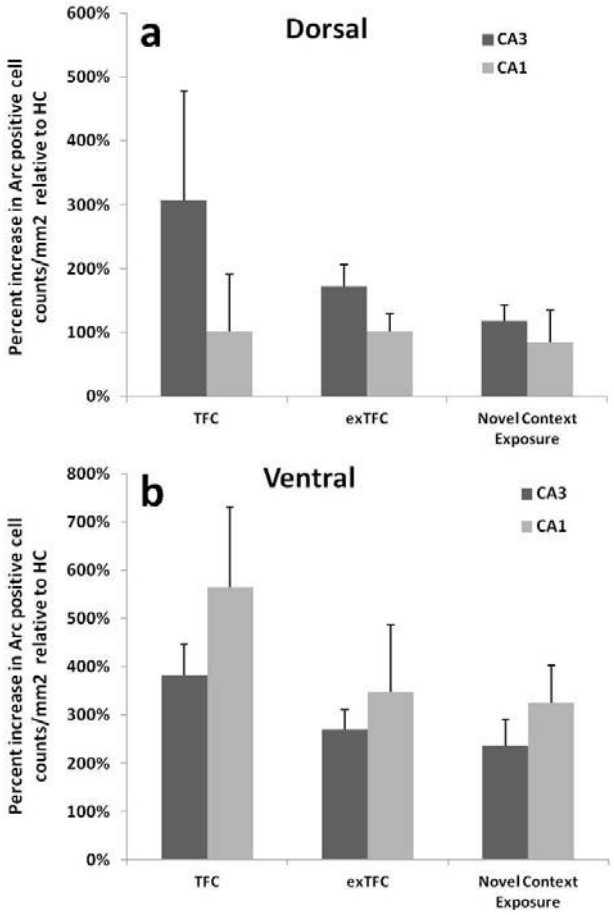

Figure 7. Arc positive cells counts in CA3 and CA1 subfields in dorsal and ventral hippocampus. (a) Mean ± SEM percent increase in Arc positive cells per mm^2 over home cage subjects in the dorsal hippocampus. (b) Mean ± SEM percent increase in Arc positive cells per mm^2 over home cage subjects in the ventral hippocampus.

Discussion

Previously published data from our laboratory demonstrated that Arc expression within the hippocampus is significantly enhanced in animals trained in trace fear conditioning relative to home cage control subjects as determined through Western Blotting protein analysis on fresh hippocampal tissue (Czerniawski et al., 2011; Chia & Otto, 2013). The present results extend these previous findings in a variety of important ways. The current data also include additional behavioral groups designed to better characterize the specific aspects of the trace fear conditioning paradigm which result in enhancement of Arc protein expression.

Animals trained in trace fear conditioning (TFC, exTFC) and animals receiving novel context exposure demonstrated substantial increases in Arc expression in both the dorsal and ventral hippocampus compared to home cage subjects. Within the dorsal hippocampus, trace fear conditioning (TFC, exTFC groups) and novel context exposure (NCE) enhanced arc expression in CA3, but not CA1, relative to home cage control subjects. Within the ventral hippocampus, trace fear conditioning (TFC, exTFC) and novel context exposure (NCE) enhanced Arc protein expression in both CA3 and CA1 compared to home cage control subjects. Finally, inherent variability in immunohistochemical data, using our current immunohistochemical procedure, makes identifying statistically significant differences in Arc expression between various hippocampal regions within and across training conditions difficult. Each of these issues is discussed in more detail below.

Behavioral Measures of Freezing did not differ between TFC and exTFC Subjects

In contrast to our predictions, behavioral measures of freezing did not differ between animals trained in TFC compare to exTFC (Figure 2d). It was predicted that

extended pre-exposure to the training context would reduce context-shock associations and, in turn, contextually-elicited fear during later testing. The lack of a significant behavioral effect of context pre-exposure for exTFC subjects may have contributed to the lack of difference in levels of Arc expression between exTFC, TFC, and Novel Context Exposure subjects (see below).

Dorsal Hippocampus Arc Protein Expression

The regional distribution of Arc positive cells is generally consistent with our initial hypotheses when the CA1 and CA3 subfields are combined. We initially hypothesized that the TFC group would exhibit the largest increases in Arc protein expression relative to other behavioral groups, as these animals were exposed to both the tone-shock pairing and a novel context during training. We further hypothesized that the exTFC group would exhibit less Arc protein expression than the TFC group as we expected that Arc expression due to novel environment exposure would have peaked prior to training and returned to baseline levels. The Novel Context Exposure group was expected to exhibit Arc expression but at lower levels than both TFC and exTFC. This hypothesis is supported when Arc expression is totaled across subfields of theoretical interest (CA1, CA3) for each animal group (Figure 6a). For statistical analysis, non parametric analyses suggest that only exTFC subjects differed from home cage subjects (Figure 6b). While only exTFC subjects were significantly different than home cage subjects, TFC and Novel Context Exposure subjects demonstrated consistently higher levels of Arc expression relative to home cage subjects.

