Role and plasticity of prefrontal engrams of long-term fear memory in aggravating on-going pain and pain chronicity

Authors: Alina Stegemann[1,‡], Sheng Liu[1,‡], Oscar Andrés Retana Romero[1,‡], Manfred Josef Oswald[1], Yechao Han[1], Carlo Beretta[1], Linette Liqi Tan[1], William Wisden[2], Johannes Gräff[3], Rohini Kuner[1,*]

List of supplementary information items:

Supplementary notes:

Supplementary note 1: Use of viral approach for activity-dependent tagging neurons for fluorescent identification or optogenetic manipulation

To identify prelimbic neuronal assemblies that are selectively recruited during fear recall or tonic pain with tight spatio-temporal control, we performed bilateral prelimbic injections of adeno-associated virus (AAV) particles expressing a binary, tetracycline-inducible activity mapping system (Extended Data Fig. 1A). Herein, expression of tetracycline-controlled transactivator (tTA) in the mouse prelimbic cortex under control of the activity-dependent promoter of the immediate early gene *c-fos* enables expression of a fluorescent reporter, mCherry, via binding of tTA to the tTA-responsive promoter element (TRE) (Extended Data Fig. 1A, B). This is inhibited by Doxycycline, a tetracycline. Thus, keeping the mice on Doxycycline (Dox ON) represses background expression (Extended Data Fig. 1C), and taking the mice off Doxycycline (Dox OFF) opens a finite time window to label neuronal populations with mCherry expression in response to a given external stimulus or behavioral state (Extended Data Fig. 1D,E). Mice are placed on Doxycycline again to close the window and, at a later time point, the cells that are activated by exposing the mice to the second test stimulus of choice can be identified 90 minutes thereafter via anti-Fos immunohistochemistry (examples shown in Extended Data Fig. 1F), Fos protein being the product of the activity-induced expression of the immediate early gene *c-fos*.

Using a single electroshock given to the mouse hindpaw as a stimulus, the system was first validated for signal-to-background optimization and specific labeling of recruited prefrontal neurons with the mCherry reporter in a Dox OFF time window as compared to homecage control (Fig. 1A). These results validate the labeling strategy and show that the prefrontal cortex is an appropriate target for *c-fos*-based engram-labelling approaches, as shown by the low levels of background expression in the naïve state and the adequate increase in labeled cells following a brief but salient stimulus. Another group of mice was put through a classical fear conditioning paradigm under Dox supplementation, in which they learned to associate a 30 s auditory tone with succeeding acutely painful foot shocks. Re-exposing the mice later to the same auditory cue or spatial context in the absence of foot shock elicits freezing behavior indicative of fear. Since the prelimbic cortex participates in encoding remote fear memory (Kitamura et al., 2017), we presented the auditory cue and the context without the foot shock at 28 days post-fear conditioning during a Dox OFF window, thus tagging the prelimbic population that is active during pain anticipation by the remote recall of a fear memory. In a distinct group of mice, the activity-based labeling paradigm was employed in conjunction with capsaicin injection into the hind paw to elicit tonic pain ongoing for several minutes during a Dox OFF window. As explained above, the labeling system enables taking 'activity snapshots' of neural populations engaged at two different time points in the same animal via expression of the marker mCherry over a transient Dox OFF time window in response to the first stimulus, followed by immunohistochemical analysis of activity-induced Fos protein in response to a second stimulus. We validated this strategy by giving two distinct electroshock stimuli 24 h apart to the hindpaw, which led to reactivation of 40% of prefrontal neurons (Fig. 1A, Extended Fig. 1F), thus reflecting the relative stability of engrams expected from previous studies in other fields (e.g., see Kitamura et al., 2017, DeNardo et al. 2019). We then sought to discern and delineate cells specifically activated in fearful anticipation from those selectively recruited during tonic pain. In mice subjected to labeling of the remote fear memory engram with mCherry, we observed an overlap of about 15 % with the neuronal assemblies activated by capsaicin-induced tonic pain detected by anti-Fos-immunofluorescence (Fig. 1A). Conversely, by presenting the capsaicin stimulus first to label cells recruited during tonic pain with mCherry, followed by cued fear recall at 28 days post-fear conditioning to label cells with Fos, we observed that about 10 % of cells were commonly recruited (Fig. 1A). In a parallel group of mice, presenting the same fear recall stimulus twice revealed an overlap of about 30 % (Fig.

