Memories are thought to be represented by discrete physiological changes in the brain, collectively referred to as an engram, that allow patterns of activity present during learning to be reactivated in the future. During the formation of a conditioned fear memory, a subset of principal (excitatory) neurons in the lateral amygdala (LA) are allocated to a neuronal ensemble that encodes an association between an initially neutral stimulus and a threatening aversive stimulus. Previous experimental and computational work suggests that this subset consists of only a small proportion of all LA neurons, and that this proportion remains constant across different memories. Here we examine the mechanisms that contribute to the stability of the size of the LA component of an engram supporting a fear memory. Visualizing expression of the activity-dependent gene Arc following memory retrieval to identify neurons allocated to an engram, we first show that the overall size of the LA engram remains constant across conditions of different memory strength. That is, the strength of a memory was not correlated with the number of LA neurons allocated to the engram supporting that memory. We then examine potential mechanisms constraining the size of the LA engram by expressing inhibitory DREADDS (designer receptors exclusively activated by designer drugs) in parvalbumin-positive (PV+) interneurons of the amygdala. We find that silencing PV+ neurons during conditioning increases the size of the engram, especially in the dorsal subnucleus of the LA. These results confirm predictions from modeling studies regarding the role of inhibition in shaping the size of neuronal memory ensembles and provide additional support for the idea that neurons in the LA are sparsely allocated to the engram based on relative neuronal excitability.

1. Introduction

Memories are represented by distinct changes in the brain (Eichenbaum, 2016; Josselyn, Köhler, & Frankland, 2015; Tonegawa, Liu, Ramirez, & Redondo, 2015). These learning-induced changes, which together constitute the engram, or memory trace, are thought to be widely distributed and include a broad range of alterations, from epigenetics to synaptic connectivity and neural excitability. Although little is known about how information is encoded within these engrams (for instance, how the strength of a memory is encoded), recent experiments examining components of engrams in the amygdala, hippocampus and retrosplenial cortex have confirmed that the reactivation of neuronal ensembles active during learning is both necessary and sufficient for memory retrieval (Cowansage et al., 2014; Han et al., 2009; Kim, Kwon, Kim, Josselyn, & Han, 2013; Liu et al., 2012; Yiu et al., 2014).

A critical component of the engram supporting an auditory fear memory can be identified within the lateral nucleus of the amygdala (LA). Auditory information from the thalamus and auditory cortex converges with pain signals from the thalamus, spinal cord and somatosensory cortex (Tovote, Fadok, & Lüthi, 2015) in the LA, allowing an association to be formed between a motivationally neutral tone (conditioned stimulus, CS) and an aversive footshock (unconditioned stimulus, US). In agreement with computational theories of memory (Kanerva, 1988; Krieg & Triesch, 2014), physiological data indicate that fear memories in the LA are sparsely encoded. For instance, although >70% of principal (excitatory) LA neurons...
neurons are tone-responsive (Repa et al., 2001) and most are footshock-responsive (Barot, Kyono, Clark, & Bernstein, 2008; Lanuza, Moncho-Bogani, & Ledoux, 2008; Romanski, Clugnet, Bordi, & LeDoux, 1993), electrophysiological recordings from LA neurons before and after auditory fear conditioning show that only a modest subset (~25%) of excitatory neurons show increased CS-responsiveness after conditioning (Quirk, Repa, & LeDoux, 1995). Additional studies using different methods [single-unit recordings (An, Hong, & Choi, 2012; Ghosh & Chattarji, 2015; Herry et al., 2008), immediate early genes (Gouty-Colomer et al., 2015; Kim, Kwon, et al., 2013; Reijmers, Perkins, Matsuo, & Mayford, 2007) and molecular tagging (Rumpel, LeDoux, Zador, & Malinow, 2005)] agree that only a small proportion (15–30%) of principal neurons in the LA become part of a fear memory trace supporting any one memory. Together, these findings suggest that only a small subpopulation of eligible neurons, which receive appropriate sensory innervation, are allocated to a specific engram in the LA.