Trends in the regional distribution of Arc positive cell counts within individual subfields are generally consistent with our initial hypotheses. The current results are consistent with anatomical evidence suggesting dissociable functional roles of

different regions of the hippocampus that likely support trace fear conditioning. More specifically, in the dorsal hippocampus CA3 provides the major pathway for amygdala inputs to the dorsal hippocampus via ventral CA1, and is required to support uncompromised contextual fear conditioning (Hunsaker & Kesner, 2008). This ultimately suggests that plasticity and Arc expression within dorsal CA3, and not CA1, may reflect contextual components of trace fear conditioning as trained animals also demonstrate freezing to the training context (Figure 2d). This supports and extends previous data from our laboratory on the role of plasticity and Arc expression in the dorsal hippocampus for contextual fear conditioning (Czerniawski et al. 2012). While there has yet to be a systematic investigation of the relative roles of Arc expression in the dorsal hippocampal subfields in the acquisition of trace fear conditioning, the current results are consistent with previous evidence in that Arc expression in CA3, but not CA1, of the dorsal hippocampus tended to be greater in trace fear conditioned subjects (TFC, exTFC) (Figure 7a). These results are consistent with evidence supporting the differential role of dorsal CA3 versus CA1 in contextual fear. Moreover, the significant difference in Arc expression in dorsal CA3 versus CA1 overall further supports the specific role of dorsal CA3 in modulating contextually elicited fear (TFC, exTFC) (Figure 2d). While these trends are present in TFC and exTFC trained subjects, and not Novel Context Exposure subjects, levels of Arc expression did not differ significantly between groups. The lack of a significant effect of training condition precluded a more in-depth statistical comparison of CA3 versus CA1 across different animal groups.

Trends in the regional distribution of Arc positive cell counts within subfields and between different behavioral groups are generally consistent with our initial hypotheses. The current data partially support hypothesized differences in Arc protein expression between different behavioral groups. Arc expression in the dorsal CA3

region in was greater in TFC subjects relative to exTFC subjects (Figure 7a), although this trend did not reach statistical significance. This difference supports our hypothesis as TFC subjects were exposed to both tone-shock paring and a novel context during training. For exTFC subjects the present data are then partially consistent with previous observations in that both dorsal CA3 and CA1 have previously been identified to demonstrate an increase in Arc expression due to novel context exposure (Ramirez-Amaya et al. 2005). This suggests that pre-exposure to the training context for exTFC subjects was sufficient to drive down Arc protein expression within dorsal CA3 but was not sufficient to reduce expression in dorsal CA1 relative to TFC subjects. The current data regarding dorsal CA1 expression suggests similar effects of trace fear conditioning (TFC, exTFC) and novel context exposure groups as there was no apparent trend toward decreasing expression as seen in dorsal CA3 reported above. While these findings are contrary to other data which identify both dorsal CA3 and CA1 Arc expression due to novel context exposure, these data do support the differential role of dorsal CA3 versus CA1 in mediating contextual fear mentioned above. This notion is further supported in that Novel Context Exposure subjects do not show a similar trend of greater Arc expression in CA3 versus CA1 as these subjects did not receive fear conditioning and hence would not exhibit contextually elicited fear.

Ventral Hippocampus Arc Protein Expression

The regional distribution of Arc positive cells is generally consistent with our initial hypotheses when the CA1 and CA3 subfields are combined. Hypothesized differences between TFC, exTFC, and Novel Context Exposure subjects are only partially supported when Arc protein expression is totaled across subfields of theoretical interest (CA1, CA3) (Figure6c). TFC subjects exhibited a significant increase in Arc protein expression over home cage subjects, and differences between Novel Context

Exposure subjects and home cage subjects approach significance (p=0.054). There was no significant difference in Arc protein expression between the TFC group and the ExTFC group, as predicted, as both of these animal groups were trained in trace fear conditioning. Yet, there was also not a significant difference between Novel Context Exposure subjects and trace fear conditioned subjects (TFC, exTFC) which does not support our initial hypothesis of a substantial decrease in Arc expression for animals which did not receive fear conditioning. The increase in Arc protein expression in the ventral hippocampus was unexpected in Novel Context Exposure subjects as these animals were not trained in our fear conditioning protocol and as such amygdala input to the ventral hippocampus should have been minimized. While these effects were inconsistent with our initial predictions, this is the only study to date which has investigated Arc protein expression in the ventral hippocampus after novel context exposure; the Arc expression data regarding novel context exposure will be discussed in more detail below.

