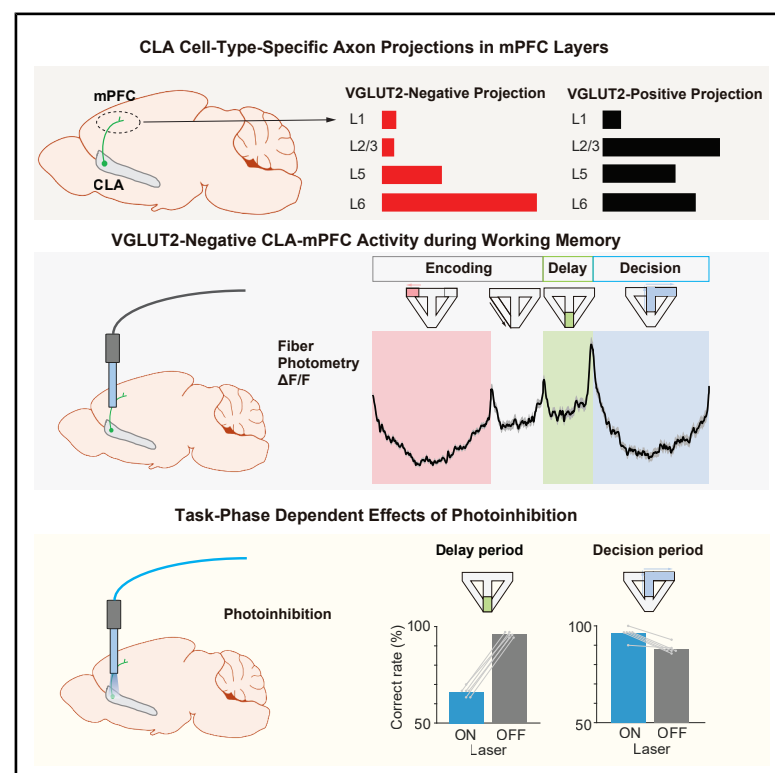


Current Biology

Essential role of VGLUT2-negative claustrum-prefrontal projections in working memory

Graphical abstract



Authors

Jia Yun Tan, Huee Ru Chong, Malcolm Zheng Hao Ho, ..., Martin Graf, George J. Augustine, Tsukasa Kamigaki

Correspondence

tsukasar@ntu.edu.sg

In brief

Tan et al. identify VGLUT2-negative claustrum neurons projecting to the medial prefrontal cortex that preferentially target layer 6 and support successful working memory. These neurons show task-phase-specific activity and causally modulate behavior, impairing performance when silenced during maintenance but enhancing performance when inhibited during decision-making.

Highlights

- VGLUT2-negative claustrum→medial prefrontal cortex neurons are identified
- These neurons preferentially innervate layer 6 of the medial prefrontal cortex
- They are active during successful working memory maintenance
- Silencing during delay impairs behavior, but during decision-making enhances behavior

Report

Essential role of VGLUT2-negative claustrum-prefrontal projections in working memory

Jia Yun Tan,¹ Huee Ru Chong,¹ Malcolm Zheng Hao Ho,^{1,2} Xuan Ouyang,¹ Priya Ramesh,^{1,2} Martin Graf,³ George J. Augustine,³ and Tsukasa Kamigaki^{1,4,*}

¹Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232, Singapore

²IGP-Neuroscience, Interdisciplinary Graduate Programme, Nanyang Technological University, Singapore 308232, Singapore

³Temasek Life Sciences Laboratory, Singapore 117604, Singapore

⁴Lead contact

*Correspondence: tsukasar@ntu.edu.sg

<https://doi.org/10.1016/j.cub.2026.04.067>

SUMMARY

The claustrum (CLA) is a key integrative hub with extensive bidirectional connectivity to higher-order regions, particularly the medial prefrontal cortex (mPFC).^{1–6} Such connectivity supports diverse cognitive functions,⁷ including attention,^{8–12} sensory integration,^{6,13} salience detection,^{14–16} and memory.^{17–23} A recent review highlights the growing evidence that the CLA contributes to cognitive control, including working memory (WM), by coordinating activity across distributed cortical networks.⁷ Consistent with this, recent work implicates the CLA in WM,^{20,22} although the specific CLA-to-mPFC pathways mediating this function remain unclear. The CLA comprises heterogeneous neuronal subpopulations with divergent molecular, anatomical, physiological, and projection profiles.^{24–27} VGLUT2-positive neurons constitute a major population providing excitatory input to the mPFC.²⁵ However, non-specific CLA activation paradoxically produces net mPFC inhibition via local interneuron recruitment.²⁴ This suggests that VGLUT2-negative neurons—the remaining projection type—form distinct pathways that contribute to inhibitory effects²⁸ and exert unique cognitive influences. We identified the previously uncharacterized population of VGLUT2-negative neurons within the CLA that project to the mPFC in mice. These neurons are enriched ventral to the parvalbumin-dense CLA core and preferentially innervate mPFC layer 6, unlike the broader laminar projections of VGLUT2-positive neurons. Fiber photometric calcium measurements revealed that these neurons were selectively active during maintenance of a spatial WM and less active during error trials. Optogenetic silencing of these neurons during WM maintenance, but not during encoding, severely impaired performance. Silencing during decision-making modestly improved performance, suggesting that disengagement of this pathway facilitates optimal processing. These findings reveal a claustrum-prefrontal circuit crucial for WM maintenance and phase-dependent behavioral modulation.

RESULTS

Anatomical validation of VGLUT2-negative projection neurons in the CLA

To examine the brain-wide connectivity of the VGLUT2-negative projection neurons in the claustrum (CLA), VGLUT2-Cre mice were injected with two adeno-associated virus (AAV) constructs: (1) Flp-encoding retrograde AAV (AAVrg) injected into the medial prefrontal cortex (mPFC), enabling Flp recombinase (Flippase) to be retrogradely transported to mPFC-projecting neurons (AAVrg-Flp; [Figure 1A](#)), and (2) CreOFF/FlpON-oScarlet-encoding AAV injected into the CLA. This drives expression of oScarlet only in the presence of Flp and the absence of Cre.²⁹ This intersectional virus strategy allowed selective labeling of mPFC-projecting, VGLUT2-negative neurons in the CLA.

To validate the viral strategy, we compared wild-type and VGLUT2-Cre mice. In both, AAV-CreOFF/FlpON-oScarlet was injected into the CLA with or without AAVrg-Flp into the mPFC. In wild-type mice, where Cre was absent, oScarlet expression

was observed throughout the CLA and adjacent areas only when AAVrg-Flp was injected into the mPFC ([Figure S1B](#), bottom, white arrows). No oScarlet expression was detected without AAVrg-Flp injection ([Figure S1B](#), top), confirming that oScarlet expression required Flp and that there was no leaked expression in its absence. Additionally, this demonstrated retrograde transport of Flp from the mPFC to the CLA. In VGLUT2-Cre mice, where Cre was expressed in VGLUT2-positive neurons, oScarlet expression was observed when AAVrg-Flp was injected into the mPFC ([Figure 1B](#), bottom, white arrow). However, oScarlet expression in the CLA was more restricted and focal compared with wild-type mice. This difference likely reflects Cre in VGLUT2-positive neurons within the CLA and surrounding regions, such as the dorsal endopiriform and insular cortices, which are rich in VGLUT2-positive neurons.³⁰ Consistent with findings in wild-type mice, no oScarlet expression was detected without AAVrg-Flp injection ([Figure 1B](#), top). To confirm the identity of the labeled neurons, we performed immunohistochemistry for Cre in VGLUT2-Cre mice injected with AAVrg-Flp in the mPFC

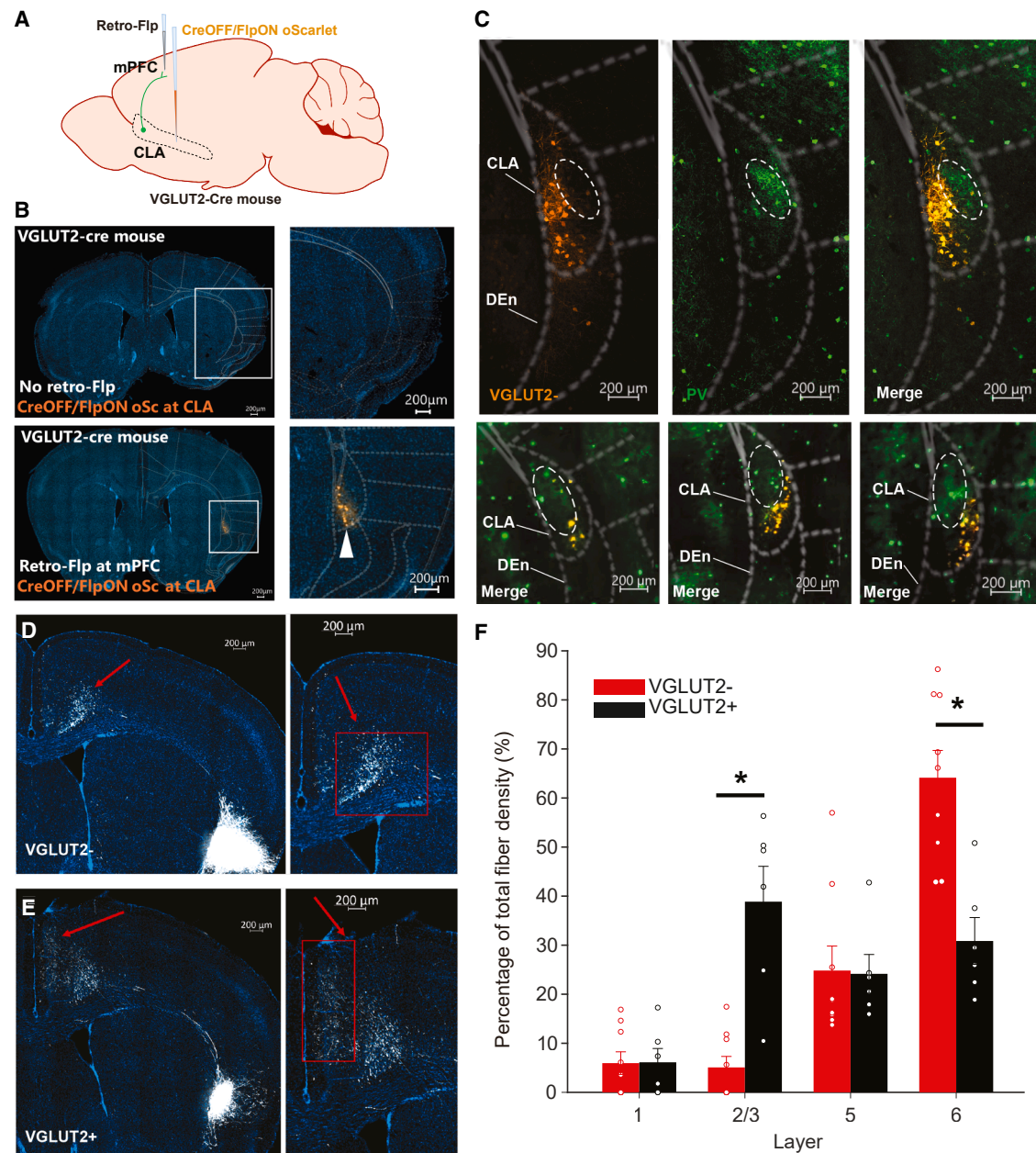


Figure 1. Distinct projection patterns of claustral VGLUT2-negative and VGLUT2-positive neurons to the mPFC