It has previously been shown that highly excitable LA neurons are more likely to be allocated to an engram (Han et al., 2007; Hsiang et al., 2014; Yiu et al., 2014; Zhou et al., 2009). However, artificially increasing excitability in a large number of LA neurons does not influence the size of the engram, consistent with the interpretation that memory allocation is a competitive process in which only a portion of the most excitable cells are selected. Computational modeling studies suggest that inhibitory interneurons may play an important role in this process by allowing highly excitable principal neurons to inhibit their neighbors and exclude them from becoming part of the memory trace (Feng, Samarth, Paré, & Nair, 2016; Kim, Paré, & Nair, 2013). By magnifying the difference in excitability between ‘winner’ and ‘loser’ neurons, the activity of interneurons during learning may “cap” the number of neurons allocated to the engram, thereby promoting the specificity of learned associations (Kim, Samarth, Feng, Pare, & Nair, 2016).

Here we examine the process of neuronal allocation to the LA component of an engram underlying auditory fear memory. We first investigate whether the size of the LA engram varies with memories of different strength. Second, we test whether inhibitory interneurons in the amygdala constrain the size of the LA engram by inhibiting their activity during learning.

2. Results

2.1. The number of amygdala neurons expressing Arc increases after fear conditioning

To identify neurons that were active during memory encoding or retrieval (and therefore neurons that may be allocated to the engram) we visualized expression of the activity-dependent gene, Arc, similar to previous studies (Gouty-Colomer et al., 2015; Reijmers et al., 2007; Tayler, Tanaka, Reijmers, & Wiltgen, 2013). Specifically, we trained mice in auditory fear conditioning (Kim, Samarth, Feng, Pare, & Nair, 2016; Kim, Paré, & Nair, 2013). By magnifying the difference in excitability between ‘winner’ and ‘loser’ neurons, the activity of interneurons during learning may “cap” the number of neurons allocated to the engram, thereby promoting the specificity of learned associations (Kim, Samarth, Feng, Pare, & Nair, 2016).

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2.2. Memory retrieval activates a constant proportion of cells in the amygdala despite varying memory strength

In order to assess the relationship between memory strength and the size of the Arc+ engram, we trained groups of mice with different US intensity (0.3 mA, 0.5 mA and 0.75 mA footshock). Following a memory test 24 h later, we examined the number of neurons (NeuN+, Duan et al., 2016) positive for Arc+ using systematic stereological counting procedures.

Regardless of memory strength, a stable, sparse proportion of Arc+ neurons in the LA (10–15%) was observed. Varying footshock intensity produced different levels of freezing during testing (Fig. 2a; F(2, 14) = 4.39, P > 0.05), consistent with different memory strengths. However, there was no difference in the proportion of Arc+ neurons between these groups (Fig. 2b; F(2, 14) = 0.22, P > 0.05). The proportion of Arc+ neurons increased in all trained groups over homecage controls (Tukey’s post-hoc). Linear regression analysis revealed no relationship between the amount of time a mouse spent freezing to the tone during the test session and Arc+ proportion in the LA (Fig. 2c, R^2 = 0.003). Together, these data suggest that a similar number of neurons are allocated to the engram regardless of the intensity of training conditions or the strength of learned fear associations.

In order to gain a more temporally precise indication of which neurons in the LA were reactivated as part of an engram, we measured Arc mRNA with cellular compartmental analysis of temporal activity by fluorescence in situ hybridization (catFISH). Localization of Arc mRNA in the nucleus is a molecular signature of a neuron that was active in the previous 5 min (Guzowski et al., 2005). Therefore, visualization of Arc mRNA allowed us to identify neurons active during memory retrieval with a high degree of temporal accuracy.

Similar to the previous experiment, we fear conditioned mice with different training intensities (1 × 0.4 mA, 1 × 0.7 mA and 3 × 0.7 mA footshock). As expected, these different conditions produced varying degrees of freezing to the tone during a memory test (Fig. 3a; F(2, 7) = 5.30, P < 0.05), indicating different memory strengths. However, there was no difference in the proportion of Arc+ cells between any fear conditioned group (Fig. 3b; F(4, 10) = 0.99, P > 0.05). Furthermore, no difference was observed in the proportion of Arc+ neurons detected between groups examined after training or testing. All trained groups showed higher levels of Arc than the group trained with an immediate shock control, which exhibited very low Arc signal (all groups vs immediate shock, P < 0.001). One again, no relationship was observed between Arc+ proportion and percentage time spent freezing (Fig. 3c, R^2 = 0.096), suggesting that the proportion of LA neurons in an engram is unrelated to the strength of learned associations.

