An organization of visual and auditory fear conditioning in the lateral amygdala

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ABSTRACT

Pavlovian fear conditioning is an evolutionary conserved and extensively studied form of associative learning and memory. In mammals, the lateral amygdala (LA) is an essential locus for Pavlovian fear learning and memory. Despite significant progress unraveling the cellular mechanisms responsible for fear conditioning, very little is known about the anatomical organization of neurons encoding fear conditioning in the LA. One key question is how fear conditioning to different sensory stimuli is organized in LA neuronal ensembles. Here we show that Pavlovian fear conditioning, formed through either the auditory or visual sensory modality, activates a similar density of LA neurons expressing a learning-induced phosphorylated extracellular signal-regulated kinase (p-ERK1/2). While the size of the neuron population specific to either memory was similar, the anatomical distribution differed. Several discrete sites in the LA contained a small but significant number of p-ERK1/2-expressing neurons specific to either sensory modality. The sites were anatomically localized to different levels of the longitudinal plane and were independent of both memory strength and the relative size of the activated neuronal population, suggesting some portion of the memory trace for auditory and visually cued fear conditioning is allocated differentially in the LA. Presenting the visual stimulus by itself did not activate the same p-ERK1/2 neuron density regulating some portion of the memory trace for auditory and visually cued fear conditioning. Together, these findings reveal an anatomical distribution of visual and auditory fear conditioning at the level of neuronal ensembles in the LA.

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1. Introduction

It is widely thought that only a subset of neurons in a whole population encodes any given memory (Rumpel, LeDoux, Zador, & Malinow, 2005; Han et al., 2007; Bergstrom, McDonald, & Johnson, 2011; Chapeton, Fares, Lasota, & Stepaniants, 2012; Liu et al., 2012). What is not known is precisely which neurons in a population are allocated for memory encoding, and which neurons are not (Johnson, LeDoux, & Doyere, 2009). Localizing memory in neuronal subsets is a formidable research challenge (Krupa, Thompson, & Thompson, 1993) and is of clinical relevance for understanding disorders of learning and memory, such as post-traumatic stress disorder (PTSD) (Johnson, McGuire, Lazarus, & Palmer, 2011) and addictions (Hyman, 2005).

Pavlovian fear conditioning is an extensively used behavioral paradigm for studying learning and memory in the brain (Davis, 1992; LeDoux, 2000; Johnson et al., 2011; McGuire, Coyner, & Johnson, 2012). In Pavlovian fear conditioning, a previously innocuous sensory stimulus, such as a tone or light, quickly acquires negative valence (conditioned stimulus, CS) after being paired with a naturally fearful stimulus (unconditioned stimulus, US). As a result of CS and US pairing, a stable and lasting fear-evoking memory about the CS is formed. The molecular, physiological
and anatomical framework of Pavlovian fear conditioning is well-characterized, with the lateral nucleus of the amygdala (LA) a key hub for the establishment of long lasting fear memory (Sah, Faber, Lopez De Armentia, & Power, 2003; Johnson & Ledoux, 2004; Lamprecht & LeDoux, 2004; Rodrigues, Schafe, & LeDoux, 2004; Pape & Pare, 2010). This makes the study of Pavlovian fear conditioning at the level of neuronal ensembles in the LA particularly advantageous because a significant portion of the engram has been localized (Schafe, Doyere, & LeDoux, 2005; Kwon & Choi, 2009).

We previously demonstrated that neurons activated after auditory fear conditioning are topographically organized in the LA (Bergstrom et al., 2011, 2013; Bergstrom, McDonald, Dey, Fernandez, & Johnson, 2013). The objective of the present study was to determine whether a redundant or new distribution of neurons is activated in the LA after visual fear conditioning. We selected the LA for mapping visual and auditory fear conditioning because it receives both visual and auditory sensory input (Doron & Ledoux, 1999; Pitkänen, 2000) and has been directly linked with the generation of new visually cued fear memories (Ledoux, Romanski, & Xagoraris, 1989; Campeau & Davis, 1995; Shi & Davis, 2001), although see (Tazumi & Okaichi, 2002). Precise topographic measures of an activated neuronal population following visual fear conditioning have never been conducted or compared with the topography of neurons activated after auditory fear conditioning. Detailed mapping of an activated neuron population in the LA following auditory or visual fear conditioning is an important preliminary step towards decoding the anatomical organization of more complex, multimodal associative fear memories in the brain.

The study of two different types of fear conditioning in the LA required we first experimentally control for differences in the strength of the expressed memory so the underlying neuron population was of equivalent size, and thus comparable. Memory strength was modified by calibrating the intensity of auditory and visual CS salience to produce equivalent levels of a conditioned defensive “fear” response (freezing). Topographic measurements of the activated neuronal population were conducted by mapping the 3D coordinates of LA neurons expressing the phosphorylated form of extracellular signal-regulated kinase 1/2 (p-ERK1/2), a well-validated molecular marker of learning-induced synaptic plasticity following fear conditioning (Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999; Schafe et al., 2000; Radwanska, Nikolaev, Knapska, & Kaczmarek, 2002; Paul et al., 2007; Schafe, Swank, Rodrigues, Debiec, & Doyere, 2008; Kim, Hamlin, & Richardson, 2009; Kim, Li, Hamlin, McNally, & Richardson, 2012; Olausson et al., 2012; Besnard, Laroche, & Caboche, 2013; Cestari, Rossi-Arnaud, Saraulli, & Costanzi, 2013) for review.

We found both a common and distinct anatomical organization of p-ERK1/2-expressing neurons in the LA after auditory and visual fear conditioning. This organization was independent of both the size of the total activated neuron population and the relative strength of the memory, suggesting that some portion of the auditory and visual fear memory trace is allocated differently based on the anatomical distribution of p-ERK1/2-expressing neurons in the LA. These data provide the first insight into how Pavlovian fear conditioning, formed through different sensory modalities, is represented and organized at the level of neuronal ensembles in the LA.