(A) Injection schematics to label CLA VGLUT2-negative neurons projecting to the mPFC. Retro-Flp virus (AAVrg-Flp) was injected into the mPFC region in VGLUT2-Cre mice. This was followed by the CreOFF/FlpON-oScarlet injection into the CLA. This strategy was used to conditionally express the oScarlet fluorophore specifically in the mPFC-projecting VGLUT2-negative projection neurons. For labeling VGLUT2-positive projection neurons, CreON/FlpON-oScarlet was used. CLA, claustrum; mPFC, medial prefrontal cortex.

(B) Histological confirmation of targeting CLA VGLUT2-negative projection neurons. CreOFF/FlpON oScarlet-encoding virus (CreOFF/FlpON oSc) was injected. In one condition, flippase (Flp) was not injected into the mPFC (top), while in another case, Flp was injected into the mPFC (bottom). VGLUT2-negative projection neurons are labeled in orange, as indicated by the white arrows.

(C) (Top) Histological images showing the location of VGLUT2-negative projection neurons relative to the PV-rich core of the CLA. (Bottom) Three additional sample images demonstrate that the labeled cells do not fully overlap with the PV-enriched core of the CLA. The dotted gray margin marks the boundaries from the reference brain atlas. PV-staining revealed the PV-rich neuropil (CLA core) and the surrounding PV-negative region (CLA shell). The dashed white ellipse outlines the boundary of the CLA core. DEn, dorsal endopiriform.

(legend continued on next page)

and AAV-CreOFF/FlpON-GCaMP6m in the CLA. We quantified the overlap between GCaMP6m-expressing neurons and Cre-immunopositive neurons (85 GCaMP6m-expressing neurons and 182 Cre-positive neurons across 7 slices from 2 mice; [Figures S1C and S1D](#)). Among Cre-positive neurons, only a small fraction overlapped with GCaMP6m expression (mean $\pm$ SEM across slices: $8.3 \pm 3.3\%$), consistent with the CreOFF design effectively excluding Cre-positive cells. Conversely, among GCaMP6m-expressing neurons, the vast majority were Cre-negative ($86.7 \pm 5.1\%$), indicating that these labeled neurons predominantly lack Cre expression. This low degree of overlap is consistent with previous work using a similar intersectional viral strategy.³¹ While this analysis is based on a limited number of animals ($n = 2$ mice) and sections primarily sampled from the anterior CLA (anteroposterior [AP] +0.9–1.4 mm) and thus may not fully capture variability across the rostro-caudal extent of the CLA, these results nonetheless provide validation that our intersectional viral strategy selectively targets VGLUT2-negative neurons projecting to the mPFC.

To prove that the labeled neurons were located inside the CLA, sections were stained with anti-parvalbumin (PV) antibody, which labels the PV-rich CLA core ([Figure 1C](#)).^{26,32–34} To validate their anatomical localization, we performed a systematic spatial registration analysis by defining the centroid of the PV-dense CLA core in each section and mapping the positions of labeled neurons relative to this reference ([Figures S1E and S1F](#)). VGLUT2-positive neurons were clustered around the PV-core centroid, whereas VGLUT2-negative neurons were distributed ventrally and medially relative to this core. Importantly, while VGLUT2-negative neurons extended beyond the PV-defined core, their distribution remained within the CLA shell region, defined in previous work as the collective spatial extent of CLA neurons labeled via projections to multiple cortical areas.^{34,35} Collectively, these results demonstrate that mPFC-projecting VGLUT2-negative neurons reside within the CLA (hereafter termed “VGLUT2-negative CLA-mPFC” neurons).

3D quantification of the cell-type-specific projections: VGLUT2-positive and VGLUT2-negative claustral neurons

CLA neurons are known for their broad projection patterns, typically innervating a wide range of distinct regions.^{5,34,36} To investigate the anatomical connections and specific projection targets of VGLUT2-negative CLA-mPFC neurons, as well as to quantify their axonal projections across various brain regions, histological images were analyzed using the QUINT pipeline, a workflow for atlas-registered brain-wide quantification and mapping of rodent brain microscopy data.^{34,37} 3D imaging showed that VGLUT2-negative CLA-mPFC neurons prominently project to regions within the mPFC, including the anterior cingulate and prelimbic cortices ([Figures S1G and S1H](#)). These findings underscore the existence of a strong anatomical connection between VGLUT2-negative CLA neurons and the mPFC. This

projection pattern also suggests a role for VGLUT2-negative CLA-mPFC neurons in influencing mPFC function, potentially serving as an anatomical substrate for claustral contributions to higher cognitive processes mediated by the mPFC.

To compare the projection patterns of VGLUT2-positive and VGLUT2-negative CLA-mPFC neurons, we examined brain sections with labeled VGLUT2-positive claustral neurons projecting to the mPFC (termed “VGLUT2-positive CLA-mPFC” neurons) or with labeled VGLUT2-negative CLA-mPFC neurons ([Figures 1D and 1E](#)). To label VGLUT2-positive CLA-mPFC neurons, we used a strategy similar to that used for labeling VGLUT2-negative CLA-mPFC neurons, except that AAV-CreON/FlpON-oScarlet was injected into the CLA rather than AAV-CreOFF/FlpON ([Figure 1A](#)). VGLUT2-negative CLA-mPFC neurons most densely projected to the mPFC, while also projecting to other regions, including the motor, gustatory, dorsal peduncular, and agranular insular areas ([Figure S1H](#)). VGLUT2-positive CLA-mPFC neurons showed a broadly similar projection pattern ([Figure S1H](#)). Overall, the brain-wide connectivity profiles of these cell types were largely comparable.

Differential projection patterns to the mPFC layers

We next compared their projections within the mPFC. VGLUT2-negative CLA-mPFC neurons exhibited a notable preference for deeper layers, specifically targeting layer 6 (L6) more extensively than VGLUT2-positive CLA-mPFC neurons ([Figures 1D and 1E](#)). A line scan analysis ([STAR Methods](#)) revealed significant differences in fiber density across mPFC layers between these cell types ($p = 4.7 \times 10^{-9}$; layer $\times$ cell-type interaction with two-way mixed-effects ANOVA). Notably, VGLUT2-negative CLA-mPFC axons were denser in L6 compared both to other mPFC layers and to the axons of VGLUT2-positive CLA-mPFC neurons across all layers ([Figure 1F](#); $p < 0.01$, post hoc Tukey’s honestly significant difference test). In addition, VGLUT2-negative CLA-mPFC axons were sparser than VGLUT2-positive CLA-mPFC axons in L2/3 ($p = 7.0 \times 10^{-5}$).

Activity of VGLUT2-negative neurons during spatial WM

We showed that VGLUT2-negative CLA-mPFC neurons preferentially target layer 6 while only sparsely innervating L2/3 of the mPFC. L6 is primarily an output layer, characterized by prominent corticothalamic projections.^{38,39} A study in the visual cortex has shown that L6 neurons can modulate the gain of activity in upper-layer neurons through intracortical and corticothalamic projections.⁴⁰ While these findings are limited to the visual cortex, by analogy, CLA inputs to L6 may similarly influence mPFC circuits, suggesting a potentially unique and significant role in modulating prefrontal activity. To explore their behavioral relevance, we used GCaMP6m to detect the activity of these neurons during a working memory (WM) task. To express GCaMP6m in VGLUT2-negative CLA-mPFC neurons, we modified the viral strategy used above by employing AAV-CreOFF/FlpON-GCaMP6m ([Figure 2A](#)). Optical fibers were further

(D and E) Histological images of the mPFC showing the distribution of the axonal projection of CLA VGLUT2-negative (VGLUT2[−]) projection neurons (D) and CLA VGLUT2-positive (VGLUT2⁺) projection neurons (E).

(F) The mean percentages of fiber density across each neuronal layer in the mPFC ($n = 9$ sections, 3 mice for VGLUT2-negative; $n = 6$ sections, 2 mice for VGLUT2-positive). * $p < 0.0001$; two-way mixed-effects ANOVA with post hoc Tukey’s honestly significant difference test.

See also [Figure S1](#).