2.3. Inhibition of PV+ interneurons during conditioning increases the size of lateral amygdala engram

In both the LA and the basal amygdala (BA), local inhibition is predominantly mediated by GABAergic parvalbumin-positive (PV+) interneurons (Ehrlich et al., 2009; Spampanato, Polepalli, & Sah, 2011), which form a broad, inter-connected inhibitory
network that tightly controls the activity of LA principal neurons through plastic, perisomatic synapses (Freund & Katona, 2007; Trouche, Sasaki, Tu, & Reijmers, 2013). Therefore, PV+ interneurons are a promising candidate for mediating inhibitory interactions between principal neurons.

To determine whether PV+ interneurons constrain the size of the LA engram, we used inhibitory DREADDS (designer receptors exclusively activated by designer drugs) to silence the activity of PV+ interneurons during training. DREADDS are modified G-protein coupled receptors activated by the synthetic ligand clozapine-N-oxide (CNO) that allow neuronal activity to be modulated over prolonged periods (Armbruster, Li, Pausch, Herlitze, & Roth, 2007; Nichols & Roth, 2009). We expressed the inhibitory DREADD hM4Di in amygdalar PV+ cells by bilaterally microinjecting a Cre recombinase-dependent adeno-associated viral vector (AAV-DIO-hM4Di-mCherry) into the amygdali of transgenic mice expressing Cre recombinase specifically in PV+ cells (PV-Cre knockin) (Tanahira et al., 2009). We systemically administered either CNO or a vehicle (VEH) 1 h before auditory fear conditioning. The next day, mice were tested for memory and 90 min later, perfused for Arc protein immunohistochemistry, as above.

Inhibiting PV+ interneurons during auditory fear conditioning increased the number of neurons allocated to the LA component of the engram (Arc+ following memory retrieval). Although there was no difference in percent time spent freezing to the tone in mice treated with CNO or VEH (Fig. 4a; unpaired t test, P > 0.5), there was an increase in the proportion of Arc+ cells in mice treated with CNO (Fig. 4b; P < 0.01; Mann-Whitney U test, P < 0.05). Once again, no relationship was observed between percentage of time spent freezing and Arc+ proportion (Fig. 4c; CNO, R2 = 0.015; VEH R2 < 0.001). These findings suggest that reduced PV+ interneuron activity during auditory fear conditioning increases the number of cells allocated to the engram without influencing memory strength.

The LA can be subdivided into distinct subnuclei on the basis of extra-amygdalar connectivity (Romanski et al., 1993; Sah, Faber, Armentia, & Power, 2003). To explore whether the allocation of neurons to the engram in our experiments varied between subnuclei, we divided our stereological estimates of Arc+ proportion among the dorsal (LAd), ventral-medial (LAvm) and ventral-lateral (LAvl) regions of the LA (Fig. 5a). This analysis revealed that the LAd was the only subnucleus in which there was an increased proportion of Arc+ neurons in CNO-treated mice (unpaired t test, P < 0.01), suggesting that the observed increase in the size of the engram may have been driven exclusively by changes in the recruitment of LAd neurons.

3. Discussion

Although the mechanisms of memory encoding in the amygdala are not fully understood, several lines of evidence suggest that memories are sparsely encoded in ensembles of neurons in the LA (An et al., 2012; Han et al., 2007; Herry et al., 2008; Quirk et al., 1995; Reijmers et al., 2007; Rumpel et al., 2005). Here we
show that only a small portion of LA neurons (10–15%) are active during the encoding and retrieval of auditory fear memories and that the size of this portion remains constant regardless of the memory strength. Additionally, we find that this proportion is increased if PV+ inhibitory interneurons are silenced during memory formation, suggesting that inhibition may play a crucial role in constraining the overall size of neuronal memory ensembles in the LA.