1A), which is consistent with previous reports (DeNardo et al. 2019). Thus, about a third of fear engram cells that were reactivated with fear recall were also activated by capsaicin.

To address functional implications, we employed next generation versions of the binary labeling system (Tre Tight; Zhang et al. 2015). We generated a virus expressing Archeorhodopsin (ArchT), which silences neuronal activity upon illumination with yellow light (Mattis et al. 2012), in the TRE-Tight system. The Dox ON/Dox OFF dynamics and dosage were technically optimized to yield specific ArchT expression (Extended Data Fig. 2A) and ArchT-mediated silencing *in vivo*, as verified via Fos expression (Extended Data Fig. 2B; Fig. 1C), paving the way for functional behavioral analyses. Mice in each group were divided into two cohorts, which were subjected to optogenetic silencing (laser ON) or mock treatment (laser OFF) on alternate days in behavioral assays in a cross-over design. Similarly, we generated a TRE-tight variant expressing Channelrhodopsin-YFP and again established the optimum conditions for dose and period of Dox treatment in vivo to yield optimal levels of Dox-controlled expression in the prefrontal cortex (Extended Data Fig. 2A) and ArchT-mediated silencing *in vivo*, as verified via Fos expression (Extended Data Fig. 2B; Fig. 1D).

To manipulate a random, population of prelimbic neurons (Extended Data Fig. 4A), we established conditions such that the number of neurons expressing ArchT randomly using the murine synapsin promoter was comparable to number of neurons expressing ArchT in an activity-dependent manner (Extended Data Fig. 8B). Similar conditions were established to express ChR2 in a random manner.

References:

Kitamura, T.*, et al.* Engrams and circuits crucial for systems consolidation of a memory. *Science* **356**, 73-78 (2017).

DeNardo, L.A.*, et al.* Temporal evolution of cortical ensembles promoting remote memory retrieval. *Nature Neuroscience* **22**, 460-469 (2019).

Zhang, Z.*, et al.* Neuronal ensembles sufficient for recovery sleep and the sedative actions of alpha2 adrenergic agonists. *Nat Neurosci* **18**, 553-561 (2015).

Mattis, J.*, et al.* Principles for applying optogenetic tools derived from direct comparative analysis of microbial opsins. *Nat Methods* **9**, 159-172 (2011).

Supplementary note 2:

We directly studied changes in neuronal activity with high temporal precision via *in vivo* 64-channel recordings in the prelimbic cortex of behaving mice. Activity of 261 prelimbic units was tested in multiple paired recording sessions over phases of remote fear recall (using the same auditory-cued fear conditioning paradigm employed in the cell engramming approach described above) and capsaicin-induced tonic pain in a total of 7 mice. We observed units with globally altered rates of firing in either fear or pain conditions compared to neutral context activity (Extended Data Fig. 3A), To test for significance of global firing rate changes between these states, we also recorded global firing rates in the neutral context and compared this to activity recorded when mice were put back into the neutral context a day later. Likewise, the mice undergoing fear recall in the cued context were also recorded in two repeated sessions 1 day apart. In subsequent analyses, rate changes exceeding two standard deviations were considered to be significant. Analysis of significant mean firing rate changes in either fear or pain conditions revealed that a considerable proportion of prefrontal neurons demonstrated an increase in the global firing rate during the periods of assessment of fear recall or tonic pain (15% each), comprising state-specific (about two-thirds) and commonly recruited (about one third) populations (examples of representative units shown in binned heat maps in Extended Data Fig. 3B and quantitative summary in Extended Data Fig. 3C). Analogous to recent reports on 'fear-up' and 'fear-down' as well as state-specific neurons in the basolateral amygdala (Grewe et al. 2017, Gründemann et al. 2019), about 15% prefrontal neurons were found to be suppressed during fear recall, while a higher proportion (nearly 30%) were suppressed during tonic pain (Extended Data Fig. 3C).

These were further delineated into putative principal neurons (n = 231) and inhibitory interneurons (n = 30) based upon waveform and firing rate analyses. Both putative principal neurons and inhibitory interneurons showed increase or decrease in activity during fear recall or tonic pain (Extended Data Fig. 3D). Amongst neurons commonly activated during fear recall and tonic pain, both interneurons and principal neurons showed decreased activity over the neutral context; however, remarkably, units showing increased activity commonly across fear recall and tonic pain largely comprise putative principal (excitatory) neurons (Extended Data Fig. 3D).