2. Materials and methods

2.1. Subjects

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by the Uniformed Services University Institutional Animal Care and Use Committee (IACUC). Subjects were experimentally naïve male Sprague–Dawley rats (Taconic Farms, Derwood, MD). Rats weighing 225–250 g on arrival to the vivarium were group housed (2/cage) on a 12 h light:dark cycle (lights on 06:00; lux 15) with food and water provided without restriction. Bedding was changed 2/week. The vivarium humidity (55%) and temperature (20.5 °C) was constantly maintained. Rats were allowed at least seven days of acclimation to the vivarium and handled on three days prior to testing. All experiments were conducted during the light phase. Rats weighed 413 ± 6.7 g (342.8–512.8 g) at time of testing. Disclosure of housing and husbandry procedures was in accordance with recommendations for standard experimental reporting in behavioral neuroscience research (Praeger, Bergstrom, Grunberg, & Johnson, 2011).

2.2. Pavlovian fear conditioning

Sprague–Dawley rats (N = 44) were randomized into two experimental conditions (auditory fear conditioning, aFC, n = 14; visual fear conditioning, vFC, n = 17) and two control conditions (Shock alone, Shock, n = 6; box alone, Box, n = 7). All rats were allowed to explore (habituated) both the fear conditioning (Context A) and testing (Context B) chambers in counterbalanced order for 30 min each on three consecutive days prior to fear conditioning. Context A and B were distinguished by olfactory, visual, and tactile cues (background lux 1.0 and db < 50 for both chambers). On the training day, following three min of acclimation in Context A, rats were presented either two pairings of an auditory CS (2 kHz, 55 dB, 20 s) or visual CS (1 Hz: 0.5 s On/0.5 s Off for 20 s, 35 lux) that co-terminated with a mild foot shock US (0.6 mA, 500 ms). The mean random intertrial interval (ITI) duration was 120 s. Rats were removed from the chamber 60 s after the final stimulus presentation and returned to the vivarium. There were two control conditions. In the Shock alone condition (Shock), rats were presented the US without the auditory or visual CSs. Rats in the Box alone control condition (Box) were handled, habituated and exposed to Context A for the same duration of time as the experimental conditions but did not undergo fear conditioning.

Twenty-four hours later, a randomized subset of rats in the aCS (n = 7) and vCS (n = 8) conditions were placed into Context B for three minutes and then were replayed either the auditory CS or visual CS three times for 20 s each to test the expression of the auditory or visual cued fear memory. The mean ITI was 120 s. An experimenter blind to the experimental condition of the animals scored freezing behavior from digitized videos. Freezing is a behavioral index of conditioned fear (Blanchard & Blanchard, 1969). For the CS test, freezing was scored during the three min prior to the CS and during the CSs (20 s intervals). A mean freezing value was calculated during the presentation of the CS and transformed into a percentage freezing. Mean freezing percentage was the dependent variable for all behavioral analyses.

2.3. p-ERK1/2 immunohistochemistry

The presence of p-ERK1/2 in LA neurons served as a molecular marker of neuroplasticity associated with Pavlovian fear conditioning consolidation (Schafe et al., 2000) and see (Davis & Laroche, 2006) for review. The expression of p-ERK1/2 following auditory fear conditioning is predominantly localized to principal cell-type neurons in the LA (Bergstrom, McDonald, Dey, Tang, et al., 2013).

2.3.1. Tissue preparation

Rats were anesthetized for perfusion exactly 60 min after fear conditioning (Schafe et al., 2000). Rats were anesthetized with an intraperitoneal (i.p.) injection of a ketamine/xylazine (100 mg/kg,
10 mg/kg) cocktail and transectially perfused through the ascend-
ing aorta with ice-cold saline followed by ice-cold 4% paraformal-
dehyde/0.1 M phosphate buffer (PB) at pH 7.4 (250 mL). Brains
were removed and stored in the fixative overnight (4°C) then
stored in phosphate buffered saline (PBS). Free-floating serial coro-
nal brain sections containing the amygdala were sliced on a vibra-
tome at 60 μm. All sections were thoroughly washed (PBS) prior to
processing for p-ERK1/2 immunohistochemistry.

2.3.2. Immunohistochemistry

Sections were first blocked in PBS containing 1% bovine serum
albumin (BSA)/0.02% Triton X-100 for 1 h. Next, sections were
incubated in a 1:250 dilution rabbit polyclonal antibody to phos-
pho-p44/42 MAPK (p-ERK1/2) (Thr202/Tyr204, Cell Signaling
Technology, Boston, MA) in PBS-1% BSA/0.02% Triton X-100 for
24 h at room temperature. Following washing (PBS), slices for p-
ERK1/2 immunoreactivity were subsequently incubated with bio-
tinilated goat anti-rabbit IgG (1:200 dilution in PBS, Vector Labs,
Burlingame, CA) in PBS-1% BSA/0.02% Triton X-100 for 1 h. Slices
were then washed (PBS) again and incubated in avidin-biotin
HRP complex (ABC Elite, Vector Laboratories, Burlingame, CA) for
2 h. After a final wash (PBS), activated neurons were developed
in 5G chromagen for <10 min (Vector Laboratories, Burlingame,
CA). Serial sections were mounted on gelatin-coated slides, air-
dried, then dehydrated in a graded series of alcohol then xylene,
and cover-slipped for bright field microscopy.

2.4. Amygdala alignment

Spatial analysis requires precise brain alignment to ensure the
same neuron population is compared across individuals
(Bergstrom et al., 2011; Bergstrom, McDonald, Dey, Fernandez,
et al., 2013; Bergstrom, McDonald, Dey, Tang, et al., 2013). To
quantitatively match sections in a common group stereotaxic
space, the morphology of the lateral ventricle (LV) was digitally
reconstructed in four consecutive serial sections (l.7.1.5 to 3.48
Bregma) (Neulrolucida v10, MBF Biosciences, VT). The area of
the LV was calculated (NeuroExplorer, MBF Biosciences, VT), and
sections matched based on LV area. Methods for this procedure have
been described in detail previously (Bergstrom, McDonald, Dey,
Tang, et al., 2013).