Previous research shows that LA neurons expressing high levels of the transcription factor CREB are more likely to be allocated to an engram supporting a conditioned fear memory (Han et al., 2007). CREB, in addition to promoting synaptic plasticity (Silva, Kogan, Frankland, & Kida, 1998), increases intrinsic neuronal excitability (Zhou et al., 2009). Thus, it has been hypothesized that excitability plays a major role in determining which neurons are allocated to the LA component of an auditory fear engram. However, increasing the excitability of the LA by overexpressing CREB in up to 20% of neurons does not increase the number of neurons recruited to an engram (Han et al., 2007; Yiu et al., 2014). By manipulating memory strength with various intensities of training, we find that increasing the strength of the memory also has no effect on the overall size of the LA engram. Together, these results suggest that the mechanisms of memory allocation constrain neuronal memory ensembles to a sparse proportion of all cells capable of responding to the appropriate stimuli. Although a relationship between the size of the reactivated engram population and memory strength may emerge when memories are extinguished (Reijmers et al., 2007), information storage in small, consistently-sized neuronal ensembles is consistent with the theory of sparse distributed memory and physiological data from other brain regions (Hromadka, DeWeese, & Zador, 2008; Sanes & Donoghue, 2000; Weliky, Fiser, Hunt, & Wagner, 2003; Wixted et al., 2014). Sparse distributed coding, in which discrete units of information are distributed across small subsets of neurons in a large network, may provide a structure for high-capacity memory storage that is robust to noise and capable of being implemented in a rapidly-changing biological substrate (Ahmad & Hawkins, 2015; Druckmann & Chklovskii, 2012; Krieg & Triesch, 2014).

Computational modeling of memory formation in the LA suggests that competitive interactions mediated by inhibitory interneurons maintain the size of memory traces despite variations in excitability (Feng et al., 2016; Kim, Paré, et al., 2013). To test this hypothesis, we used DREADDs to inhibit interneurons in the amygdala during auditory fear conditioning and measured the proportion of neurons active during subsequent memory retrieval. We focused on PV+ interneurons because of their role in regulating LA principal neuron excitability (Spamparano et al., 2011) and modulating the expression of fear memories (Trouche et al., 2013). However, we leave open the possibility that other interneuron subtypes may be involved in constraining the size of the LA.

Fig. 2. Consistent proportion of Arc+ neurons in the LA following retrieval of an auditory fear memory despite different memory strengths. (a) Increasing footshock intensity during training produced higher levels of freezing during the test, consistent with greater conditioned fear memory. (b) The proportion of Arc+ neurons was higher following memory testing (compared to homecage, HC) in all trained groups, but there was no difference in proportion of Arc+ neurons between groups trained with varying intensities of shock. (c) No relationship between time spent freezing to tone (strength of memory) and Arc+ proportion (size of LA engram). (d) Examples of Arc (green) and NeuN (red) staining in the LA of HC mice and those trained for auditory fear conditioning using different intensities of shock. Scale bars, 50 μm. (HC, n = 7; 0.3 mA footshock intensity, n = 5; n = 7, 0.5 mA; n = 5, 0.75 mA). *P < 0.05 with Tukey’s post hoc test for multiple comparisons. Data is expressed as mean ± sem.
component of an engram (Stefanelli, Bertollini, Lüscher, Muller, & Mendez, 2016). We observed that inhibiting PV+ interneurons during fear conditioning increased the number of neurons that were active during memory retrieval 24 h later. This increase was specific to the dorsal subnucleus of the LA. Although our study is unable to confirm that this effect was due to increased recruitment of tone-responsive neurons during the formation of a tone-shock association, the present results do suggest that PV+ interneurons play an important role in shaping plasticity related to memory formation in the LA. Interestingly, we did not observe any effect of increased engram size on the strength of memory expression, suggesting that the changes that take place within the neuronal ensembles of an engram (such as increased synaptic efficacy) are more relevant to the strength of stored memories than the size of the ensembles themselves.

