Identification of a functional network for long-term fear memory in mice

Supplementary Text 1

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Supplementary Methods

Relationship between magnitude and variance of regional Fos signal and correlation strength

We additionally evaluated the relationship between the magnitude and variance of our regional Fos signal and the tendency to see correlations with Fos expression in other brain regions. The mean and coefficient of variation (standard deviation/mean) were computed for the Fos counts in each of the 84 brain regions in the four groups of animals (WT/1 day, WT/36 days, α-CaMKII$^{+/-}$/1 day and α-CaMKII$^{+/-}$/36 days). The Pearson correlation coefficient was calculated to assess the association between (i) the mean Fos level and the mean squared Pearson correlation coefficient (mean r^2) and (ii) the coefficient of variation of the Fos signal and the mean r^2 by region across all 4 conditions.

Evaluation of reuniens thalamic nucleus anatomical connectivity

The direct anatomical connectivity of the reuniens thalamic nucleus was assessed by mining published data that investigated reuniens connectivity with infusion of tracers into the rodent brain. These studies were identified with the aid of an online connectivity database (The Brain Architecture Management System[1] [BAMS; [http://brancusi.usc.edu/bkms/]). Since the BAMS database remains sparsely populated, in addition this information was supplemented with studies identified through a PubMed search of the literature (http://www.ncbi.nlm.nih.gov/pubmed).

Context specificity of memory at short and long retention delays

In the main experiment, mice were trained and tested in context A. In order to assess whether the precision of the fear memory changes over time, we trained additional groups of mice using an identical protocol and then assessed their freezing in the training context (context A) and an alternate context (context B) either 1 or 36 days later. Context A was identical to the main experiment. For context B, a white plastic floor covered the shock grid bars, and a white, plastic, triangular insert was placed inside the conditioning chamber. As in the main experiment, mice were handled prior to training (3 days, 2 min/day). One day following the completion of handling, mice were placed in context A for seven minutes. After two minutes they were presented with five unsignalled footshocks (2 s duration, 0.75 mA, 1 minute apart). Following the last footshock mice remained in the context for an additional minute, and were returned to their home cage. Either 1 day (n = 12) or 36 days (n = 14) later, freezing was assessed in contexts A and B. Tests were 3 min in duration, spaced ~5 h apart, and presented in a counter-balanced order. To compare discrimination across groups, we used the freezing scores to compute the following index: $[\text{freezing}_A - \text{freezing}_B]/[\text{freezing}_A + \text{freezing}_B]$.

Supplementary Results

Relationship between magnitude and variance of regional Fos signal and correlation strength

We found that functional connectivity was influenced both by retention delay and by genotype (Figure 2 and Figure 8). Importantly, these differences were independent of any group differences in Fos activation. For example, overall Fos levels were elevated in WT mice at the remote time-point (planned t-tests, WT/36 day > WT/1 days, α-CaMKII$^{+/-}$/1 day, α-CaMKII$^{+/-}$/36 days; Ps < 0.05) (**Figure S4A**). However, regional correlation strength was not dependent upon Fos levels (or signal strength) (r = 0.021; P = 0.71) (**Figure S4B**), and therefore increased network connectivity in WT mice at the remote time-point is not simply a consequence of generally increased levels of activation. Moreover, while correlation strength typically increased as a function of variance (or coefficient of variation) (r = 0.198; P < 0.001), variance was equivalent across groups and therefore cannot account for increased network connectivity in WT mice at the remote time-point (planned t-tests, all comparisons Ps > 0.05) (**Figure S4C-D**).

Patterns of inter-regional correlations derived from Fos and Egr-1 expression are similar

A number of other immediate early genes are regulated by neural activity, including *egr-1* [2]. In order to explore the generality of our effects we additionally quantified Egr-1 expression in a subset of brain regions in the WT/36 d group. We found that Fos- and Egr-1-derived patterns of inter-regional correlations were similar (**Figure S5**): Overall correlation strength did not differ in the Fos vs. Egr-1 matrices (by permutation testing; P = 0.76), nor were any individual inter-regional correlations different (P > 0.05 for all comparisons, corrected for multiple comparisons using the False Discovery rate set at 5%). These results are consistent with previous studies showing that immediate early genes are typically expressed in the same or largely overlapping neuronal populations[3].

Time-dependent changes in context generalization

Mice were trained in context A and then tested in contexts A and B either 1 or 36 days later. At the short delay, mice froze more in context A compared to context B. In contrast, at the long delay, mice exhibited robust, but equivalent levels of freezing in either context (context × delay ANOVA; significant context × delay interaction; $F(1,54) = 7.89$, $P < 0.005$; planned paired t-tests indicated that freezing was greater in context A vs. B at the short [$t(11) = 4.30$, $P < 0.005$] but not long [$t(13) = 0.31$, $P > 0.05$] delay) (**Figure S14B**). Reflecting this time dependent increase in context generalization, discrimination declined as a function of retention delay (unpaired t-test, $t(24) = 3.37$, $P < 0.005$]) (**Figure S14C**). These time-dependent changes in context generalization are consistent with the idea that the contextual fear memory is transformed from a precise, detailed form into a less precise, generalized form[4].

Supplementary Notes

Defining functional connections on the basis of inter-regional analysis of Fos expression. In brain imaging studies, two regions are said to be functionally connected if their activity co-varies. Co-variance may be computed either within subjects (which is typically the case in human imaging studies) or between subjects (more typical in experimental animal studies where 'activity' is inferred post-mortem by changes in expression of activity-regulated genes, for example). Functional connections therefore reflect a statistical (rather than physical) relationship between two regions. As a purely statistical construct, functional connections may therefore be defined on multiple timescales, and the timescale depends on how 'activity' is being measured. For example, in electrophysiological studies, correlated patterns of spiking between two regions would define functional connections on the millisecond or second timescale. In contrast, correlated increases in the expression of an activity-regulated gene such as *c-fos* across two regions would define functional connections on the minutes to hours timescale, as Fos is induced by sustained neural activation and Fos expression peaks after 60-90 minutes.

Fos expression reflects sustained neural activation. To study the relationship between stimulation and Fos expression *in vivo*, we previously electrically stimulated of the entorhinal cortex, and examined Fos expression in granule cells in the dentate gyrus [3](see Figure 1). Importantly, we found that increases in Fos expression in dentate granule cells were 1) similar in magnitude to those following behavioral testing (e.g., placement of mouse in context previously paired with shock), 2) anatomically-specific (limited to dentate granule cells ipsilateral to stimulation site, consistent with predominantly unilateral efferent connections from the entorhinal cortex to the dentate gyrus) and 3) localized to the same subpopulations of granule cells expressing other activity-regulated genes (e.g., Arc). These *in vivo* data suggest that Fos induction reflects sustained neuronal activation.

Control networks. Since the emergence of applying graph theoretical approaches to study real life networks there has been much discussion and research into the choice of approaches for generating appropriate control networks (e.g.[5,6]). There are many ways in which random graphs may be constructed, and the choice of method will result in markedly different connection properties. For example, one could simply generate a network in which a set number of connections between nodes are randomly assigned. For the purposes of providing appropriate controls that reflect the connection distributions of the network being studied, a standard method (and the one we adopt here) is to shuffle the connections between nodes, while maintaining the same number of connections for each node [7]. This not only preserves the overall degree distribution of the network, but also ensures that each node has the same number of connections (albeit to different partners) as the original network. In this way we can examine how the global properties of the network vary from our so-called 'random networks' in which local properties of organization are preserved.

Supplementary References

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