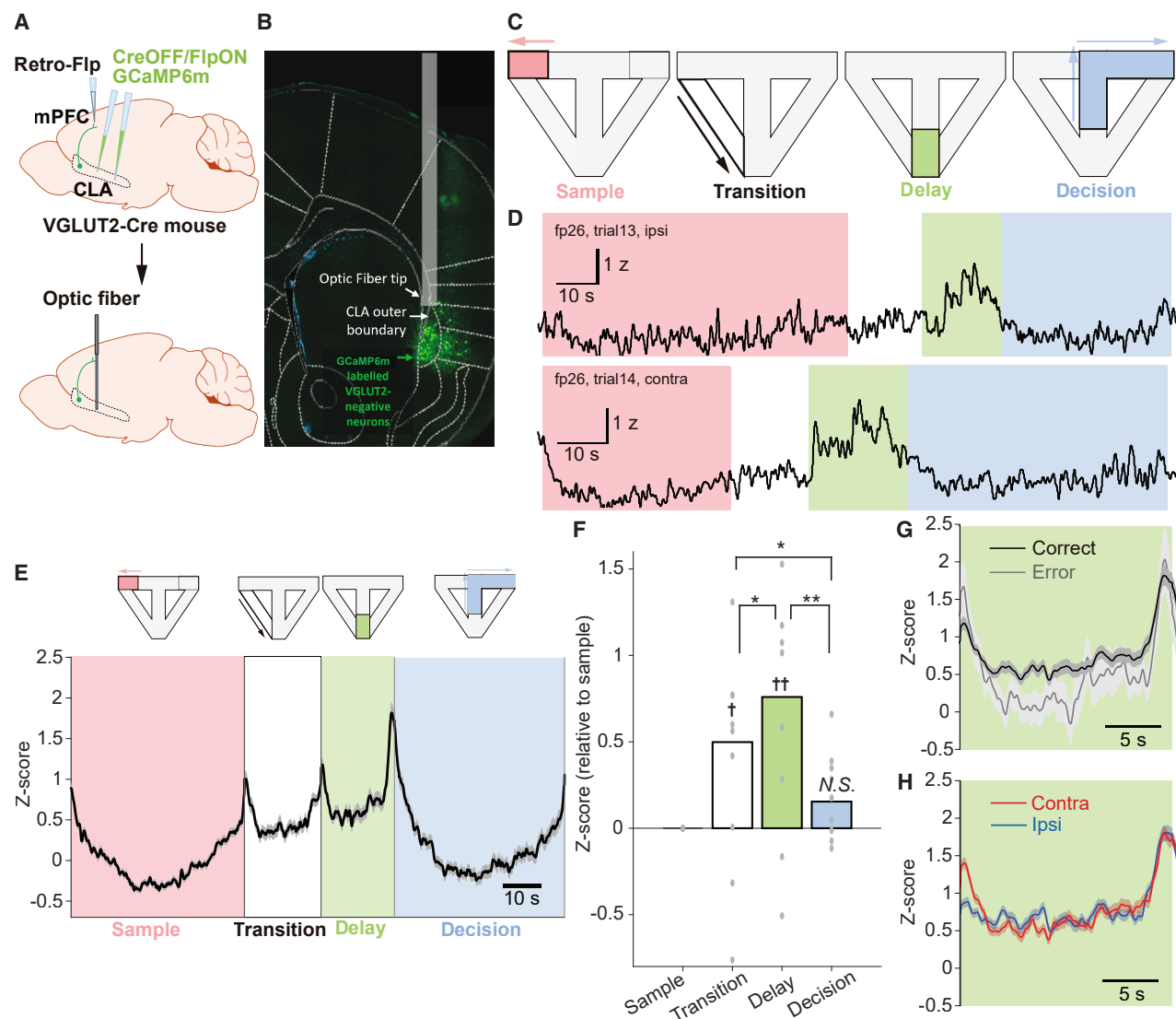


Figure 2. Measurement of activity of VGLUT2-negative CLA-mPFC neurons during a T-maze task via fiber photometry

(A) Injection schematics to target VGLUT2-negative CLA-mPFC neurons with GCaMP6m. Retro-Flip was injected into the mPFC, followed by the GCaMP6m injection into the CLA.

(B) Histological images of CreOFF/FlpON GCaMP6m-labeled VGLUT2-negative CLA-mPFC neurons with an implanted fiber optic for fiber photometry.

(C) Schematic of the automated T-maze setup, illustrating the distinct periods of the task: sample (pink), transition (white), delay (green), and decision (blue).

(D) Z scored GCaMP6m fluorescence traces from two example trials, with task phases color-coded as in (C).

(E) Time course of trial-averaged neuronal activity across all mice (n = 9 mice, 335 correct trials). Gray shaded area, mean ± SEM. Insets above each region of the T-maze illustrate how the mice progress through each phase of the T-maze task.

(F) Mean activity for each phase averaged across animals (n = 9 mice). Activity was Z scored relative to the sample period in each trial. *p < 0.0005 and **p < 10⁻¹⁷; one-way mixed-effects ANOVA with Bonferroni-corrected post hoc test. NS (non-significant), p = 0.25, †p < 10⁻⁴, and ‡p < 10⁻¹¹; comparison with zero (sample activity). Gray points, individual mice.

(G and H) Time course of trial-averaged neuronal activity during the delay period, separated by trial outcome (correct vs. error; G) and by the spatial relationship between the sample location and the recording hemisphere (contra vs. ipsi; H). n = 335 correct trials, 33 error trials.

See also Figure S2.

implanted in the CLA (Figure 2B), enabling measurement of population-level activity of VGLUT2-negative CLA-mPFC neurons.

After the implantation, mice were trained for a delayed non-match-to-sample spatial task (Figures 2C, S2A, and S2B). In each trial, mice were first forced to enter a randomly assigned open arm to obtain food reward (sample period) and then

returned to the start zone (transition period). The delay period began upon re-entry into the start zone, during which the gate was closed for 10 s and persisted until the mice exited the start zone, requiring them to maintain the sampled spatial location in WM. Upon gate opening, mice were required to choose the opposite arm to receive a food reward (decision period). After

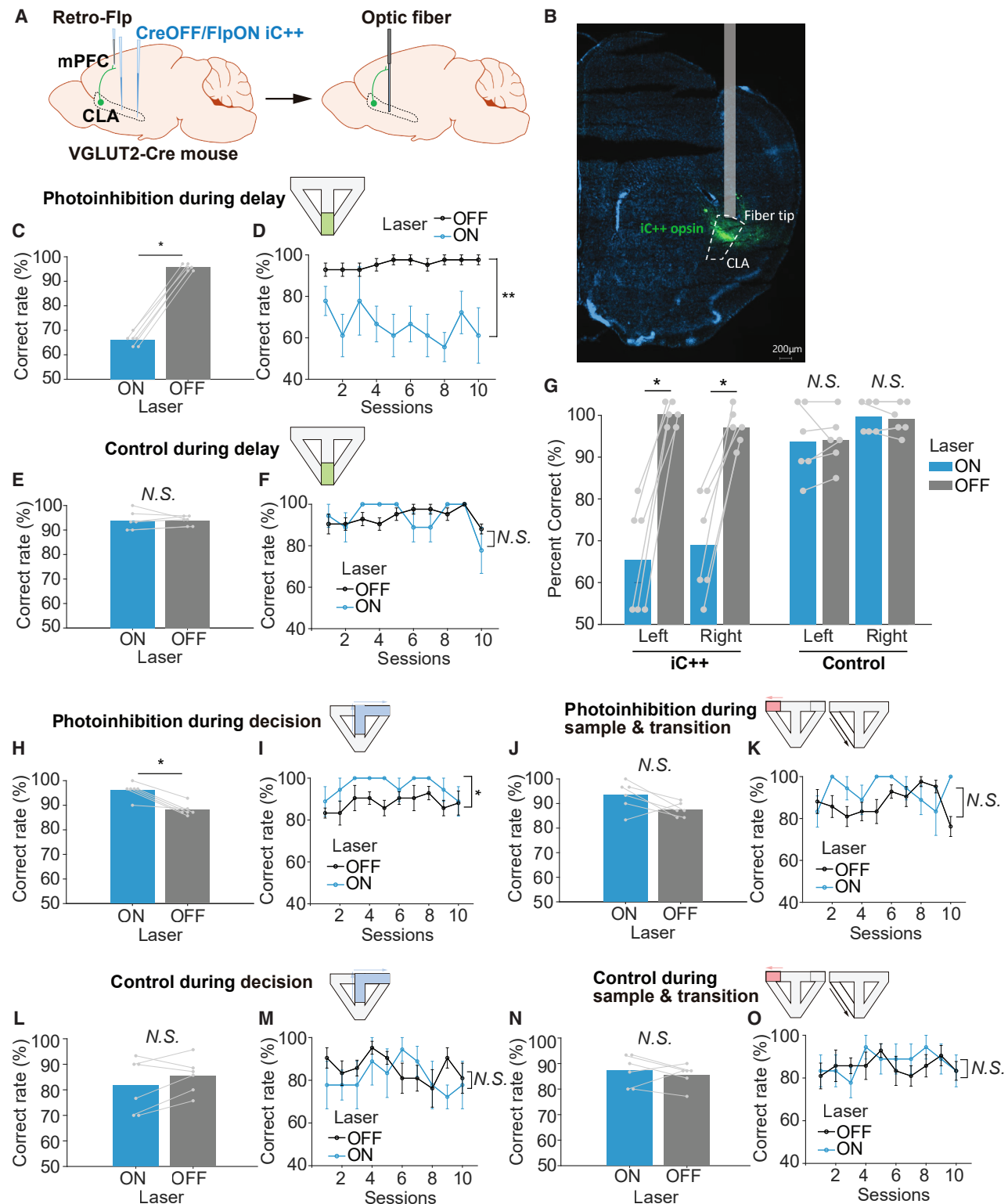


Figure 3. Photoinhibition of VGLUT2-negative CLA-mPFC neurons during a T-maze task

(A) Injection schematics targeting VGLUT2-negative CLA-mPFC neurons with iC++. Retro-Flp was injected into the mPFC, and CreOFF/FlpON iC++ was injected into the CLA region of VGLUT2-Cre mice.

(B) Histological images of CreOFF/FlpON iC++-expressing VGLUT2-negative CLA-mPFC neurons and the implanted fiber optic.

(legend continued on next page)

meeting the performance criterion ($\geq 80\%$ accuracy in two consecutive sessions), GCaMP6m fluorescence was recorded. As illustrated by the representative $\Delta F/F$ traces (Figure 2D), the activity of GCaMP6m-expressing neurons changed during different periods. Responses averaged across trials were temporally resampled to the median duration of each task period across trials, enabling comparison of activity across trials despite variability in period length (Figure 2E; STAR Methods).⁴¹ For the delay period, although the gate was closed for 10 s, the median duration exceeded this due to variability in when mice exited the start zone (Figure 2E). During the sample period, activity was low but gradually increased during the subsequent transition period, peaking in the delay period before returning to low levels during the decision period. Activity during the delay period was significantly elevated compared with other task periods ($p < 0.0005$, one-way mixed-effects ANOVA with Bonferroni test; Figure 2F). This activity modulation across task periods provides compelling evidence for period-specific engagement of VGLUT2-negative CLA-mPFC neurons in WM processing. In addition to this period-specific modulation, there were transient peaks in activity just before and during task-period boundaries (Figure 2E): for all boundaries examined (sample-to-transition, transition-to-delay, and delay-to-decision), activity in the boundary center (-2 to $+2$ s) was significantly higher than in both pre- (-6 to -2 s) and post-boundary ($+2$ to $+6$ s) windows ($p < 0.001$; Bonferroni test following repeated-measures ANOVA), potentially reflecting anticipatory attention to salient events such as gate openings associated with transitions between periods.