Computational studies have suggested that the sparsity of the LA engram contributes to the stimulus specificity of fear memories (Kim et al., 2016). Indeed, memories encoding highly salient threat-related information must exist in a delicate balance between generalizability and specificity in order to allow organisms to both rapidly respond to potential threats and avoid pathological fear and anxiety caused by the overactive retrieval of such memories. It would be interesting for future studies to investigate whether engram size influences memory generalization and whether memories encoded in an artificially increased engram might be retrieved by a broader range of contextual and sensory triggers. Additionally, it may be fruitful to examine whether an artificially expanded engram population in the LA influences the sparsity of engram components in downstream areas such as the BNST.

Although more information is required to fully characterize the role of inhibitory interneurons in memory formation, the present findings corroborate predictions made by modeling studies regarding the role of inhibitory connectivity in the LA in regulating plasticity auditory fear conditioning (Feng et al., 2016) and provide novel evidence for the involvement of LA interneurons in memory allocation.

4. Methods

4.1. Mice

Except where noted, adult (at least 8 weeks of age) male and female F1 hybrid (C57BL/6NTac X 129S6/SvEvTac) wild-type (WT) mice were used in all experiments. Mice were bred at the Hospital for Sick Children, provided with food and water ad libitum, and group housed (4 per cage) on a 12 h light/dark cycle. All mice were briefly singly housed, transported and handled once daily for 6 days prior to experiments. All experiments were performed...
during light-phase. All procedures were conducted in accordance with policies of the Hospital for Sick Children Animal Care and Use Committee and conformed to both Canadian Council on Animal Care (CCAC) and National Institutes of Health (NIH) Guidelines on Care and Use of Laboratory Animals.

For experiments involving PV+ interneuron inhibition, heterozygous PV-Cre knockin driver mice (B6;129P2-Pvalbtm1(cre)Arbr/J) maintained on a C57BL/6 genetic background were used. These mice were obtained from Jackson Labs and were originally generated by Silvia Arber, FMI (Hippenmeyer et al., 2005).

4.2. Auditory fear conditioning

Auditory fear training involved placing mice into a conditioning chamber and, 2 min later, presenting a tone (2800 Hz, 85 dB, 30 s) that terminated in a 2 s footshock. Testing was performed 24 hr later by placing mice in a novel context and, after a 2 min baseline period, presenting an identical tone for 1 min. During testing an overhead camera captured the animal’s activity and was used to assess percentage time spent freezing during the tone [defined as an immobilized, crouched position, with an absence of any movement except respiration (Fanselow & Bolles, 1979)].

4.3. Immunohistochemistry

We used the expression of Arc protein in neurons following training or a memory test as a proxy for identifying active neurons that compose part of an engram supporting that memory. Arc protein is reliably elevated in excitatory neurons 90 min after activity (Guzowski et al., 2005) and has been used as a proxy for visualizing neuronal components of an engram (Gouty-Colomer et al., 2015). 90 min after tone delivery (during training or testing), mice were transcardially perfused with 4% paraformaldehyde (PFA) and brains removed. Brains remained in PFA for 1 d and were then transferred into cryoprotectant solution (20% sucrose) at 4°C. Three d later, brains were sectioned into 50 μm sections using a cryostat with an inter-slice interval of 100 μm. Slices were then washed and incubated overnight with Arc polyclonal rabbit antibody (1:500, Synaptic Systems, Cat. No. 156 003) at room temperature. When investigating shock intensity, slices were also concurrently incubated with NeuN polyclonal mouse antibody (1:1000, Millipore, MAB377). Staining was visualized either with 3,3′-Diaminobenzidine (DAB) or fluorescent secondary goat anti-rabbit Alexa 488 (1:1000, Life Technologies, Cat. No. A11034) and goat anti-mouse Alexa 568 (1:1000, Life Technologies, Cat. No. A11004) antibodies.

Measures of Arc+ cell density by area (in the first experiment using DAB) were obtained by imaging sections under a 10x Nikon light microscope and manually counting from these images with ImageJ software (NIH) (Schneider, Rasband, & Eliceiri, 2012) by experimenters unaware of the treatment condition. ANOVAs with post-hoc Fisher’s LSD tests were conducted to compare Arc+ cell density between different treatment groups within each amygdala nucleus.