2.5. Neuron counting

For all neuronal counting the experimenter was blind to exper-
imental condition. Cell counting was conducted at five matched
locations (Bregma −2.52 mm, −2.64 mm, −2.76 mm, −3.24 mm
and −3.36 mm) spanning 0.84 mm (Paxinos & Watson, 2006). At
these coronal levels, the LA and LAd were well represented. Only
neurons from the right brain hemisphere were included into the
analyses. To segregate the various amygdala subdivisions, a digital
rat brain atlas (Paxinos & Watson, 2006) was scaled, superimposed
and aligned with a representative immunostained section of the
amygdala. The anatomical boundaries of the LA subnuclei (LAd,
LAvm, LAvl) were traced to create a generic contour at 2x magni-
fication (Neulrolucida, MBF Biosciences, VT) using the superim-
posed digital image at the corresponding Bregma coordinate. For
counting neurons from each subject and at each Bregma coordi-
nate, the respective digital contours were positioned over the LA
using various amygdala-centric anatomical features for alignment
including the distance/presence of the lateral ventricle, rhinal fis-
sure, central amygdala and external capsule. Therefore, the dimen-
sions and position of the generic LA contour was identical between
experimental groups. At the rostrocaudal level selected for analy-
sis, the following subdivisions were chosen for quantitative analy-
sis: LA nuclei, dorsal (LAd), ventromedial (LAvm) and ventrolateral
(LAvl). The (XYZ) coordinates of individually immunopositive-
labelled p-ERK1/2 neurons in the LA were marked under a 100X/
1.4 NA oil immersion objective. It was unnecessary to correct for
double counting because sections were non-consecutive. For all
bright field microscopy, Koehler illumination principles were applied.
Markers (XYZ coordinates) and contours were quantified (NeuroExplorer, MBF Biosciences, VT). The density of p-ERK1/2
neurons was calculated as the ratio of p-ERK1/2 neurons and the
contour area (mm²) of each nucleus from 2 to 5 matched sections
(Bregma −2.52 mm to −3.36 mm) from each subject.

2.6. Neuron mapping

Serial coronal rat brain sections were stereotaxically aligned at
Bregma −3.36 mm (see above). To retain stereotaxic alignment at a
distal coronal plane (Bregma −2.76 mm) we measured 600 μm (10
sections) rostral from the anatomical matched section (Bregma −3.36 mm). Therefore, initial mapping was conducted at two rela-
tively distal locations in the LA (Bregma −3.36 mm and −2.76 mm). The boundaries of the LA were delineated using the
procedures described above. We employed a combination of plot-
ting and analytic procedures for visualizing the topography of neu-
rons activated after auditory or visually cued fear conditioning (1.
raw data heat maps, 2. difference heat maps and 3. stability heat
maps).

2.6.1. Raw heat maps

Raw heat maps provide an important plotting tool for visualizing
multivariate neuronal distributions (Bergstrom et al., 2011; Bergstrom, McDonald, Dey, Fernandez, et al., 2013; Bergstrom,
McDonald, Dey, Tang, et al., 2013). To build the heat maps, the XY coordinates for individual neurons were binned (Origin v 8,
OriginLab, Northampton, MA). The spatial dimension for each bin
was 100 × 100 μm. Each bin represented the total number of acti-
vated neurons contained in the 60 μm thick section. Thus, each bin
represented an equal sized portion of the LA XYZ axis (e.g., 100 × 100 × 60 μm). Values within each bin were assigned a color
for visualization purposes (a “heat” map) (SigmaPlot v 12, Systat
Software, San Jose CA).

2.6.2. Difference maps

Difference maps provide another tool for visualizing the loca-
tion of neurons specific to fear memory formation. Difference maps
were generated by subtracting the mean neuron distribution asso-
ciated with the control conditions (Box and Shock) from the neu-
ron distribution of activated neurons associated with each of the
experimental conditions (aFC and vFC). We defined the spatial dis-
tribution of neurons activated after Box and Shock control proce-
dures as representing a “baseline”. A subtraction of “baseline” was
used to isolate neuronal activation related to cued associative
fear learning from non-specific neuronal activation. Correlation
analysis of the values obtained for the difference maps provided
a measure for the degree of relationship between aFC and vFC
memory maps. We interpreted R² (coefficient of determination)
as the degree of shared or “overlapping” variance between mem-
ory maps.

2.6.3. Stability maps

Stability maps provide a visualization of the variation in the
activated neuronal topography within experimental condition. Sta-
bility maps were generated by first calculating the coefficient of
variation (CV) for each bin in the matrix. The CV was determined
by the standard deviation and the mean values for each bin
(CV = SD/mean). CV can be conceptualized as the opposite of signal
to noise; it is the noise in the signal. The CV was particularly useful
in this study for estimating relative variability in the distribution of
activated neurons within group and across the sampling area because the CV is normalized to the mean of the cell population contained in each bin. Therefore, the relative variability across all bins in the matrix was equivalently compared. Distributions with CV < 1.0 were considered to possess low variation and termed “stable.” Heat maps of the CV were generated to visualize the distribution of variance across different brains in the visual and auditory fear conditioning groups. Because all brains were aligned in a common group space (see 2.4. Amygdala alignment), corresponding “Difference” and “CV” maps provided a useful tool for visualizing sites that contained the greatest number of activated neurons specific to encoding either auditory or visual fear conditioning and were stable across individuals.

2.7. Statistical analyses

2.7.1. Univariate ANOVA

All statistical comparisons among experimental conditions for the analysis of neuronal density were conducted using one-way ANOVA. Homogeneity of variance was checked using Levene’s test and corrected if warranted using the Brown-Forsythe F. A significant F statistic was followed by a Fisher’s LSD post hoc test.