We next examined whether neuronal activity differed between correct and error trials and whether activity was influenced by laterality, i.e., the recording hemisphere relative to the sample location. Delay-period activity was significantly modulated by trial outcome ($p = 0.037$; two-way ANOVA with Trial Outcome and Laterality), with higher activity in correct compared with error trials ($p = 0.039$, Wilcoxon signed-rank test; Figure 2G). However, there was no significant effect of laterality (contra vs. ipsilateral; $p = 0.076$, two-way ANOVA; Figure 2H) nor a significant interaction between trial outcome and laterality ($p = 0.095$, two-way ANOVA). These results suggest that the delay activity of VGLUT2-negative CLA-mPFC neurons reflects WM retention success.

One potential confounding factor is that mobility differed across trial periods, which could have influenced neural activity. Video analysis revealed that movement speed was significantly

modulated by the task period ($p = 7.4 \times 10^{-6}$, one-way repeated ANOVA; Figure S2C). During the sample period, when the mice consumed the food reward, the movement speed was minimal. During the transition period, when the mice returned to the start zone, the speed was highest. During the subsequent delay period, the speed was slower than the transition period and comparable to the decision period. These results are consistent with the literature showing that animals generally reduce locomotor activity during delay periods of spatial WM tasks but are not completely immobile.^{41–43} To disentangle the contributions of task-related and behavioral variables, we modeled the neural activity of each session using a general linear model (GLM) that included multiple regressors capturing distinct aspects of task structure and behavior: task phase (sample, transition, delay, and decision periods), choice (left or right), trial outcome (correct or error), reward expectation, running direction (outbound or inbound), speed, and acceleration (Figure S2D; STAR Methods). Notably, task phase emerged as the dominant contributor to neural activity (Figure S2E), with a significant effect observed in 95% of imaging sessions (Figure S2F) even after the model accounted for movement-related factors such as running direction and kinematics. These results indicate that modulation by task phase was the primary driver of activity in VGLUT2-negative CLA-mPFC neurons and that this activity cannot be explained by movement alone.

Causal role of VGLUT2-negative neurons in WM

Because VGLUT2-negative CLA-mPFC neurons are active during the delay period and their activity is associated with successful WM retention, we next determined whether these neurons causally contribute to WM performance. We selectively photoinhibited them by activating iC⁺⁺, an anion-conducting channelrhodopsin, with blue light.⁴⁴ Following injection of iC⁺⁺-expressing virus into both hemispheres (Figure 3A), optical fibers were bilaterally implanted to deliver blue light (Figure 3B).

Bilateral photoinhibition during the delay period drastically reduced behavioral performance to nearly chance levels (Figure 3C; $p = 0.031$, Wilcoxon signed-rank test). This impairment was consistently observed regardless of whether the sample location was on the left or right (Figure 3G; $p = 0.031$). Photoinhibition during the delay period tended to shorten reaction times but not significantly (median: 1.59 s vs. 1.40 s for laser OFF vs. ON; $p = 0.063$). To assess whether the observed

(C and D) Mean correct performance across all sessions (C) and across consecutive 10-day sessions (D) during laser ON and OFF conditions targeting the delay period in iC⁺⁺-injected mice ($n = 6$). Gray lines, individual mice. $^*p = 0.031$, Wilcoxon signed-rank test (C). Error bars, mean $\pm$ SEM across mice. $^{**}p = 0.0059$; main effect of photoinhibition in two-way mixed-effects ANOVA: photoinhibition $\times$ session (D). There was no significant effect of session ($p = 0.15$) or interaction ($p = 0.12$).

(E and F) Same as (C) and (D) but in control mice ($n = 6$). NS, $p > 0.50$; Wilcoxon signed-rank test (E). There were no significant effects of photoinhibition, session, or their interaction ($p = 0.14$, 0.17 , and 0.097 ; two-way mixed-effects ANOVA) (F).

(G) Mean correct rate in iC⁺⁺-injected and control mice, separated by left and right sample trials. $^*p = 0.031$, NS, $p > 0.50$; Wilcoxon signed-rank test.

(H and I) Same as (C) and (D) but when photoinhibition was applied during the decision period in iC⁺⁺-injected mice ($n = 6$). $^*p = 0.031$, Wilcoxon signed-rank test (H). $^*p = 0.011$, main effect of photoinhibition, two-way mixed-effects ANOVA (I). There was no significant effect of session ($p = 0.94$) or interaction ($p = 0.46$).

(J and K) Same as (H) and (I) but when photoinhibition was applied during the sample and transition periods in iC⁺⁺-injected mice ($n = 6$). NS, $p = 0.22$; Wilcoxon signed-rank test (J). There were no significant effects of photoinhibition, session, or their interaction ($p = 0.15$, 0.82 , and 0.71) (K).

(L and M) Same as (H) and (I) but in control mice ($n = 6$). NS, $p = 0.13$; Wilcoxon signed-rank test (L). There were no significant effects of photoinhibition, session, or their interaction ($p = 0.28$, 0.83 , and 0.55 ; two-way mixed-effects ANOVA) (M).

(N and O) Same as (J) and (K) but in control mice ($n = 6$). NS, $p = 0.66$; Wilcoxon signed-rank test (N). There were no significant effects of photoinhibition, session, or their interaction ($p = 0.90$, 0.33 , and 0.62) (O).

impairment was attributed to altered mobility, we examined movement speed during the delay period. However, neither mean nor maximum speed differed significantly by photoinhibition ($p = 0.16$ and 0.31), suggesting that photoinhibition did not affect overall motor activity.

To assess potential non-specific laser effects, we also conducted experiments in control mice in which VGLUT2-negative CLA-mPFC neurons expressed GCaMP6m instead of iC++. In these control mice, laser illumination did not affect WM performance (Figure 3E; $p = 0.63$), demonstrating that the reduced performance of mice expressing iC++ was a specific consequence of photoinhibiting VGLUT2-negative CLA-mPFC neurons.

To confirm the reliability of photoinhibition effects across days, we examined task performance over daily sessions (each comprising 10 trials). In iC++-injected mice, performance during laser-ON trials was consistently lower across daily sessions (Figure 3D). Performance was significantly affected by the photoinhibition factor ($p = 0.0059$, two-way mixed-effects ANOVA) but not session ($p = 0.15$) or their interaction ($p = 0.12$). These indicate that photoinhibition reduced performance consistently across sessions. Conversely, illumination in control mice consistently yielded high performance across sessions (Figure 3F). There was no significant effect of illumination, session, or their interaction.

Next, to determine whether the photoinhibition effect depends on the task period, we photoinhibited VGLUT2-negative CLA-mPFC neurons during two other periods: (1) the decision period and (2) the sample and transition periods. Photoinhibition during the decision period significantly improved performance (Figure 3H; $p = 0.031$, Wilcoxon signed-rank test), whereas inhibition during the sample and transition periods had no effect (Figure 3J; $p = 0.22$). Across 10 sessions, performance was consistently higher in laser-ON trials than laser-OFF trials during decision-period photoinhibition (Figure 3I; main effect of photoinhibition, $p = 0.011$), with no effects of session or their interaction. By contrast, for photoinhibition during the sample and transition periods, there were no significant effects of photoinhibition, session, or their interaction (Figure 3K). No effects were observed in control mice during any period (Figures 3L–3O), indicating that the improvement during the decision period in iC++-expressing mice is not due to non-specific effects.

Overall, these results indicate that the activity of VGLUT2-negative CLA-mPFC neurons is essential for WM retention during the delay period but may be dispensable or even disruptive during decision period.

DISCUSSION

We have examined the function of a previously unexplored population of VGLUT2-negative neurons within the CLA that project to the mPFC, distinct from the well-characterized VGLUT2-positive neurons. We identified (1) the anatomical projection patterns of these neurons, (2) their activity during spatial WM, and (3) their necessity for WM, as revealed by optogenetic photoinhibition. These findings establish VGLUT2-negative CLA-mPFC projections as a critical component of WM circuits.

The CLA has previously been implicated in memory processes, particularly episodic memory.^{6,13,17–19,21–23} Our study, however, focuses on WM, which is a form of memory that is

distinct from episodic memory. While recent work has begun to implicate the CLA in WM,^{20,22} the circuit mechanisms underlying this role remain largely unknown. By demonstrating that VGLUT2-negative CLA-mPFC neurons are selectively engaged during the delay period and are necessary for WM performance, our findings complement and extend prior studies, providing pathway-specific insight into how the CLA contributes to WM maintenance.

VGLUT2-negative CLA-mPFC neurons were primarily localized in the CLA shell, ventral to the PV-rich core, in contrast to VGLUT2-positive neurons, which are more dorsally distributed around the core.^{45,46} While the molecular identity of these VGLUT2-negative neurons remains to be fully characterized, they likely include Gng2-expressing neurons, which project to the mPFC²⁵ and are located in the ventral CLA.^{47–49} Other candidates include VGLUT1-positive⁵⁰ or Gnb4-positive neurons,^{5,10,11,25,33,51} both of which are additional claustral projection populations.

Although both VGLUT2-positive and VGLUT2-negative neurons project to the mPFC, they appear to have different functions. Our findings indicate that VGLUT2-negative neurons preferentially target deeper layers, with higher axonal density in L6. This laminar specificity may underlie functional differences between the two projection types. The deeper layers of the mPFC house interneurons critical for cognitive processes, particularly the regulation of feedforward inhibition.^{24,52} Notably, CLA projections preferentially activate PV-expressing and neuropeptide Y (NPY)-expressing interneurons in the mPFC, with NPY interneurons playing a key role in feedforward inhibition.²⁴ Given the dense VGLUT2-negative projections to L6, their photoinhibition likely disrupts this inhibitory balance, thereby altering mPFC activity and impairing WM maintenance.

