

Supplementary Note 1. To our knowledge there are no prior peer-reviewed published reports investigating mPFC-amygdala connectivity in mice using viral tracers to distinguish well-resolved vmPFC and dmPFC projections that have been validated by connectivity patterns, although there are reports using viral tracers and optogenetics that used injections that encompass both vmPFC and dmPFC^{16, 17}. The majority of prior anatomical tracing studies in rodents that studied vmPFC and dmPFC projections to the amygdala separately were conducted in rats. These studies show that in rats the vmPFC projects strongly to the BMA, but only weakly to the BLA; see Fig. 4 of Vertes et al.,⁹ Fig. 2 of Takagishi et al.⁴⁷, and Fig. 3 of Hurley et al.³³. Similarly, in agreement with these studies Cassel et al.,³² also studied mPFC-amygdala connectivity, and stated that “The infralimbic area does not project to the basolateral nucleus”, noting “the almost total absence of labeling in the central, basolateral, medial nuclei and the intercalated cell masses”. Cassell also noted that their “findings support previous studies in failing to find evidence of projections from the infralimbic area to the basolateral nucleus”. Furthermore, Vertes et al.⁹ reported “an essential absence of labeling in the lateral and basolateral nuclei”. Strong connections to the BLA from the vmPFC have been reported by McDonald et al 1996⁵¹. However, it should be noted that a later study from McDonald (Pinard et al.⁸) acknowledged that the observed fibers in the BLA in their 1996 paper may be due to leakage of the tracer from the infralimbic cortex (IL) to the prelimbic cortex (PL), writing that “As expected from previous studies of prefrontal-amygdalar projections (McDonald et al., 1996), injections of the IL that also involved significant portions of the dorsally adjacent prelimbic cortex (e.g. case R40, see Fig. 2) produced projections to the dorsal portion of the BLA”. Bukalo et al.,⁵² also observed dense mPFC innervation in the BLA following injections in the infralimbic cortex. However, similarly to the report by McDonald et al.,⁵¹ Bukalo et al.,⁵² also noted that “it remains likely that at least some portion of PL neurons were infected in the current study”. This has been our own experience as well, as we also observe prominent mPFC fibers in the BLA following even minor leakage of virus into the prelimbic cortex. An important methodological distinction between our study and other reports in mice is that our vmPFC injections were made below the infralimbic cortex, in the ventral portion of the dorsal peduncular cortex (depth 3.3 mm from bregma, see Methods). Spread of virus along the cannula tract is sufficient to induce infection in the infralimbic, but not prelimbic cortex. To our knowledge, in

other reports in mice, injections targeting the infralimbic region are centered in the middle of the infralimbic cortex (depths of 2.1¹⁶, 2.6¹⁷, 2.8⁵² and 2.0⁵³ mm) producing spread into the prelimbic cortex and a consequent dense innervation in the BLA (likely originating from the dmPFC), which was observed in all these four studies^{16, 17, 52, 53}. Similarly to Bukalo et al.,⁵² these other reports also used injections spanning both pre and infralimbic cortices. Cho et al.,¹⁷ stated that “the spread of virus to PL has inevitably occurred”, Hubner et al.¹⁶ mentioned that “Viral injections into the mPFC either infected neurons mainly located in PL or IL, or a larger area of the mPFC, encompassing PL and IL”, and clear viral expression in the prelimbic cortex can be seen in Strobel et al.,⁵³ Supplementary Fig. 1A. In contrast, prior anatomical studies which specifically targeted vmPFC^{9,33,47} without affecting dmPFC demonstrated that in rodents vmPFC has only sparse connections to the BLA, as can be clearly seen in Supplementary Video 1, while the vmPFC-BMA connection has been reported to be strong and dense in every study on vmPFC-amygdala connectivity^{9,32,33,47,51}