2.7.2. Mass univariate ANOVA

To statistically evaluate the memory matrix, mass univariate analysis of variance (ANOVA) with a false discovery rate (FDR) correction for multiple comparisons was conducted across all bins in each memory matrix. The total number of activated neurons corresponding with each bin in the matrix was considered the dependent variable in the mass ANOVA. The FDR correction for multiple comparisons has been well described (Benjamini & Hochberg, 1995) and applied previously to a similar type of data set (Bergstrom, McDonald, Dey, Fernandez, et al., 2013). We set the FDR cutoff at q < 0.10. Bins with q < 0.10 were termed micro-regions of interest (MROIs). Subsequent post hoc comparisons were conducted on statistically significant bins using a Bonferroni-corrected post hoc FDR corrected p-values were generated using R software v. 2.13.0 (Storey & Tibshirani, 2003). All values embedded in the text are the mean ± standard error of the mean (SEM).

3. Results

3.1. Behavior

Twenty-four hours following fear conditioning, presentation of the auditory or visual CS resulted in a statistically equivalent freezing response (p = 0.42, aCS 55% and vCS 68%) (Fig. 1). These data indicate the fear conditioning protocol employed was sufficient to produce a lasting auditory or visual conditioned fear memory. INDICATE the fear conditioning protocol employed was sufficient to produce a lasting auditory or visual conditioned fear memory. There was also no difference between the Shock and Box (p = 0.864) or Auditory and Visual fear conditioning groups (p = 0.701). Together, these data implicate the ERK/MAPK intracellular signaling pathway in visual fear conditioning. These results also suggest the acquisition of two different fear memories of equivalent strength, as assessed by freezing in response to the relevant visual and auditory CS, was associated with a similar density of p-ERK1/2 neurons in the LA.

Multivariate ANOVA (MANOVA) conducted on the p-ERK1/2-expressing neuronal density across the LAd, LAvm and LAvl subnuclei revealed a significant effect of experimental condition (Pillai’s trace $\Lambda = 0.42, F[9, 46.4] = 2.2, p = 0.04$) (Fig. 1). Subsequent one-way ANOVAs on the LAd, LAvm and LAvl revealed significant effects of the experimental conditions in the LAd ($F[3, 24] = 4.8; p = 0.009$) and LAvl ($F[3, 24] = 3.8; p = 0.02$) only (Fig. 1). In the LAd, a post hoc test revealed a significantly greater density of p-ERK1/2 neurons in the aFC group relative to the Box (p = 0.01) and Shock (p = 0.01) control conditions and in the LAvl, there was significantly greater density of p-ERK1/2 neurons in the aFC group relative to the Box (p = 0.01) and Shock groups (p = 0.03). For the vFC group, there was a greater density of p-ERK1/2 neurons relative to Box (p = 0.03) and Shock groups (p = 0.04) for the LAd and a greater density of p-ERK1/2 neurons relative to the Box (p = 0.04) group for the LAvl. There was a nearly significant difference in the vFC versus Shock group (p = 0.09) for the LAvl. There were no other statistically significant effects detected. Together, these results align with previous work indicating the LAd and LAvl subnuclei as important sites for neuronal plasticity underlying fear learning and memory (Schafe et al., 2000; Wilson & Murphy, 2009; Trogrlic, Wilson, Newman, & Murphy, 2011; Bergstrom, McDonald, Dey, Tang, et al., 2013).

3.3. Neuron mapping

3.3.1. Bregma – 3.36 mm spatial analyses

Prior to spatial analysis, one outlier from the FCv group was detected (1.5× the interquartile range) and removed from subsequent analysis. Spatial analyses were conducted on a total of 2381 activated neurons and 107 bins across all subjects (aCS n = 7, vCS n = 8, Shock n = 6 and Box n = 7). Results from correlation analysis revealed a topographic relationship ($r(26) = 0.34, R^2 = 0.12, p < 0.001$) between auditory and visual difference maps at Bregma –3.36 mm (Fig. 2). From this statistic we estimate a 12% overlap in variance for auditory and visual fear memory maps in the LA.

Next, we statistically evaluated the topography of p-ERK1/2 neurons between experimental conditions by applying FDR corrected mass multiple comparisons. FDR corrected multiple comparisons testing revealed 3/107 bins significantly different (q < 0.10) between experimental conditions (Fig. 2). Post hoc analysis revealed significantly more activated neurons in the aFC group (mean of three sig. bins, 3.5 ± 0.6) relative to the vFC group and all controls (mean of three sig. bins, 0.9 ± 0.4) (p < 0.001, ~3:1 ratio) (Fig. 2). These MROIs (100 μm$^3$) were localized in the superior and inferior LAd and LAd – LAvl transition (Fig. 2).

In the last step of the spatial analysis, the stability of the distribution of activated neurons in the aFC and vFC groups was determined by calculating the coefficient of variance (CV) for each bin in the matrix (107 bins). Results indicated stability, as indicated by low CV values (<1.0) that coincided with the regions of greatest difference (difference maps) in the number of activated neurons in the aFC group (Fig. 2). The CV values for each MROI were <0.5, sug-
gesting a particularly high degree of stability in the number of activated neurons specific to individuals encoding an auditory conditioned fear memory. There were few, if any, prominent areas of activated neurons exhibiting stability within the vFC group. Overall, these results suggest the pattern of activated neurons specific to encoding auditory fear conditioning groups. Freezing prior to the presentation of the auditory or visual CS in the novel environment (pre-CS) was low, indicating the freezing response was specific to the auditory CS. (B) Neuron counting in the LA revealed a nearly identical mean density (neurons/mm²) of p-ERK1/2 immunopositive neurons after auditory and visual fear conditioning, that was significantly greater than after Shock and Box control procedures (67.6% change). (C) When the LA was subdivided into LAd, LAvm and LAvl subnuclei, the greatest difference in the density of p-ERK1/2 neurons related to auditory and visual fear conditioning was found in the LAd and LAvl subnuclei. The stereotaxic locations of neurons specific to auditory cued fear conditioning at Bregma /C0 3.24 mm was highly consistent with the location of neurons found at Bregma /C0 3.36 mm (Fig. 3). Like Bregma /C0 3.36 mm, several of the MROIs at Bregma /C0 3.24 mm were located in the LAd