Research in the visual cortex has revealed that L6 neurons modulate the gain of visually evoked activity in upper-layer neurons without changing their tuning to orientation.⁴⁰ L6 is recognized as a hub integrating inputs from multiple brain regions to regulate cortical processing.^{38,39} It should be noted that these studies are limited to the visual cortex, and direct evidence is currently lacking for the mPFC. Nevertheless, by analogy, similar mechanisms may exist in the mPFC, suggesting that VGLUT2-negative projection neurons could potentially contribute to gain modulation within prefrontal circuits.

VGLUT2-negative CLA-mPFC neurons were most active during the delay period of the spatial WM task. This elevated activity likely reflects a role in supporting or stabilizing cognitive processes that sustain WM, rather than directly encoding or driving decision-related processes. Their intermediate activity during the transition period may represent a functional bridge between encoding and maintenance, consistent with the notion that WM retention begins to emerge even before the formal onset of the delay period. Crucially, this activity was stronger in correct trials than error trials, reinforcing its role in successful WM maintenance.

It is important to consider what the animal is doing during the delay period. In our task, mice remained alert and occasionally mobile, making head turns or postural adjustments, rather than entering a state of complete immobility. Video analyses confirmed that locomotor speed was significantly modulated across trial epochs, with delay-period mobility reduced relative

to the transition period but not absent. These results are consistent with previous findings that rodents generally reduce locomotion during delay phases of spatial WM tasks but are not entirely quiescent.^{41–43} This behavioral profile could be linked to a shift from externally oriented sensory engagement to internally generated mnemonic activity, supported by reduced acetylcholine tone and the emergence of hippocampal sharp-wave ripples.⁴² The elevated delay-period activity of VGLUT2-negative CLA-mPFC neurons may therefore reflect a contribution to sustaining or coordinating these internally organized states of memory maintenance, rather than processing external sensory stimuli.

No laterality bias in this activity was observed during the delay period, in contrast to the contralateral bias typically seen in frontoparietal areas,^{53–55} suggesting that these neurons may contribute more broadly to WM retention rather than representing spatial content per se. This could be attributed to the extensive reciprocal connectivity of the CLA with sensory, motor, and association areas,^{2,36,56} facilitating bilateral integration. Alternatively, population-level fiber photometry measurements may mask subtle lateralization of individual CLA neurons. Future studies using single-neuron resolution will be valuable for uncovering potential heterogeneity and functional specialization within this population.

To directly test the role of VGLUT2-negative CLA-mPFC activity in WM performance, we employed optogenetic photoinhibition during task performance. Remarkably, photoinhibition of VGLUT2-negative CLA-mPFC neurons during the delay period severely reduced performance, whereas photoinhibition during the decision period produced a modest improvement. These findings suggest that while VGLUT2-negative CLA-mPFC neurons are critical for WM retention during the delay, their activity may be nonessential or even detrimental during decision-making, consistent with their low activity in this period (Figures 2E and 2F). These findings point to a dynamic, task-period-specific contribution of VGLUT2-negative CLA-mPFC neurons to WM. While the activity of these neurons is critical for maintaining information during the delay period, it may need to be suppressed or disengaged during the decision period to optimize behavioral output. Such temporal specialization highlights the importance of coordinated CLA-mPFC circuit dynamics across distinct task phases in supporting effective WM processing. Comparable task-phase specificity has been observed in other WM-related circuits. For example, the mediodorsal thalamus (MD), which shares reciprocal connectivity with the mPFC, plays a causal role in sustaining mPFC activity during the delay period. Inhibition of MD-to-mPFC projections during the delay, but not during sample or decision periods, disrupted delay-period spiking in mPFC neurons and impaired WM performance, while activating MD enhanced it.⁵⁷ Conversely, inhibiting mPFC-to-MD projections impaired performance during the decision period, highlighting the bidirectional yet temporally distinct functions of this circuit. By contrast, hippocampal inputs to the mPFC are essential during the sample period but are not required during the delay or decision periods.⁵⁸ Although recordings from MD-projecting and hippocampus-projecting neurons were not obtained in these studies, they collectively suggest a division of labor across circuits subserving distinct WM components. In this context, our findings indicate that the VGLUT2-negative

CLA-mPFC pathway may represent a complementary modulatory system, supporting delay-period maintenance while contributing differently—or even oppositely—during decision-making. This raises the possibility that the CLA helps fine-tune prefrontal computations in a task-phase-specific manner, in coordination with other WM-related circuits such as the MD and hippocampus. Previous studies have shown that excitotoxic lesions of the CLA impair spatial WM⁵⁹ and that inhibiting VGLUT2-positive neurons within the CLA disrupts WM performance in delayed non-match-to-sample paradigms.²⁰ By examining a distinct population—VGLUT2-negative CLA-mPFC neurons—we extend these prior findings by uncovering a novel circuit essential for WM. Future studies will be needed to determine whether distinct, cell-type-specific CLA-mPFC pathways support different aspects of cognitive function.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tsukasa Kamigaki (tsukasara@ntu.edu.sg).

Materials availability

This study did not generate any new reagents.

Data and code availability

- The data have been deposited at Zenodo and are publicly available as of the date of publication at <https://doi.org/10.5281/zenodo.19755049>.
- All original code has been deposited at Zenodo and is publicly available at <https://doi.org/10.5281/zenodo.19755049> as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

ACKNOWLEDGMENTS

This research is supported by the Ministry of Education – Singapore, under its MOE AcRF Tier 2 awards MOE-T2EP30224-0004 and MOE-T2EP30220-0003, MOE AcRF Tier 1 award RG110/24 to T.K., and MOE AcRF Tier 3 award MOE2017-T3-1-002 to G.J.A.

AUTHOR CONTRIBUTIONS

J.Y.T. conducted all the experiments. H.R.C., M.Z.H.H., X.O., and P.R. performed behavioral experiments, immunohistochemistry, and image scanning. J.Y.T., M.G., G.J.A., and T.K. conceived, designed, and wrote the manuscript. J.Y.T. and T.K. analyzed the data. G.J.A. and T.K. supervised the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
- **METHOD DETAILS**
 - Surgical procedures
 - Immunohistochemistry
 - Behavior-measuring apparatus and training
 - Fiber photometry
 - Optogenetics

● **QUANTIFICATION AND STATISTICAL ANALYSIS**

- Image acquisition and processing
- Atlas-based registration and quantification of axonal projections
- Laminar boundary delineation in the mPFC
- Spatial distribution analysis of mPFC-projecting CLA neurons
- Behavioral analysis
- Fiber photometry calcium signal preprocessing
- General Linear Modeling (GLM) of fiber photometry signal
- Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cub.2026.04.067>.