Validating these findings, work in rats^{9,33,47} has found that the vmPFC projects strongly to the dorsomedial, but not ventromedial hypothalamus, while the dmPFC does not project substantially to these regions⁹. These studies are in agreement with our results, despite differences in method (*phaseolus vulgaris* leucoagglutinin vs. viral AAV5 tools) and species (rats vs. mice). The most comparable experiment is from the Allen Brain Institute; a search for “infralimbic area” in the Institute's Mouse Connectivity database (<http://connectivity.brain-map.org/>) locates an experiment in which AAV5 was injected in an area spanning the infralimbic and dorsal peduncular cortices, in wild-type C57BL mice, as in the current work (Extended Data Fig. 1b). The resulting pattern of fiber innervation seen in the amygdala in that experiment is virtually identical to ours, displaying numerous vmPFC fibers in the BMA and dorsomedial hypothalamus, but not in the BLA. We observe sparse vmPFC fibers in the BLA, and find a low (12%) but nonzero population of BLA cells to be responsive to vmPFC fiber stimulation (Fig. 4c). These data indicate that vmPFC-BLA functional connectivity in the mouse exists, although it is weak.

Supplementary Note 2. The control animals in Fig. 1k and 1o do not show an increase in heart rate because the entire experiment was done in the safe home cage environment, whereas in Fig. 1c and 1g animals were only in the home cage in the first epoch. In the 2nd and 3rd epochs of Fig. 1c and g animals were placed in the anxiety-provoking open field. For this reason, the respiratory

rates of control animals have different profiles in Fig. 1c,g and Figure 1k,o. In both Fig 1c,g and 1k,o data from a 15-minute session are plotted as three 5-minute consecutive epochs.

Supplementary Note 3. Currently there are no optimal methods to robustly, specifically, and safely target BMA-projecting vmPFC neurons. While rabies-ChR2 could be used for such purpose, this method would induce robust expression of opsin in all inputs to the BMA. It is likely that some of these inputs have fibers of passage through the vmPFC. Consequently, delivery of blue light to BMA-projecting neurons would also activate other inputs to the BMA. Other approaches to target BMA-projecting vmPFC neurons involve the injection of two viral vectors (one in the BMA, and another in the vmPFC). Compared to using rabies, these approaches are anatomically more selective, but produce weaker pre-synaptic labelling, as the inefficiencies of two viral vectors are involved. Thus, with a two-viral approach, negative results are more likely to occur due to the weaker targeting. Due to the higher anatomical specificity of the two viral approach we chose to inject the retrogradely propagating canine adenovirus (CAV) encoding cre in the BMA and an adeno-associated virus (AAV5) encoding cre recombinase dependent ChR2 in the vmPFC. As expected from the high incidence of negative results of the double-viral approach, indeed, optical stimulation did not alter anxiety in vmPFC-amy:CAV-ChR2 mice. Nevertheless, despite the sparse labelling in vmPFC cells, delivery of blue light to these cells decreased freezing in cued fear conditioning (Fig. 5c).

Supplementary Note 4. Activation of BMA-projecting vmPFC cell bodies will also activate other output targets of these cells that are outside the BMA. It is possible that one or more of these targets counteract the anxiolytic effect induced by BMA activation. Activation of vmPFC projection cells (without specifically targeting those that project to the vmPFC) decreased cued freezing, but not anxiety (Extended Data Fig. 2a-b), similarly to the results observed with vmPFC-amy:CAV-ChR2 mice. Taken together these results support the idea that direct activation of the vmPFC may recruit targets that mask the anxiolytic effect of the vmPFC-BMA projection. Other vmPFC targets that may increase or modulate anxiety in unexpected ways include the bed nucleus of the stria terminalis, the substantia innominata, the septum and the periaqueductal grey⁹. These targets are interconnected to the amygdala, potentially resulting in complex behavioral effects.

Supplementary Note 5. To assess if there are differences in transmitter type between open and closed-arm preferring cells, we tested if these two populations of cells differed in spike waveform features which are used to classify cells in glutamatergic projection cells and GABAergic interneurons^{54,55}. We find that open- and closed-arm cells do not differ significantly in spike half-width ($p < 0.31$), peak-to-trough ratios ($p < 0.92$), peak-to-valley ratios ($p < 0.92$) or overall firing rates ($p < 0.67$). Additionally, EPM scores are not correlated with spike half-width ($r = -0.28$, $p < 0.08$), time from spike peak-to-trough ($r = -0.21$, $p < 0.20$) and peak-to-valley ratio ($r = 0.08$, $p < 0.59$). These results suggest that firing patterns in the BMA during exploration of the elevated plus maze are not strongly associated with glutamatergic or GABAergic subtype.

Supplementary References

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