3.3.2. Bregma /C0 3.24 mm spatial analyses

The reliability of the auditory fear memory map was tested by conducting another spatial analysis at another coronal plane (Bregma /C0 3.24 mm) at a location proximal to the original mapped location (Bregma /C0 3.36 mm) to retain stereotaxic alignment (Fig. 3). A visual inspection of the mean distribution of activated neurons at Bregma /C0 3.24 mm revealed a clear relationship in topography with the auditory fear memory map at Bregma /C0 3.36 mm. FDR corrected multiple comparisons revealed several discrete locations (3/103) in the LA that were significantly different among experimental conditions (q < 0.10). Post hoc analysis revealed significantly more activated neurons in the aFC group (mean of three sig. bins, 3.2 ± 0.7) relative to the vFC group and all controls (mean of three sig. bins, 0.7 ± 0.3)(p < 0.001, ~3:1 ratio). The stereotaxic locations of neurons specific to auditory cued fear conditioning at Bregma /C0 3.24 mm was highly consistent with the location of neurons found at Bregma /C0 3.36 mm (Fig. 3). Like Bregma /C0 3.36 mm, several of the MROIs at Bregma /C0 3.24 mm were located in the LAd...
and LAvl transition area and possessed highly similar D-V (700-800 µm) and M-L (250 µm) coordinates (Fig. 3). These results support previous findings showing a reliable functional topography of auditory fear memory storing cells in the (Bergstrom, McDonald, Dey, Tang, et al., 2013).

### 3.3.3. Spatial analyses at Bregma –2.76 mm

Next, we mapped the LA at a distal location approximately 600 µm rostral to Bregma –3.36 mm. Spatial analyses were conducted on a total of 1969 activated neurons and 69 bins across all subjects (aCS n = 6, vCS n = 9, Shock n = 6 and Box n = 7). Results from correlation analysis revealed a topographic relationship between auditory and visual maps for Bregma –2.76 mm (Fig. 4). The relationship (r(26) = 0.43, R² = 0.18, p < 0.001) is also indicated by a visual comparison of the neuronal distribution in the topographic heat maps for visual and auditory fear conditioning (Fig. 4). From this statistic, we estimate 18% overlap in variance for auditory and visual fear memory maps at Bregma –2.76 mm in the LA.

A spatial analysis of p-ERK1/2 neurons across all of the experimental conditions was conducted using mass multiple comparisons. FDR corrected comparisons revealed a single discrete location (1/69) in the LA that was significantly different among experimental conditions (q < 0.10) (Fig. 4). Post hoc analysis showed significantly more activated neurons in the visual fear conditioning group (3.2 ± 0.4) relative to the auditory fear conditioning group (p = 0.02, 1.6 ± 0.4) and controls (Box p = 0.002, 1.0 ± 0.4) and Shock (p = 0.001, 0.8 ± 0.3). Interestingly, the MROI specific to visual fear conditioning...
was located in the LAvm subnucleus of the amygdala. To test whether the location of neurons specific to visual fear conditioning was reliable, neuronal mapping was conducted on a section proximal to Bregma −2.76 mm (Bregma −2.64 mm) (Fig. 5). FDR corrected multiple comparisons revealed a single discrete location (1/58) in the LA that was significantly different among experimental conditions (q < 0.10) (Fig. 5). Post hoc analyses revealed significantly more activated neurons in the visual fear conditioning group (2.6 ± 0.5) relative to all controls (Box p = 0.002, 0.3 ± 0.2; Shock p = 0.001, 0.2 ± 0.2) and a nearly significant increase in the number of neurons compared with the auditory fear conditioning group (p = 0.076, 1.3 ± 0.3). The MROI at Bregma −2.64 mm were highly consistent with the location of neurons found at Bregma −2.76 mm. Both MROIs were located in the LAvm and possessed highly consistent D-V (800–1000 μm) and M-L (150 μm) coordinates (Fig. 6). These results suggest the MROI specific to visual fear conditioning was reliable and non-erroneous.

3.4. Light alone control group

In the next experiment we set out to determine the contribution of non-associative neuronal activation as a result of the light CS alone by including a group of rats that were presented with the identical visual CS (1 Hz: 0.5 s ON/0.5 s OFF for 20 s, 35 lux) as used in the previous experiments but without the US (n = 4). A tone alone control condition was not included into the design because the presentation of an auditory CS alone has not been previously associated with an increase in p-ERK1/2 neurons beyond baseline in the LA (Schafe et al., 2000). Further, unpaired fear conditioning using an auditory CS has not been shown to increase the density of p-ERK1/2 neurons beyond Box controls or produce stable patterning in the LAd (Schafe et al., 2000; Bergstrom et al., 2011; Bergstrom, McDonald, Dey, Tang, et al., 2013). Inclusion of a light alone control group into an ANOVA revealed a significant effect (F[3, 22] = 4.15; p = 0.02) with an increase in the density of activated LA neurons in the vFC group relative to the Light alone (p = 0.01), Box alone (p = 0.02) and Shock alone (p = 0.01) controls. For the LA at Bregma −2.76 mm (p = 0.04, data not shown) where the visual map was localized (Fig. 5), results showed a significant increase in the density of activated neurons in the visual fear conditioning group relative to the Light control group (unpaired t-test, p = 0.01). This result suggests the visual CS alone was insufficient to induce synaptic plasticity in the LA when using p-ERK1/2 as a molecular marker. Next, we asked of the cells activated as a result of the visual CS alone, were they in the same location as the visually conditioned fear group? In particular, we wanted to determine whether the discrete site of activated neurons that was localized in the LAvm after visual fear conditioning might simply reflect a site of plasticity related to the non-associative visual stimulus. We used a difference map of visual fear conditioning minus the spatial
distribution of activated cells after light alone to visualize the contribution of the light by itself to the visual fear conditioning map (Fig. 6). Spatial analyses conducted on the difference maps clearly confirmed that the spatial distribution of activated neurons following the light alone could not account for the geometry of the visual fear conditioning map (Fig. 6).