Received: July 21, 2025

Revised: April 10, 2026

Accepted: April 28, 2026

REFERENCES

1. Smith, J.B., and Alloway, K.D. (2010). Functional specificity of claustrum connections in the rat: interhemispheric communication between specific parts of motor cortex. *J. Neurosci.* 30, 16832–16844. <https://doi.org/10.1523/jneurosci.4438-10.2010>.
2. Smith, J.B., and Alloway, K.D. (2014). Interhemispheric claustral circuits coordinate sensory and motor cortical areas that regulate exploratory behaviors. *Front. Syst. Neurosci.* 8, 93. <https://doi.org/10.3389/fnsys.2014.00093>.
3. Zingg, B., Hintiryan, H., Gou, L., Song, M.Y., Bay, M., Bienkowski, M.S., Foster, N.N., Yamashita, S., Bowman, I., Toga, A.W., et al. (2014). Neural networks of the mouse neocortex. *Cell* 156, 1096–1111. <https://doi.org/10.1016/j.cell.2014.02.023>.
4. Torgerson, C.M., Irimia, A., Goh, S.Y.M., and Van Horn, J.D. (2015). The DTI connectivity of the human claustrum. *Hum. Brain Mapp.* 36, 827–838. <https://doi.org/10.1002/hbm.22667>.
5. Wang, Q., Ng, L., Harris, J.A., Feng, D., Li, Y., Royall, J.J., Oh, S.W., Bernard, A., Sunkin, S.M., Koch, C., et al. (2017). Organization of the connections between claustrum and cortex in the mouse. *J. Comp. Neurol.* 525, 1317–1346. <https://doi.org/10.1002/cne.24047>.
6. Zahacy, R., Ma, Y., Winship, I.R., Jackson, J., and Chan, A.W. (2024). Claustrum modulation drives altered prefrontal cortex dynamics and connectivity. *Commun. Biol.* 7, 1556. <https://doi.org/10.1038/s42003-024-07256-5>.
7. Madden, M.B., Stewart, B.W., White, M.G., Krimmel, S.R., Qadir, H., Barrett, F.S., Seminowicz, D.A., and Mathur, B.N. (2022). A role for the claustrum in cognitive control. *Trends Cogn. Sci.* 26, 1133–1152. <https://doi.org/10.1016/j.tics.2022.09.006>.
8. Goll, Y., Atlan, G., and Citri, A. (2015). Attention: the claustrum. *Trends Neurosci.* 38, 486–495. <https://doi.org/10.1016/j.tins.2015.05.006>.
9. Bayat, A., Joshi, S., Jahan, S., Connell, P., Tsuchiya, K., Chau, D., Syed, T., Forcelli, P., and Koubeissi, M.Z. (2018). A pilot study of the role of the claustrum in attention and seizures in rats. *Epilepsy Res.* 140, 97–104. <https://doi.org/10.1016/j.epilepsyres.2018.01.006>.
10. White, M.G., Mu, C., Qadir, H., Madden, M.B., Zeng, H., and Mathur, B.N. (2020). The Mouse Claustrum Is Required for Optimal Behavioral Performance Under High Cognitive Demand. *Biol. Psychiatry* 88, 719–726. <https://doi.org/10.1016/j.biopsych.2020.03.020>.
11. Ollerenshaw, D.R., Davis, J., McBride, E.G., Shelton, A., Koch, C., and Olsen, S.R. (2021). Anterior claustrum cells are responsive during behavior but not passive sensory stimulation. *bioRxiv*, 2021.2003.2023.436687. <https://doi.org/10.1101/2021.03.23.436687>.
12. Atlan, G., Matosevich, N., Peretz-Rivlin, N., Marsh-Yvgi, I., Zelinger, N., Chen, E., Kleinman, T., Bleistein, N., Sheinbach, E., Groysman, M., et al. (2024). Claustrum neurons projecting to the anterior cingulate restrict engagement during sleep and behavior. *Nat. Commun.* 15, 5415. <https://doi.org/10.1038/s41467-024-48829-6>.
13. Jankowski, M.M., and O'Mara, S.M. (2015). Dynamics of place, boundary and object encoding in rat anterior claustrum. *Front. Behav. Neurosci.* 9, 250. <https://doi.org/10.3389/fnbeh.2015.00250>.
14. Atlan, G., Terem, A., Peretz-Rivlin, N., Sehwat, K., Gonzales, B.J., Pozner, G., Tasaka, G.I., Goll, Y., Refaeli, R., Zviran, O., et al. (2018). The Claustrum Supports Resilience to Distraction. *Curr. Biol.* 28, 2752–2762.e7. <https://doi.org/10.1016/j.cub.2018.06.068>.
15. Smith, J.B., Watson, G.D.R., Liang, Z., Liu, Y., Zhang, N., and Alloway, K.D. (2019). A Role for the Claustrum in Salience Processing? *Front. Neuroanat.* 13, 64. <https://doi.org/10.3389/fnana.2019.00064>.
16. Atilgan, H., Doody, M., Oliver, D.K., McGrath, T.M., Shelton, A.M., Echeverria-Altuna, I., Tracey, I., Vyazovskiy, V.V., Manohar, S.G., and Packer, A.M. (2022). Human lesions and animal studies link the claustrum to perception, salience, sleep and pain. *Brain* 145, 1610–1623. <https://doi.org/10.1093/brain/awac114>.
17. Kitanishi, T., and Matsuo, N. (2017). Organization of the Claustrum-to-Entorhinal Cortical Connection in Mice. *J. Neurosci.* 37, 269–280. <https://doi.org/10.1523/jneurosci.1360-16.2016>.
18. Terem, A., Gonzales, B.J., Peretz-Rivlin, N., Ashwal-Fluss, R., Bleistein, N., Del Mar Reus-Garcia, M., Mukherjee, D., Groysman, M., and Citri, A. (2020). Claustral Neurons Projecting to Frontal Cortex Mediate Contextual Association of Reward. *Curr. Biol.* 30, 3522–3532.e6. <https://doi.org/10.1016/j.cub.2020.06.064>.
19. Mutel, S., Renfer, J., Rodriguez, I., Carleton, A., and Salazar, R. (2023). The claustrum drives large-scale interactions of cortical circuits relevant to long-term memory. *bioRxiv*, 2023.2003.2002.530783. <https://doi.org/10.1101/2023.03.02.530783>.
20. Bhattacharjee, A.S., Huber, C., Unsal, B., Renfer, J.-R., Rodriguez, I., and Carleton, A. (2024). The claustrum is critical for maintaining working memory information. *bioRxiv*, 2024.2010.2028.620649. <https://doi.org/10.1101/2024.10.28.620649>.
21. Medina, C., Ojea Ramos, S., Depino, A.M., Romano, A.G., Krawczyk, M. C., and Boccia, M.M. (2024). The role of the claustrum in the acquisition, consolidation and reconsolidation of memories in mice. *Sci. Rep.* 14, 24409. <https://doi.org/10.1038/s41598-024-74419-z>.
22. Stewart, B.W., Cormie, M.A., Keaser, M.L., Moayed, M., Mathur, B.N., and Seminowicz, D.A. (2025). The human claustrum initiates networks for externally and internally driven task demands. *bioRxiv*, 2025.06.26.661853. <https://doi.org/10.1101/2025.06.26.661853>.
23. Yamawaki, N., Login, H., Feld-Jakobsen, S.Ø., Molnar, B.M., Kirkegaard, M.Z., Moltesen, M., Okrasa, A., Radulovic, J., and Tanimura, A. (2025). Endopiriform neurons projecting to ventral CA1 are a critical node for recognition memory. *eLife* 13, RP99642. <https://doi.org/10.7554/eLife.99642>.
24. Jackson, J., Kamani, M.M., Zemelman, B.V., Burdakov, D., and Lee, A.K. (2018). Inhibitory Control of Prefrontal Cortex by the Claustrum. *Neuron* 99, 1029–1039.e4. <https://doi.org/10.1016/j.neuron.2018.07.031>.
25. Fodoulou, L., Gschwend, O., Huber, C., Mutel, S., Salazar, R.F., Leone, R., Renfer, J.-R., Ekundayo, K., Rodriguez, I., and Carleton, A. (2020). The claustrum-medial prefrontal cortex network controls attentional set-shifting. *bioRxiv*, 2020.2010.2014.339259. <https://doi.org/10.1101/2020.10.14.339259>.
26. Graf, M., Nair, A., Wong, K.L.L., Tang, Y., and Augustine, G.J. (2020). Identification of Mouse Claustral Neuron Types Based on Their Intrinsic Electrical Properties. *eNeuro* 7, ENEURO.0216-20.2020. <https://doi.org/10.1523/eneuro.0216-20.2020>.
27. Graf, M., Sadeh, S., and Augustine, G.J. (2026). Mapping the functional connectome of the claustrum: Noise filtering via local inhibitory circuits. *Cell Rep.* 45, 116821. <https://doi.org/10.1016/j.celrep.2025.116821>.

28. de La Torre-Martínez, R., Chia, Z., Baxendale, J., Tokarska, A., Frost-Nylén, J., Augustine, G.J., and Silberberg, G. (2025). Presynaptic and postsynaptic determinants of claustrum-cortical connectivity. *Curr. Biol.* 35, 3174–3190.e5. <https://doi.org/10.1016/j.cub.2025.05.056>.
29. Fenno, L.E., Ramakrishnan, C., Kim, Y.S., Evans, K.E., Lo, M., Vesuna, S., Inoue, M., Cheung, K.Y.M., Yuen, E., Pichamoorthy, N., et al. (2020). Comprehensive Dual- and Triple-Feature Intersectional Single-Vector Delivery of Diverse Functional Payloads to Cells of Behaving Mammals. *Neuron* 107, 836–853.e11. <https://doi.org/10.1016/j.neuron.2020.06.003>.
30. Hur, E.E., and Zaborszky, L. (2005). Vglut2 afferents to the medial prefrontal and primary somatosensory cortices: a combined retrograde tracing in situ hybridization study [corrected]. *J. Comp. Neurol.* 483, 351–373. <https://doi.org/10.1002/cne.20444>.
31. Barbano, M.F., Wang, H., Zhang, S., Shevelkin, A.V., Yu, K.J., Richie, C.T., Liu, B., Hahn, S., Ye, R., and Morales, M. (2025). VTA monosynaptic connections by local glutamate and GABA neurons and their distinct roles in behavior. *Nat. Commun.* 16, 8500. <https://doi.org/10.1038/s41467-025-63396-0>.
32. Mathur, B.N., Caprioli, R.M., and Deutch, A.Y. (2009). Proteomic analysis illuminates a novel structural definition of the claustrum and insula. *Cereb. Cortex* 19, 2372–2379. <https://doi.org/10.1093/cercor/bhn253>.
33. White, M.G., Panicker, M., Mu, C., Carter, A.M., Roberts, B.M., Dharmasri, P.A., and Mathur, B.N. (2018). Anterior Cingulate Cortex Input to the Claustrum Is Required for Top-Down Action Control. *Cell Rep.* 22, 84–95. <https://doi.org/10.1016/j.celrep.2017.12.023>.
34. Ham, G.X., and Augustine, G.J. (2022). Topologically Organized Networks in the Claustrum Reflect Functional Modularization. *Front. Neuroanat.* 16, 901807. <https://doi.org/10.3389/fnana.2022.901807>.
35. Marriott, B.A., Do, A.D., Zahacy, R., and Jackson, J. (2021). Topographic gradients define the projection patterns of the claustrum core and shell in mice. *J. Comp. Neurol.* 529, 1607–1627. <https://doi.org/10.1002/cne.25043>.
36. Zingg, B., Dong, H.W., Tao, H.W., and Zhang, L.I. (2018). Input-output organization of the mouse claustrum. *J. Comp. Neurol.* 526, 2428–2443. <https://doi.org/10.1002/cne.24502>.
37. Yates, S.C., Groenboom, N.E., Coello, C., Lichtenthaler, S.F., Kuhn, P.H., Demuth, H.U., Hartlage-Rübsamen, M., Roßner, S., Leergaard, T., Kreshuk, A., et al. (2019). QUINT: Workflow for Quantification and Spatial Analysis of Features in Histological Images From Rodent Brain. *Front. Neuroinform.* 13, 75. <https://doi.org/10.3389/fninf.2019.00075>.
38. Vélez-Fort, M., and Margrie, T.W. (2012). Cortical circuits: layer 6 is a gain changer. *Curr. Biol.* 22, R411–R413. <https://doi.org/10.1016/j.cub.2012.03.055>.
39. Vélez-Fort, M., Rousseau, C.V., Niedworok, C.J., Wickersham, I.R., Rancz, E.A., Brown, A.P.Y., Strom, M., and Margrie, T.W. (2014). The stimulus selectivity and connectivity of layer six principal cells reveals cortical microcircuits underlying visual processing. *Neuron* 83, 1431–1443. <https://doi.org/10.1016/j.neuron.2014.08.001>.
40. Olsen, S.R., Bortone, D.S., Adesnik, H., and Scanziani, M. (2012). Gain control by layer six in cortical circuits of vision. *Nature* 483, 47–52. <https://doi.org/10.1038/nature10835>.
41. Wilhelm, M., Sych, Y., Fomins, A., Alatorre Warren, J.L., Lewis, C., Serratos Capdevila, L., Boehringer, R., Amadei, E.A., Grewe, B., O'Connor, E.C., et al. (2023). Striatum-projecting prefrontal cortex neurons support working memory maintenance. *Nat. Commun.* 14, 7016. <https://doi.org/10.1038/s41467-023-42777-3>.
42. Zhang, Y., Cao, L., Varga, V., Jing, M., Karadas, M., Li, Y., and Buzsáki, G. (2021). Cholinergic suppression of hippocampal sharp-wave ripples impairs working memory. *Proc. Natl. Acad. Sci. USA* 118, e2016432118. <https://doi.org/10.1073/pnas.2016432118>.
43. Vogel, P., Hahn, J., Duvarci, S., and Sigurdsson, T. (2022). Prefrontal pyramidal neurons are critical for all phases of working memory. *Cell Rep.* 39, 110659. <https://doi.org/10.1016/j.celrep.2022.110659>.
44. Berndt, A., Lee, S.Y., Wietek, J., Ramakrishnan, C., Steinberg, E.E., Rashid, A.J., Kim, H., Park, S., Santoro, A., Frankland, P.W., et al. (2016). Structural foundations of optogenetics: Determinants of channelrhodopsin ion selectivity. *Proc. Natl. Acad. Sci. USA* 113, 822–829. <https://doi.org/10.1073/pnas.1523341113>.
45. Real, M.A., Dávila, J.C., and Guirado, S. (2006). Immunohistochemical localization of the vesicular glutamate transporter VGLUT2 in the developing and adult mouse claustrum. *J. Chem. Neuroanat.* 31, 169–177. <https://doi.org/10.1016/j.jchemneu.2005.12.002>.
46. Druga, R. (2014). Chapter 2 The Claustrum (Elsevier), pp. 29–84. <https://doi.org/10.1016/B978-0-12-404566-8.00002-7>.
47. Pirone, A., Cozzi, B., Edelstein, L., Peruffo, A., Lenzi, C., Quilici, F., Antonini, R., and Castagna, M. (2012). Topography of Gng2- and NetrinG2-expression suggests an insular origin of the human claustrum. *PLoS One* 7, e44745. <https://doi.org/10.1371/journal.pone.0044745>.
48. Watakabe, A. (2017). In situ hybridization analyses of claustrum-enriched genes in marmosets. *J. Comp. Neurol.* 525, 1442–1458. <https://doi.org/10.1002/cne.24021>.
49. Dillingham, C.M., Mathiasen, M.L., Frost, B.E., Lambert, M.A.C., Bubbs, E. J., Jankowski, M.M., Aggleton, J.P., and O'Mara, S.M. (2019). The Anatomical Boundary of the Rat Claustrum. *Front. Neuroanat.* 13, 53. <https://doi.org/10.3389/fnana.2019.00053>.
50. Geisler, S., Derst, C., Veh, R.W., and Zahm, D.S. (2007). Glutamatergic afferents of the ventral tegmental area in the rat. *J. Neurosci.* 27, 5730–5743. <https://doi.org/10.1523/jneurosci.0012-07.2007>.
51. Narikiyo, K., Mizuguchi, R., Ajima, A., Shiozaki, M., Hamanaka, H., Johansen, J.P., Mori, K., and Yoshihara, Y. (2020). The claustrum coordinates cortical slow-wave activity. *Nat. Neurosci.* 23, 741–753. <https://doi.org/10.1038/s41593-020-0625-7>.
52. Naka, A., and Adesnik, H. (2016). Inhibitory Circuits in Cortical Layer 5. *Front. Neural Circuits* 10, 35. <https://doi.org/10.3389/fncir.2016.00035>.
53. Kagan, I., Iyer, A., Lindner, A., and Andersen, R.A. (2010). Space representation for eye movements is more contralateral in monkeys than in humans. *Proc. Natl. Acad. Sci. USA* 107, 7933–7938. <https://doi.org/10.1073/pnas.1002825107>.
54. Li, N., Chen, T.W., Guo, Z.V., Gerfen, C.R., and Svoboda, K. (2015). A motor cortex circuit for motor planning and movement. *Nature* 519, 51–56. <https://doi.org/10.1038/nature14178>.
55. Tsutsui, K.I., Oyama, K., Nakamura, S., and Iijima, T. (2016). Comparative Overview of Visuospatial Working Memory in Monkeys and Rats. *Front. Syst. Neurosci.* 10, 99. <https://doi.org/10.3389/fnsys.2016.00099>.
56. Chia, Z., Augustine, G.J., and Silberberg, G. (2020). Synaptic Connectivity between the Cortex and Claustrum Is Organized into Functional Modules. *Curr. Biol.* 30, 2777–2790.e4. <https://doi.org/10.1016/j.cub.2020.05.031>.
57. Bolkan, S.S., Stujenske, J.M., Parnaudeau, S., Spellman, T.J., Rauffenbart, C., Abbas, A.I., Harris, A.Z., Gordon, J.A., and Kellendonk, C. (2017). Thalamic projections sustain prefrontal activity during working memory maintenance. *Nat. Neurosci.* 20, 987–996. <https://doi.org/10.1038/nn.4568>.
58. Spellman, T., Rigotti, M., Ahmari, S.E., Fusi, S., Gogos, J.A., and Gordon, J.A. (2015). Hippocampal-prefrontal input supports spatial encoding in working memory. *Nature* 522, 309–314. <https://doi.org/10.1038/nature14445>.
59. Grasby, K., and Talk, A. (2013). The anterior claustrum and spatial reversal learning in rats. *Brain Res.* 1499, 43–52. <https://doi.org/10.1016/j.brainres.2013.01.014>.
60. Chong, H.R., Ranjbar-Slamloo, Y., Ho, M.Z.H., Ouyang, X., and Kamigaki, T. (2023). Functional alterations of the prefrontal circuit underlying cognitive aging in mice. *Nat. Commun.* 14, 7254. <https://doi.org/10.1038/s41467-023-43142-0>.

