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Parvalbumin interneurons constrain the size of the lateral amygdala engram

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ABSTRACT

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

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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 and assessed the number of neurons positive for Arc protein (Arc^+) 90 min following behavioral manipulation (e.g., initial training or memory recall) (Fig. 1a). Arc^+ neuron density (per 100 μm^2) was assessed in the LA, BA, central amygdala (CeA) and intercalated cell masses (ITC) (Fig. 1b). For comparison, we included non-learning control groups that were exposed to different aspects of the training experience (homecage mice, mice exposed to tone alone, chamber alone or an immediate shock) (Frankland et al., 2004; Zelikowsky, Hersman, Chawla, Barnes, & Fanselow, 2014).

The number of Arc^+ neurons throughout the amygdala increased after both fear memory training and testing (Fig. 1c). In comparison to homecage controls, groups that were perfused following training or testing showed increased Arc^+ cell density in the LA [$F(5, 36) = 10.63$, $P < 0.001$], BA [$F(5, 36) = 5.20$, $P < 0.001$] and CeA [$F(5, 36) = 4.99$, $P < 0.001$]. There was no difference in Arc^+ density between groups in the ITC [$F(5, 36) = 1.86$, $P > 0.05$]. The density of Arc^+ cells in the control tone alone, chamber alone or immediate shock groups did not differ from the homecage group in any region ($P > 0.1$). Interestingly, fear training and testing induced a similar number of Arc^+ neurons in all regions examined (LA, $P > 0.5$; BA, $P > 0.1$; CeA, $P > 0.5$; ITC, $P > 0.5$), suggesting that a similar number of neurons were active during encoding and retrieval of an auditory fear memory.

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