To better decipher behavioral encoding, we next employed simultaneous video monitoring to delineate firing rate alterations that are phase-locked to behavioral episodes of fear recall

(freezing) or tonic pain (licking, flicking or lifting of the capsaicin-injected paw), which last a few seconds each and recur intermittently (shown as white or grey bars superimposed on heat maps in Fig. 1E). For fear recall as well as tonic pain, about 23 % units each demonstrated increased firing specifically in phase with behavioral episodes as compared to the neutral context, of which a third were commonly activated across fear recall and tonic pain (Fig. 1E). These results collectively suggest that distinct prefrontal neuronal assemblies, comprising state-specific units and commonly-recruited units, come into play in encoding behavioral manifestations of fear recall and tonic pain. They largely support the findings from activity-based labeling, but also go further to reveal an even higher level of overlap across fear recall and tonic pain in terms of absolute neuronal activity.

References:

Grewe, B.F.*, et al.* Neural ensemble dynamics underlying a long-term associative memory. *Nature* **543**, 670-675 (2017).

Grundemann, J.*, et al.* Amygdala ensembles encode behavioral states. *Science* **364** (2019).

Supplementary note 3: Use of operant conditioning paradigm for testing valence encoding of appetitive stimuli

We sought to determine whether the suppression of pain perception by silencing of the fear recall engram is specific or reflective of a more general modulation of conditioned learning. Prelimbic engrams encoding other prelimbic functions, such as valence encoding of appetitive stimuli, have been shown to be distinct from those involved in aversive processing (Ye et al. 2016, Otis et al. 2017) while commonality of dentate gyrus engrams has been reported between negative (fear) and positive (reward) valences [38]. Because prelimbic activity has been suggested to modulate goal-directed behavior in operant conditioning paradigms based on reward learning (Trask et al., 2017), we employed a task seeking water as an operant reward in a touch screen chamber on mice with water restriction (learning curve is shown in Fig. 2C top panel). Once an accuracy of more than 90% was consistently reached (day 5 and 6 of learning), we proceeded with labeling of the fear recall engram. Following testing of fear recall under optogenetic modulation until day 4 after labeling, mice were placed again on water restriction and operant behavior to obtain the water reward was tested. We ascertained that despite a period of 8 days between the last day of the learning curve and operant testing, control mice maintained a high level of accuracy. In this assay, mice with ArchT expression in the fear recall

population showed equivalent operant responsivity and task accuracy in the presence or absence of yellow illumination (Fig. 2C bottom panel).

References:

Ye, L.*, et al.* Wiring and Molecular Features of Prefrontal Ensembles Representing Distinct Experiences. *Cell* **165**, 1776-1788 (2016).

Otis, J.M.*, et al.* Prefrontal cortex output circuits guide reward seeking through divergent cue encoding. *Nature* **543**, 103-107 (2017).

Trask, S., Shipman, M.L., Green, J.T. & Bouton, M.E. Inactivation of the Prelimbic Cortex Attenuates Context-Dependent Operant Responding. *J Neurosci* **37**, 2317-2324 (2017).

Supplementary movie legends:

Movie S1. Nocifensive behavior indicative of tonic, ongoing pain upon capsaicin injection unilaterally in the left hindpaw is pronounced when the Laser is OFF, i.e.**,** when there is no silencing of the long-term fear memory engram.

Movie S2. Nocifensive behavior indicative of tonic, ongoing pain upon capsaicin injection unilaterally in the left hindpaw is less pronounced when the Laser is ON, i.e. upon bilateral yellow laser light-induced and ArchT-mediated neuronal silencing of the long-term fear memory engram in the mouse prefrontal cortex**.**

Movie S3. Hypersensitivity to heat in mice with paw inflammation, seen as **short** latency to respond to heat in Laser OFF conditions**,** i.e.**,** when there is no silencing of the long-term fear memory engram.

Movie S4. Hypersensitivity to heat in mice with paw inflammation is reduced, as seen by delay in paw withdrawal to heat when the Laser is ON, i.e., upon ArchT-mediated silencing of the prefrontal long-term fear memory engram, as compared to Laser OFF conditions.