3.5. A rostrocaudal gradient for auditory and visual fear memory in the LA

The final question asked was whether differences in the density of the p-ERK1/2 neuron population across the rostrocaudal (R-C) axis might account for a spatial segregation of visual and auditory memory maps in the LA. To address this question we calculated the p-ERK1/2 neuronal density encoding auditory and visual fear conditioning across the R-C axis of the LA. ANOVA with repeated measures on the density of p-ERK1/2 neurons across the R-C axis of the LA was found to be not significant \( p = 0.27 \) (Fig. 7). This results suggests that the identified MROIs containing activated neurons specific to encoding either auditory or visual fear conditioning at different R-C levels of the LA (Bregma \(-3.36\) mm, \(-3.24\) mm, \(-2.76\) mm, \(-2.64\) mm) was independent of the size of the total activated neuronal population. Additional repeated measures ANOVA was conducted independently for the aFC and vFC conditions versus all controls. There were no condition \( \times \) R-C axis interactions detected for either test (aFC \( p = 0.71 \), vFC \( p = 0.33 \)), further confirming that the density of p-ERK1/2 neurons specific to either memory remained relatively stable across the portion of R-C axis studied (from Bregma \(-3.36\) to \(-2.64\) mm) (Fig. 7). We conclude that while the density of the neuronal population activated...
after either auditory or visual fear conditioning was similar, there was a difference in how these neuronal populations were anatomically organized.

Overall results from this series of experiments establish (1) the same density of p-ERK1/2-expressing neurons in the LA at 60 min following visual and auditory conditioning and (2) the existence of several discrete anatomical sites of the LA that contained both a stable and high proportion (3:1) of p-ERK1/2-expressing neurons activated following auditory or visually cued fear conditioning.

4. Discussion

Here we used the presence of p-ERK1/2 in neurons from the lateral amygdala after visual or auditory Pavlovian fear conditioning to indicate an active process of fear memory consolidation. Spatial analyses of the total p-ERK1/2 neuron population revealed several discrete sites that contained both a stable and high proportion (3:1) of p-ERK1/2-expressing neurons activated following auditory or visually cued fear conditioning.

4.1. A functional map for visual fear conditioning in the LA

There has been relatively limited study into how visual fear conditioning is encoded in the LA (Ledoux et al., 1989; Sananes & Davis, 1992; Campeau & Davis, 1995; Heldt, Sundin, Willott, & Falls, 2000; Shi & Davis, 2001; Tazumi & Okaichi, 2002; Sacco & Sacchetti, 2010; Kelley, Anderson, Altmann, & Itzhak, 2011; Jones, Ringuet, & Monfils, 2013). Our data show a greater density of neurons expressing p-ERK1/2 in the LA after visual fear conditioning than presentation of the non-associated stimuli, including the light CS by itself (65% change). Spatial statistics indicated a discrete site (100 μm²) localized in the LAvm that contained a high proportion (3:1) of activated neurons after visual fear conditioning (Bregma /C0 2.76 mm) (Fig. 5). There was a 184% increase in the number of p-ERK1/2-activated neurons specific to visual fear conditioning in this site relative to a 57% increase for the surrounding area. When we mapped another brain section proximal to the original location (Bregma /C0 2.64 mm) we discovered another site located in a similar area (LAvm) that contained neurons specific to visual fear conditioning. This result confirms the location of LAvm activated neurons after visual fear conditioning is generalizable and non-erroneous. The presence of these unique sites in the LAvm did not translate into a larger overall density of activated neurons in the LAvm, suggesting neuron population size alone cannot explain the location of stable sites of activated neurons following visual fear conditioning in the LA. Together, these findings point to a much higher likelihood of localizing neurons specific to encoding for visual fear conditioning in the LAvm (Fig. 5).
4.2. A map for auditory fear conditioning in the LA

The topographic map for auditory fear conditioning comprised several unique sites located in the LAd and LAvl transition area (Figs. 3 and 4). This neuronal pattern confirms previous qualitative observations (Schafe et al., 2000) and quantitative study (Bergstrom et al., 2011; Bergstrom, McDonald, Dey, Fernandez, et al., 2013; Bergstrom, McDonald, Dey, Tang, et al., 2013). There was a 266% increase in the density of activated neurons specific to auditory fear conditioning in the identified sites relative to the surrounding area (72%). This finding aligns with evidence from rodent models (Rumpel et al., 2005; Reijmers, Perkins, Matsuo, & Mayford, 2007; Bergstrom et al., 2011) and humans (Bach, Weiskopf, & Dolan, 2011) for a sparse neuron network encoding fear conditioning in the amygdala. A matching neuron population density and sites specific to auditory fear conditioning in the identified sites relative to the surrounding area (72%). This finding aligns with evidence from rodent models (Rumpel et al., 2005; Reijmers, Perkins, Matsuo, & Mayford, 2007; Bergstrom et al., 2011) and humans (Bach, Weiskopf, & Dolan, 2011) for a sparse neuron network encoding fear conditioning in the amygdala. A matching neuron population density and sites specific to auditory fear conditioning was found at a proximal, (Bregma /C0 3.24 mm) but not distal (Bregma /C0 2.76 mm), coronal plane (Fig. 6), suggesting a rostrocaudal gradient for neurons specific to encoding auditory fear conditioning in the LA. However, within-subject analysis of the activated neuronal density across the longitudinal axis did not bear this conclusion out (Fig. 7), indicating some degree of stability in the density, but perhaps not topography, of the activated neuronal population. These are first data to indicate that a stable topography for neurons activated after auditory fear conditioning may not be completely maintained across the entire extent of the rostrocaudal axis of the LA. More work will be required to determine the precise anatomical boundaries for fear memory maps across the longitudinal axis of the LA.