Trends in the regional distribution of Arc positive cell counts within individual subfields are generally consistent with our initial hypotheses. While data from our laboratory has previously demonstrated deficits in trace fear conditioning when the ventral hippocampus is infused with either Arc ODNs or APV prior to training (Czerniawski et al., 2011; Czerniawski et al., 2012), as well as ventral hippocampus excitotoxic lesions (Czerniawski et al., 2009), there has yet to be a systematic investigation of the role of the transverse ventral hippocampal subfields in the acquisition of trace fear conditioning. Anatomical evidence described above suggests that both CA1 and CA3 receive amygdala afferent input, and consistent with the well established role of the amygdala in fear conditioning (Le Doux, 1995), Arc expression in both CA1 and CA3 of ventral hippocampus in animals trained in trace fear conditioning (TFC,

exTFC) exhibit a substantial percent increase in Arc expression relative to home cage controls (Figure 7b). More specifically, higher levels of Arc expression was observed in ventral CA1 compared to CA3 in fear conditioned animals (TFC, exTFC). Though not significantly different, these trends are also largely consistent with anatomical evidence identifying reciprocal connectivity between ventral CA1, amygdala and dorsal CA3 (Figure 1), while the same reciprocal connections are not present in ventral CA3. This supports the current results identifying higher levels of Arc expression in ventral CA1 compared to CA3. Contrary to our hypothesis, levels of Arc protein expression in exTFC subjects were similar to Novel Context Exposure subjects, which was not expected (Figure 7b). Elevated levels of Arc expression for Novel Context Exposure subjects will be discussed in more detail below.

Trends in the regional distribution of Arc positive cell counts within subfields and between different behavioral groups are generally consistent with our initial hypotheses. Ventral CA1 and CA3 Arc expression was greater in TFC trained subjects compared to exTFC subjects (Figure 7b) but more so in CA1 than CA3. This relationship was predicted based on our assumption of a reduction in contextual fear for exTFC animals due to their pre-exposure to the training context prior to conditioning. A reduction in contextually-elicited fear was predicted to be supported, in part, by reduced amygdala-hippocampal plasticity, specifically in ventral CA1. This specific prediction of within ventral CA1, and not ventral CA3, in exTFC animals, is supported by anatomical evidence identifying reciprocal communication between ventral CA1 and dorsal CA3 (Figure 1), which, as outlined above, is implicated in contextual fear. Yet the lack of a significant behavioral effect of pre-exposure (described above) may account for the lack of significant differences between TFC and exTFC animals. Contrary to this notion is the lack of significant differences in CA3 and CA1 for fear conditioned subjects (TFC,

exTFC) relative to Novel Context Exposure subjects which were hypothesized to have markedly less Arc protein expression in both ventral hippocampal subfields due to the lack of explicit fear conditioning in that animal group (Figure 7b). Overall, differences in Arc expression in CA1 versus CA3 partially support anatomical evidence and training differences for fear conditioned animals (TFC, exTFC), however the high levels of Arc expression in the Novel Context Group was unexpected.

Arc expression induced by novel context exposure is similar to that induced by trace fear conditioning.

While others have investigated the effect of novel context exposure on hippocampal Arc expression, the present study is the first examination of the effect of context exposure on hippocampal Arc expression in both the dorsal and ventral hippocampus and across subfields. While others have shown that Arc protein expression is enhanced in both dorsal CA1 and CA3 following novel context exposure (Ramirez-Amaya et al., 2005), our results demonstrate Arc expression in both the CA1 and CA3 subfields of both dorsal and ventral hippocampus show a substantial, though not statistically significant, increase relative to home cage subjects.

