Supplementary Video 1. Example simultaneous in-vivo two-photon imaging of GCAMP6f in anterior cingulate (top) and CA1 (bottom) (one optical slice each shown) during behavior (right). Mesoscope objective: 5mm FOV; 2.7mm WD, 0.6NA. Resonant scan rate: 5.1 Hz, averaged over 8 frames. Playback speed: 8x. This particular example is to demonstrate the preparation but was not used in analysis.

Supplementary Note 1. The authors of a recent bioarxiv manuscript[1] (Andrianova et al.) claimed they were not able to find AC to CA1/CA3 projections. To help the authors of that study and others who may be interested in following up on the multiple studies characterizing these pathways (e.g. refs. 3,4 and the present study), we provide here a detailed guide:

1. Retrograde tracing is substantially more sensitive than currently available anterograde tracing approaches; thus we suggest beginning with retrograde tracing to characterize any new projection. However, one must be careful to perform the retrograde tracing correctly, targeting the proper location, and labeling sufficient number of starter cells. In Andrianova et al., the authors mistakenly target retrograde labeling to dentate gyrus (a region not targeted by anterior cingulate), rather than CA3/CA1 (the site of direct projections from anterior cingulate; ~5 yellow starter cells in CA1 & 0 in CA3)[1]. Furthermore, the authors used an approach (using multiple viruses to express pseudo-typed rabies), which if not properly optimized, will result in inefficient labeling of starter cells at the location of interest. We recommend using a single-virus approach when possible. Thus, a simple injection of one virus, retroAAV-tdT[2] into the correct location targeting CA1 or CA3 will result in robust labeling of afferents in anterior cingulate (Fig. 2a, ED Fig. 5a, ED Fig. 7f). This holds across various retrograde viral approaches including CAV and Rabies[3]. Cell-type specific retrograde labeling (by injecting floxed-tdT in Emx1-cre or Vglut-cre mice) also robustly identifies afferents in anterior cingulate with direct projections onto excitatory neurons in CA1 (ED Fig. 7g-i). Finally, long range inhibitory projections from prefrontal to hippocampus have also been reported[4]. All of the above known projections were missed by the Andrianova et al. study, underscoring the importance of using appropriately-sensitive expression systems and accurate viral targeting.

2. Anterograde tracing approaches are less sensitive and require significant optimization. We reported in a previous study[3] that the initial robustness of the cingulate to CA3/1 projections visualized in the retrograde direction enabled us to systematically optimize the anterograde tracing (i.e., waiting for at least 5 weeks of viral expression; using appropriately titered viruses (for which we found that in some cases attaching ChR2 to eYFP also significantly increased trafficking to terminals); and imaging the terminals in hippocampus as large-volume z-projections), which ultimately enabled patch-clamp recordings to confirm direct mono-synaptic connectivity[3]. All steps in this kind of anterograde work should be rigorously optimized (Andrianova et al. waited only three weeks for anterograde viral expression[1] , predictably resulting in difficulty visualizing even the very strong projections to MD thalamus, as well as the hippocampal projections). To increase overall robustness, we recommend performing anterograde tracing using cre/flp-amplification, which robustly identifies the hippocampal projections (ED Fig. 7a-d); importantly, these cingulate to CA1 projections are comparable in strength to the widely-characterized entorhinal projections to CA1 (ED Fig. 7a-d). Furthermore, the long-range inhibitory projections from PFC to hippocampus (extensively characterized and reported by Malik et al.[4]) were also missed by Andrianova et al.[1] in their hSyn-based anterograde studies. All of the above underscore the importance of using cre/flp-amplification and allowing sufficient expression times to ensure robustness of anterograde tracing.

3. The Allen Brain Projection Atlas, while a powerful repository, is meant to serve as a guide to the presence of some projections rather than as a proof-of-absence of others. The repository was created more than a decade ago using techniques considered state-of-the art at that time. Thus, it is understandable that it would under-estimate or completely miss many of the projections routinely identified and characterized in more recent years[5-7], including the prefrontal to hippocampus projections[3,4].

The title of the Andrianova et al. study states *"No evidence from complementary data sources of a direct projection from the mouse anterior cingulate cortex to the hippocampal formation."* Subsequent to one round of peer-review, this original claim was retracted and shifted to suggest that a direct projection does exist, but perhaps it might be entirely inhibitory: *"if an inhibitory projection from ACC does indeed exist (it appears that it does, based on informal conversations with other researchers on social media) …".* Indeed, both inhibitory and excitatory projections are functionally important. In the future, we encourage authors to contact us for technical guidance as it may also avoid exhausting valuable resources, including mice, personnel, and time.

References:

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