4.3. A common map for auditory and visual fear conditioning in the LA

The overall density of p-ERK1/2 neurons encoding auditory fear conditioning in the LA was roughly the same after visual fear conditioning (5.3% difference, 67.7% change/controls) (Fig. 1), suggesting a common neural population size underlies both forms of memory. There was some topographic overlap for neurons activated after auditory and visual fear conditioning in this population (12% caudal [Bregma −3.36 mm] and 18% rostral [Bregma −2.76 mm]). This result suggests that some portion of the neuronal population specific to encoding auditory and visual fear conditioning is intermingled in the LA. This finding also raises the possibility that some of the same neuron subsets or possibly even the same neurons might be expected to encode multimodal fear memories in the LA. This interpretation is supported by the cell assembly theory (Hebb, 1949; Johnson et al., 2009). One attribute inherent in cell assembly theory is that for any given memory representation more than one cell assembly may be allocated to it. Therefore, it is likely that different fear memory cell assemblies overlap in the LA. Overlapping functionality for individual neurons has been demonstrated in the hippocampus for visual and auditory memory processes (Sakurai, 1996) and for different contextual memory representations in the amygdala (Nomura, Nonaka, Imamura, Hashikawa, & Matsuki, 2011). Although some aspects of the neuron distribution activated after auditory and visual fear conditioning were common, here we show that some aspects were different.

In vivo physiological techniques (e.g., multi-electrode) or genetic approaches (e.g., optogenetics, viral labeling, transgenics) may help to unravel modality-specific and multi-modal fear memory encoding neuronal ensembles in the LA. Methods developed here and in previous studies (Bergstrom et al., 2011; Bergstrom, McDonald, Dey, Tang, et al., 2013) for placing the activated neuron subpopulation in a common stereotaxic group space facilitates spatial analysis of memory-storing neurons. This approach complements existing techniques for localizing memory traces at the level of neuronal assemblies in the mammalian brain.
fear conditioning in the LA. Box control groups.

significant increase in the density of p-ERK1/2 neurons relative to Shock and density of p-ERK1/2 neurons across the longitudinal axis. However, there was a

axis of the LA. (B) After auditory fear conditioning there was no difference in the

density of p-ERK1/2 neurons after the rostrocaudal axis of the LA. (A) ANOVA with repeated measures on the longitudinal axis revealed no difference in the density of p-ERK1/2 neurons after auditory or visually cued fear conditioning across the sampled rostrocaudal axis of the LA. (Doron & Ledoux, 1999) while sensory cortical inputs (Romanski & LeDoux, 1993; Doron & Ledoux, 1999). For auditory cortical inputs there appears to be a topographic organization along the dorsoventral axis (Romanski & LeDoux, 1993) with the LAd and LAvl targeted by different auditory association cortices (Romanski & LeDoux, 1993). Further, the auditory association area TE3 projects most density to the LAd (Mascagni, McDonald, & Coleman, 1993). Overall, this pattern of connectivity corresponds well with the pattern of p-ERK1/2 neuron labeling following auditory fear conditioning in the LAd and LAvl. In the visual system, the lateral posterior nucleus (LP) of the thalamus has been shown to terminate in the entire LA (Doron & Ledoux, 1999). Interestingly, in contrast to the LP, the TE2 (a predominantly visual cortical area) projects most heavily to the LAvm (Mascagni et al., 1993; Shi & Cassell, 1997) and along the rostrocaudal plane of the LAvm. Projections of TE2 appear to be localized near the same Bregma coordinate (Bregma \(-2.76 \text{ and } -2.64 \text{ mm}\)) as the location for the greatest density of p-ERK1/2 cells following visual fear conditioning (Shi & Cassell, 1997). This correspondence raises the possibility that the density of visual-related cortical afferents contributes to the observed anatomical organization of visual fear conditioning in the LA in this study. It is important to note, however, that TE2 may also be a polymodal visual/auditory cortical region (McDonald, 1998).

Generally, it appears that sensory thalamic input is widespread throughout the LA (Doron & Ledoux, 1999) while sensory cortical projections are more localized (Mascagni et al., 1993; Romanski & LeDoux, 1993; Shi & Cassell, 1997). Auditory thalamic and cortical pathways overlap in the LAd and LAvl while the LAvm receives overlapping input from the visual thalamus and cortices. These same LA subnuclei were the locations of discrete sites (100 \times 100 \mu m) containing a high number of p-ERK1/2 cells following visual or auditory fear conditioning (Figs. 2 and 4). Learning-induced plasticity in the LA can be triggered by temporally divergent sensory signals arising from spatially convergent thalamic and cortical pathways (LeDoux, Farb, & Romanski, 1991; Davis, 1992; LeDoux, 2000; Sah et al., 2003; Parse, Quirk, & Ledoux, 2004; Johnson et al., 2008; Pape & Pare, 2010). Therefore, convergent thalamic and cortical LA afferents may represent an important organizing factor determining, at least in part, the anatomical distribution of p-ERK1/2 cells following the acquisition of visual and auditory fear conditioning. To our knowledge, the precise anatomical organization of auditory and visual thalamic and cortical connectivity in the LA has not been characterized at the fine-scale resolution of our functional spatial analysis (100 \times 100 \mu m).

The findings here combined with previous anatomical work suggest that connectivity may play a role in determining not only about the visual and auditory CS to the LA are intrinsically different in the rodent brain (McDonald, 1998; Doron & Ledoux, 1999; Pitkänen, 2000) with the visual pathways less sophisticated and likely deprived relative to the auditory pathways (LeDoux, Farb, & Ruggiero, 1990; Doron & Ledoux, 1999; Prusky, Harker, Douglas, & Whishaw, 2002). Here, we experimentally controlled for differences in auditory and visual acuity by calibrating the intensity of the auditory and visual CS to produce equivalent sensory salience. We defined memory strength as the level of the conditioned freezing response. Because memory strength was equivalent, the underlying neuronal subpopulations encoding different memories were directly comparable.