Particularly surprising was the similar increase in levels of Arc expression within the ventral hippocampus in Novel Context Exposure subjects compared to fear conditioned animals (Figure 7b). It is possible that within the Novel Context Exposure group elevated levels of Arc expression in ventral hippocampus, resulting from amygdala inputs, may serve a modulatory function in the dorsal hippocampus. Arc RNA transcription in the dorsal hippocampus has been implicated in location-specific firing of CA3 and CA1 hippocampal neurons, which in turn has been related to the establishment of hippocampal place fields (Bramham et al., 2008; Guzowski et al, 1999). Importantly,

Arc mRNA translation can be subject to modulation (McIntyre et al., 2005) via posttranscriptional regulation by amygdala-dependent neuromodulatory processes (Bramham et al., 2008). This suggests a role for amygdala connections in mediating dorsal, and perhaps ventral, hippocampal Arc protein expression seen in Novel Context Exposure groups. Yet, the involvement of the amygdala in modulating Arc expression in the hippocampus, in the absence of explicit fear conditioning, is unclear. There is evidence, however, to suggest a role of the amygdala in responding to novel objects and contexts (Moses et al., 2002). Specifically, rats with amygdala lesions have shown attenuated neophobic responses to novel food stimuli (Burns et al., 1996; Dunn & Everitt, 1988; Rolls & Rolls, 1973; Sutherland & McDonald, 1990). This evidence, coupled with general neophobia observed in rats within a novel context, may suggest a role of amygdala modification of hippocampal Arc expression in the absence of explicit trace fear conditioning or other hippocampal-dependent aversive learning experiences. As such, given the anatomical evidence identifying the ventral hippocampus as the primary pathway by which amygdala inputs would reach the dorsal hippocampus, Arc expression within the ventral hippocampus may modulate relevant dorsal hippocampal activity. This would include the establishment of dorsal hippocampal place fields in novel environments, as well as the possibility of a more general preparation for additional amygdala afferent input to modulate more explicit aversive learning events and behavioral change within a potentially aversive/fearful novel context. Hence Arc protein related plasticity in the ventral hippocampus could occur in the absence of explicit fear conditioning. Yet it is unclear, using the present techniques, which set of connections within the ventral hippocampus (reciprocal to other hippocampal regions, or afferent from the amygdala, in the case of CA3, or afferent and efferent in the case of CA1) account for observed Arc expression patterns.

Future Considerations for Behavioral Procedures and Protein Assessments

In order to further address the extent to which novel context exposure contributed specifically to the patterns of results observed here, future studies will include a group of animals that is repeatedly exposed to the novel training context prior to immunohistochemical analysis. Additional groups will include independent context and tone pre-exposure paradigms expected to result in a robust behavioral latent inhibition effect. Within these proposed behavioral control groups the conditioned responses to training context and tone CS should be reduced relative to non exposed behavioral groups. Moreover, future research will seek to combine immunohistochemical analyses with Western Blot analyses in order to obtain a more meaningful quantitative assessment of the relative levels of Arc protein within a given subfield in the dorsal and ventral hippocampus. Such analyses will allow the identification of not just where Arc protein expression changes but by how much.

Conclusions

Importantly, while Arc expression may be elevated in multiple regions following acquisition of trace fear conditioning and novel context exposure, plasticity within these regions may not be necessary to support task acquisition or experience. As previously stated, our laboratory has demonstrated that within both dorsal and ventral hippocampus, preventing Arc expression via micro injections of ODNs or APV prevents both learning related changes in Arc expression as well as compromising performance when animals are tested for the conditioned response. Yet excitotoxic lesions of the dorsal hippocampus do not affect learning within this task suggesting a unique role for Arc protein in supporting acquisition of trace fear conditioning. As such these data will guide future research in which the individual dorsal and ventral hippocampal subfields

(CA1 and CA3) are specifically targeted with micro injections of either APV or ODNs to further identify how compromising NMDA mediated Arc expression affects behavior within a variety of behavioral groups designed to more completely characterize the role of Arc protein expression in the acquisition of trace fear conditioning.

Moreover, a general lack of statistical differences in Arc expression between the TFC, exTFC and Novel Context Exposure groups could also reflect the possibility that increases in Arc protein expression in both the dorsal and ventral hippocampus were due to novel context exposure, and that Arc expression within these regions may not itself be unique to the acquisition of trace fear conditioning. Given the potential role of the amygdala and the effects of novel context exposure on Arc protein expression in the ventral hippocampus outlined above, preventing plasticity induced by novel environment exposure in either the dorsal or ventral hippocampus may interfere with the animal's ability to learn a given CS-US association within that novel context. If an animal cannot learn about the context-CS associations, or context-US association due to inhibited Arc expression effecting the formation of hippocampal place fields, then the specific acquisition of the CS-US association may be compromised as well. While this notion is speculative, it highlights the importance of and need for sophisticated behavioral controls to identify how compromising neuronal function leads to changes in behavior.

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