4.4. AAV vector

The AAV8-hSyn-DIO-hM4Di-mCherry vector was obtained from the UNC Vector Core (Chapel Hill, NC). This adeno-associated virus (AAV) contains a double floxed inverse open reading frame of hM4Di fused to mCherry that is expressed from the human synapsin (hSyn) promoter after recombination by Cre recombinase.
The average titer of the virus stocks was $4.0 \times 10^7$ infectious units/ml. Mice were allowed to recover for 4 weeks for maximal viral expression before performing behavioral experiments.

### 4.5. Surgery

Before surgery, mice were pre-treated with atropine sulfate (0.1 mg/kg, ip), anesthetized with chloral hydrate (400 mg/kg, ip) and placed in a stereotaxic frame. Skin was retracted and holes were drilled in the skull above the amygdala ($AP = -1.4$ mm, $ML = \pm 3.4$ mm, $V = -5.0$ mm from bregma). AAV-DIO-hM4Di-mCherry (1.0 $\mu$l) was infused bilaterally into the amygdala. After surgery, mice recovered for 4 hr before being returned to their standard housing.

#### 4.6. catFISH or fluorescent in situ hybridization for the activity-dependent gene arc

Mice were trained with various shock conditions ($1 \times 0.4$ mA, $1 \times 0.7$ mA, and $3 \times 0.7$ mA) in order to create memories of different strengths. A group that received a shock immediately upon entering the chamber was included as a control. 5 min after either training or testing, animals were sacrificed and brains were subsequently processed with catFISH. Brains were then imaged and analyzed with systematic stereological procedures to identify the proportion of Arc mRNA expressing neurons to the number of total neurons. In order to minimize differences in staining, sections from 4 mice were mounted together on one slide. Following hybridization and amplification of Arc signals, counterstaining with Hoechst 33258 to visualize nuclei was performed.

Following catFISH processing, sections were imaged on a laser confocal microscope (Zeiss LSM 710) to obtain optical z-stack series with a step size $\leq 1$ $\mu$m apart. Stacks were analyzed for nuclear Arc by two individuals unaware of treatment condition. Stereological counting was then performed by two individuals unaware of treatment condition, with at least four sections counted for each mouse. ANOVAs with post-hoc Tukey’s tests were conducted to compare Arc+ cell density between different treatment groups.

#### 4.7. Stereological counting

To accurately determine the proportion of neurons active in the LA during auditory fear training and testing, we used unbiased stereological principles and systematic sampling techniques to obtain the ratio of Arc-expressing neurons to the number of total neurons. In the experiment involving manipulation of shock intensity and measurement of Arc protein, we used expression of the neuron-specific protein NeuN to obtain a measure of the total neuronal population in the LA. However, for experiments involving catFISH, in which protein-based immunohistochemistry was
unfeasible, and AAV-DIO-hM4Di-mCherry expression, which occupied the marker color used previously to indicate NeuN, the nuclear stain 4,6-diamidino-2-phenylindole (DAPI) was used to obtain an estimate of the total cellular population that was later adjusted based on empirically determined estimates of the ratio of NeuN to DAPI labelling. Several sections containing both DAPI and NeuN were analyzed to obtain this estimate (78.9% ± 2.3, n = 10) and adjusting stereological counts to this ratio did not affect the relationships between groups.

Counting was performed by an experimenter unaware of the treatment condition using the Optical Fractionator probe within Stereo Investigator (version 10, MBF Bioscience, Williston, VT USA). Counting was performed on every third section (50 µm thickness, at least 5 sections per mouse) from the left hemisphere. A counting frame of 120 µm × 120 µm was randomly distributed according to a 250 µm × 250 µm grid throughout the LA. Section thickness was recorded at every sampling site to compensate for any potential thickness variation across the sections due to tissue processing. A 12 µm dissector height was used with 2 µm guard zones placed at the top and bottom of each section. Coefficient of Error values, determined by Gundersen’s method (Gundersen & Jensen, 1987) (m = 1) were <0.11 in all samples. For analysis of LA subnuclei, contours were drawn between regions as described by the stereotaxic mouse brain atlas by Paxinos and Franklin (2012). ANOVAs with post-hoc Tukey’s tests were performed to compare Arc+ cell density between treatment. All stereotaxic stain 4

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