61. Hao, M.H.Z., and Kamigaki, T. (2025). Dissociable cortical contributions to impulse control during waiting. *Sci. Adv.* *11*, ead4732. <https://doi.org/10.1126/sciadv.adx4732>.
62. Grider, M.H., Chen, Q., and Shine, H.D. (2006). Semi-automated quantification of axonal densities in labeled CNS tissue. *J. Neurosci. Methods* *155*, 172–179. <https://doi.org/10.1016/j.jneumeth.2005.12.021>.
63. Sathyanesan, A., Ogura, T., and Lin, W. (2012). Automated measurement of nerve fiber density using line intensity scan analysis. *J. Neurosci. Methods* *206*, 165–175. <https://doi.org/10.1016/j.jneumeth.2012.02.019>.
64. Wang, Q., Ding, S.L., Li, Y., Royall, J., Feng, D., Lesnar, P., Graddis, N., Naeemi, M., Facer, B., Ho, A., et al. (2020). The Allen Mouse Brain Common Coordinate Framework: A 3D Reference Atlas. *Cell* *181*, 936–953.e20. <https://doi.org/10.1016/j.cell.2020.04.007>.
65. Hoerder-Suabedissen, A., Ocana-Santero, G., Draper, T.H., Scott, S.A., Kimani, J.G., Shelton, A.M., Butt, S.J.B., Molnár, Z., and Packer, A.M. (2023). Temporal origin of mouse claustrum and development of its cortical projections. *Cereb. Cortex* *33*, 3944–3959. <https://doi.org/10.1093/cercor/bhac318>.
66. Ranjbar-Slamloo, Y., Chong, H.R., and Kamigaki, T. (2025). Aging disrupts the link between network centrality and functional properties of prefrontal neurons during memory-guided behavior. *Commun. Biol.* *8*, 62. <https://doi.org/10.1038/s42003-025-07498-x>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Primary rabbit anti-parvalbumin (PV) antibody	Swant	Cat# PV27; RRID: AB_2631173
Primary rabbit anti-Cre recombinase antibody	Abcam	Cat# ab190177; RRID: AB_2860024
Goat anti-rabbit IgG secondary antibody	Thermo Fisher Scientific	Cat# A11008; RRID: AB_143165
Bacterial and virus strains		
AAVrg-EF1a-Flpo	Addgene	55637-AAVrg
AAV8-Ef1a-Coff/Fon-oScarlet	Addgene	137138-AAV8
AAV8-Ef1a-Con/Fon-oScarlet	Addgene	137136-AAV8
AAV8-Ef1a-Coff/Fon-GCaMP6M	Addgene	137121-AAV8
AAV8-nEF-Coff/Fon-iC++-EYFP	Addgene	137157-AAV8
Chemicals, peptides, and recombinant proteins		
C&B metabond	Parkell	Cat# S380
Fluoroshield™ with DAPI	Sigma-Aldrich	Cat# F6057
Polyvinyl alcohol mounting media	Sigma-Aldrich	Cat# SGA 10981
Paraformaldehyde	Sigma-Aldrich	Cat# 158127
Deposited data		
Dataset and analysis code	This paper	https://doi.org/10.5281/zenodo.19755049
Experimental models: Organisms/strains		
Vglut2-ires-Cre mouse: Slc17a6tm2(cre)Lowl/J	The Jackson Laboratory	RRID: IMSR_JAX:016963
Software and algorithms		
MATLAB	MathWorks	SCR_001622
GraphPad Prism (v.9)	Dotmatics	https://www.graphpad.com/scientific-software/prism/
Bonsai	Bonsai Foundation	https://bonsai-rx.org/
Arduino IDE	Arduino	https://www.arduino.cc/
QUINT	EBRAINS infrastructure	https://quint-workflow.readthedocs.io/
ANY-maze video tracking software	Stoelting Co.	https://stoeltingco.com/Neuroscience/ANY-maze
ZEN Blue 3.1 acquisition software	Carl Zeiss Microscopy GmbH	https://www.zeiss.com/microscopy/en/products/software.html
Other		
Microinjector	World Precision Instruments	Micro 4
Stereotaxic frame	David Kopf Instruments	Model 900LS
Fiber-coupled LED (470 nm)	Thorlabs	M470F4
Fiber-coupled LED (405 nm)	Thorlabs	M405F3
Dichroic filter	Thorlabs	MD498
Emission filters	Thorlabs	FBH450-10, MF525-39
Photomultiplier tube (PMT)	Photon Technology International	Model 814
AC-AC Converter amplifier	Soundtech Electronics	AT-1000
Infrared (IR) video camera	Stoelting Europe	60528
Multifunction I/O device	National Instruments	DAQ USB 6002
Optogenetics patch cables	Thorlabs	Cat# M82L005
DPSS blue laser	Shanghai Laser & Optics Century	Cat# BL473T3-100FC + ADR-700A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All proposed animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University, Singapore (protocol#A19062, A24054). Experiments were performed in adult (2 to 3-month-old) wildtype C57BL/6 mice or transgenic VGLUT2-ires-cre mice (Jackson Laboratories, Slc17a6tm2(cre)Low/J, stock number 016963). Both wildtype and VGLUT2-Cre mice were used to verify that the dual injection strategy targeted VGLUT2-negative projection neurons. Animals were housed on a 12-h dark/12-h light cycle (light on between 7:00 and 19:00) at around 21°C and 62% humidity. All the experiments were carried out between 8:00 and 17:00.