4.4.2. Auditory and visual network connectivity in the lateral amygdala

Classical studies of amygdala anatomical connectivity showed both auditory and visual afferents terminate in the LA (Doron & Ledoux, 1999; Pitkänen, 2000). Auditory connectivity in the LA is well characterized with the entire LA receiving dense thalamic and cortical inputs (Romanski & LeDoux, 1993; Doron & Ledoux, 1999). For auditory cortical inputs there appears to be a topographic organization along the dorsoventral axis (Romanski & LeDoux, 1993) with the LAd and LAvl targeted by different auditory association cortices (Romanski & LeDoux, 1993). Further, the auditory association area TE3 projects most density to the LAd (Mascagni, McDonald, & Coleman, 1993). Overall, this pattern of connectivity corresponds well with the pattern of p-ERK1/2 neuron labeling following auditory fear conditioning in the LAd and LAvl. In the visual system, the lateral posterior nucleus (LP) of the thalamus has been shown to terminate in the entire LA (Doron & Ledoux, 1999). Interestingly, in contrast to the LP, the TE2 (a predominantly visual cortical area) projects most heavily to the LAvm (Mascagni et al., 1993; Shi & Cassell, 1997) and along the rostrocaudal plane of the LAvm. Projections of TE2 appear to be localized near the same Bregma coordinate (Bregma \(-2.76 \text{ and } -2.64 \text{ mm}\)) as the location for the greatest density of p-ERK1/2 cells following visual fear conditioning (Shi & Cassell, 1997). This correspondence raises the possibility that the density of visual-related cortical afferents contributes to the observed anatomical organization of visual fear conditioning in the LA in this study. It is important to note, however, that TE2 may also be a polymodal visual/auditory cortical region (McDonald, 1998).

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The findings here combined with previous anatomical work suggest that connectivity may play a role in determining not only
the anatomical organization but also stability in the location of neurons encoding different types of fear conditioning in the LA. The precise correspondence between patterns of afferent connectivity, patterns of intrinsic connectivity (Johnson & Ledoux, 2004, 2010; Johnson et al., 2008, 2009) and patterns of p-ERK1/2 neurons identified following different types of fear learning is an important endeavor for future study.

4.4.3. Lateral amygdala neuronal microcircuits

Network plasticity is not only determined by sensory inputs but also by reverberation activity in neuronal microcircuits. It is classically understood that Hebbian learning, at the level of single cells, is accomplished by coincidence detection of both temporal and spatial convergent CS and US signals (Hebb, 1949). Learning and memory also occurs at the level of neural ensembles. Both in vitro and in vivo recordings have indicated a recurrent network in the LA that reverberates and may facilitate coincidence detection of temporally incongruent thalamic and cortical neural signals (Johnson et al., 2008, 2009). It seems likely that not all of the neurons allocated to a reverberating neural network receive the same converging thalamic and cortical CS and US input. Indeed, the organization of the memory traces identified in the present study may reflect some combination of the organization of sensory inputs and reverberation activity of a recurrent microcircuit (Johnson & Ledoux, 2004, 2010; Johnson et al., 2008, 2009).

4.5. p-ERK1/2 as a molecular marker of fear learning-induced plasticity in the LA

It is important to note that the activated neuronal populations identified in this study were uncovered using a single molecular marker of fear memory consolidation (p-ERK1/2), at a single point in time during the consolidation phase of auditory or visual fear conditioning (60 min) and in only a single region of the brain (the LA). It is conceivable, and likely probable, that studying different molecular markers at different time points following learning might yield alternate stable activated neuron populations both inside and outside the lateral amygdala. Nevertheless, the data generated here represent a significant initial advancement towards identifying the organization of visual and auditory fear memory encoding in the LA.

While several studies have implicated the LA in visually cued fear conditioning (Ledoux et al., 1989; Campeau & Davis, 1995; Shi & Davis, 2001), there is some debate as to whether the LA represents a crucial site for visual fear conditioning (Tazumi & Okaihi, 2002). Here we provide the first evidence that visual fear conditioning is associated with p-ERK1/2 neuronal activation in the LA. More study is required to understand the precise role of LA neuronal plasticity in the generation of visual cued fear memory.

A single auditory CS frequency (2 kHz) or visual CS type (1 Hz blinking light) was used in these fear conditioning experiments. The intensity of the CSs was chosen because they produced an equivalent freezing (fear) response (Fig. 1). It is known that neurons in the amygdala respond to different auditory CS frequencies (Bordi & LeDoux, 1992), including in the ultrasonic range (Parsana, Li, & Brown, 2012) and there is some evidence for a tonotopic map in the amygdala (LeDoux et al., 1991). It is likely that auditory or visual CSs of different frequencies might activate different neuronal subpopulations in the LA. Under the frequency ranges tested we show parallel LA neuronal ensembles can be activated by auditory and visual input paths, producing an equivalent freezing response. Thus, the LA can associate different CS and US signals in separate ensembles to produce a similar defensive response. These data elucidate aspects of a putative functional hierarchical architecture of associative conditioning in the LA.

5. Conclusions

We conclude that a portion of an auditory or visually cued fear memory trace is encoded in different neuron subpopulations of the LA. This conclusion is based on the relative distribution of p-ERK1/2-expressing neurons in the LA after Pavlovian fear conditioning for either the auditory or visual sensory modality. These results imply that parallel neuronal ensembles encode modality-specific fear memory in the LA. An organization of auditory and visual fear conditioning in the LA is an important preliminary step toward deciphering more complex fear memory representations containing interlinked audiovisual elements (e.g., compound and second-order fear conditioning). Outside the laboratory, fear-predicting cues are complex, likely comprising more than one sensory modality. Our findings provide new insight into an organization of visual and auditory fear conditioning in the LA, which has clinical relevance for understanding the neurobiology of PTSD.

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