METHOD DETAILS

Surgical procedures

Surgical procedures were performed to inject viruses for transgene expression in the brain and to implant optical fibers, enabling light delivery for fiber photometric measurement of neural activity or optogenetic control. Mice were anesthetized using 2% isoflurane and fitted onto the stereotaxic frame (David Kopf Instruments or RWD Life Science). Animals were maintained on 1.5% isoflurane and kept warm with a heating lamp throughout the surgery. The scalps were sterilized with iodine after shaving off the fur and mice were then injected with local anaesthetic, lidocaine at 0.1 mg per mouse. After five minutes, an incision was made along the midline of the scalp and retracted to allow access to the skull. The skull was aligned to ensure a level position both between lambda and bregma, as well as between the left and right side. The Allen Mouse Brain Atlas (2008) was used to derive the coordinates of target regions: mPFC (AP +1.40 mm, ML+/- 0.30 mm, DV-1.50 mm from the pia), CLAp (posterior claustrum; AP +0.84 mm, ML+/- 2.75 mm, DV-2.55 mm), CLAA (anterior claustrum; AP +1.15 mm, ML+/- 2.30 mm, DV-2.35 mm), and CLAA for optical fibers implant (AP - +1.15 mm, ML+/- 2.30 mm, DV-2.30 mm). A craniotomy was performed above the cortex using a dental drill with a 0.5 mm tip. Next, each of the pulled pipettes was back-filled with mineral oil and loaded with one of the following viruses: Flippase (AAV Retrograde 55637-AAVrg, 50–100 nL), oScarlet (pAAV-Ef1a-Coff/Fon-oScarlet, 50 nL), oScarlet (pAAV-Ef1a-Con/Fon-oScarlet, 50 nL), GCaMP6m (pAAV-Ef1a-Coff/Fon-GCaMP6m, 50 nL) and iC++ (pAAV-nEF-Coff/Fon-iC++-EYFP, 50 nL).

To label the projection neurons for histological analysis of brain-wide connectivity, VGLUT2-Cre mice were unilaterally injected in the medial prefrontal cortex (mPFC) with 100 nL of retrograde AAV8 virus encoding flippase (AAV Retrograde 55637-AAVrg). This virus enables the retrograde expression of flippase from the mPFC to its projection source regions. On the same side of the hemisphere, mice were injected in the claustrum (CLA) with 50 nL of AAV8 virus encoding for CreOFF/FlpON oScarlet (pAAV-Ef1a-Coff/Fon-oScarlet) for labeling VGLUT2-negative projection neurons, or CreON/FlpON oScarlet (pAAV-Ef1a-Con/Fon-oScarlet) for labeling VGLUT2-positive projection neurons. For comparison, wild-type mice were also injected with 100 nL of retrograde AAV8 virus encoding flippase in the mPFC and 50 nL of AAV8 virus encoding CreOFF/FlpON oScarlet in the CLA.

After demarcating the injection locations with a surgical marker, a craniotomy was made. The dura mater was gently removed to expose the pia. The glass pipettes were lowered to the pia and mice were unilaterally injected with the viruses through these borosilicate glass pipettes using a microinjector (Micro 4, World Precision Instruments). The glass pipette remained in the mice for five minutes after each injection to allow sufficient time for the injected virus to properly diffuse into the targeted brain region and to prevent immediate backflow along the injection tract, thus facilitating a more efficient uptake of virus by the surrounding cells. After five minutes elapsed, the pipette was withdrawn at a very slow rate.

Thereafter, the scalp was sutured, and mice were transferred into a new cage for recovery from the surgery. Following surgery, mice were injected with the painkiller buprenorphine (0.1 mg/kg) and meloxicam (5 mg/kg), as well as an antibiotic baytril (12 mg/kg) to facilitate recovery from surgery.

For fiber photometric calcium imaging, 100 nL of retrograde AAV8 encoding Flp recombinase was injected into the bilateral mPFC of VGLUT2-Cre mice. Additionally, 50 nL of AAV8 carrying a Cre-off/Flp-on GCaMP6m (pAAV-Ef1a-Coff/Fon-GCaMP6m) was delivered to both anterior and posterior regions of the bilateral claustrum (CLA). Injection procedures were otherwise identical to those used for the AAV8-oScarlet virus described above. Mice were allowed to recover for two weeks before bilateral implantation of fiber optic cannulas. A second craniotomy was made at the same location, and surrounding tissue was cleared to expose the pia. The fiber optic implant was then lowered to the pia and fixed slightly dorsal to the injection site using screws and dental cement (C&B Metabond, Rayden).

For optogenetics experiments, VGLUT2-Cre mice underwent the injection of 100 nL retrograde AAV8 virus coding Flippase into the bilateral mPFC in combination with the injection of 50 nL AAV8 virus coding CreOFF/FlpON iC++ (pAAV-nEF-Coff/Fon-iC++-EYFP) in each of the posterior and anterior regions along the bilateral CLA. Except for the four-week recovery period between virus injection and fiber optic implantation, the procedure followed the same protocol used for preparing mice for fiber photometric calcium imaging.

Immunohistochemistry

The brains of the mice were histologically examined to validate the injection strategy, identify the location of VGLUT2-negative neurons, quantify and spatially analyze VGLUT2-negative and -positive projection neurons, and verify the accuracy of the viral injections and precise targeting of the CLA. For this purpose, mice were anesthetized with phenobarbital (200 mg/kg) two weeks post-injection. They were perfused transcardially with 50 mL of phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 50 mL of 4%

paraformaldehyde (PFA). For brain-wide connectivity analysis, brains were extracted and fixed in 4% PFA overnight, followed by a transfer to a cryoprotective 30% sucrose solution for an additional two days. The brains were then embedded in optimum cutting temperature compound (OCT compound, Sakura Finetek USA) for cryosectioning. Coronal sections of the brains spanning the anterior to posterior regions containing the CLA were cut into 40 μ m thick slices using a cryostat (Leica CM1950, Leica Biosystems).

Every fifth section was used for immunostaining. These sections were washed with PBS-0.25% Triton X-100 (PBSTx) and incubated in 5% goat serum (blocking solution) for one hour at room temperature. Next, the blocking solution was removed, and the sections were incubated with either primary rabbit anti-parvalbumin (PV) antibody (1:1000, Swant, PV27) or primary rabbit anti-Cre antibody (1:100, Abcam, ab190177) overnight (48 hours) at 4°C. The anti-PV antibody labels the PV-rich CLA core, providing general anatomical orientation and ensuring that the labeled neurons were located within the CLA. Afterward, the sections were washed with PBSTx and incubated with goat anti-rabbit IgG secondary antibody (1:500, Thermo Fisher Scientific, A11008) for four hours at room temperature. Sections were washed again with PBSTx before being incubated in DAPI for 20 minutes at room temperature. After a final wash, the sections were mounted onto glass slides with polyvinyl alcohol mounting media (Sigma-Aldrich, 10981).

Behavior-measuring apparatus and training

Working memory (WM) was assessed through a T-maze behavioral assay, conducted in a custom-built automated T-maze. The T-maze behavioral experiments were run using Bonsai software and custom Arduino code to control the gates of the maze. The automated T-maze featured sensor-gated control of the gates for the reward zones, return zone, and start zone (Figure S2A). Behavioral training began two weeks after mice underwent implantation surgery. Mice were first habituated to the T-maze for two days, during which they freely explored the maze with all gates open and rewards available in both arms' reward zones. This was followed by a shaping epoch, where mice were required to visit the choice arms. Food rewards (20 mg pellets) were alternated between the arms in each trial, with the gate to the unrewarded arm remaining closed. After five days of shaping, mice proceeded to training sessions, which continued until they reached a performance criterion of 80% correct choices across two consecutive sessions. Each trial consisted of three phases: encoding, maintenance, and decision. During the encoding phase, mice were allowed to enter an open arm to obtain a reward, while the opposite arm's gate remained closed. In the subsequent retrieval phase, they had to choose the arm opposite the one visited during the encoding phase. Initially, rewards alternated between the left and right arms, but later trials randomized the rewarded side, ensuring equal distribution across training and test sessions. Consecutive trials rewarding the same arm in the same direction were limited to a maximum of three. The maintenance phase began when mice returned to the start zone, where the gate remained closed for 10 seconds. This delay required the mice to retain the location of the previously rewarded arm in working memory. After the 10-second delay, the gate reopened, allowing the mice to enter the mid-arm. In the decision phase, both choice arms were opened, and the mice had to select the arm not visited during the encoding phase.

Fiber photometry

A custom-built fiber photometry apparatus was used for the experiments. Light at 470 nm from a fiber-coupled LED (M470F4, Thorlabs) was delivered to the 200 μ m diameter optical fiber implant at a power of 0.4 mW. An isosbestic light channel at 405 nm (M405F3, Thorlabs) was interleaved (5 Hz) with the 470 nm signal to account for any motion-related artifacts in the recording. A dichroic mirror (MD498, Thorlabs) and two emission filters (FBH450-10 and MF525-39, both from Thorlabs) were used to isolate the signals of interest before they were transmitted through a photomultiplier tube (PMT; model 814, Photon Technology International) sampling at 200 Hz. The recorded signals were amplified (AT-1000, Soundtech Electronics) and relayed to a data acquisition board (DAQ USB 6002, National Instruments). Whole session recordings were deinterleaved to obtain the photometric signal using MATLAB, with an effective sampling rate of 5 Hz and time bins of 200 ms.

The mice were video-recorded at 30 Hz with an infrared (IR) video camera (60528, Stoelting Europe). A custom Arduino code was used to control the automatic T-maze setup. To ensure that the photometric signal recorded by the PMT was properly synchronized with the event timings from the video analysis, time stamps were generated during the T-maze behavioral task, for both 1) the video recording at 30 Hz and for 2) the signal recorded by the PMT. These time stamps were then compared with the event markers to enable accurate temporal alignment of the photometry signals with the behavioral events.

Optogenetics

470 nm light (1 mW, 30 Hz pulses) was delivered bilaterally to the CLA through a bifurcated optical fiber bundle (200 μ m diameter, 0.50 numerical aperture) connected to a DPSS laser (BL473T3-100FC, Shanghai Laser).^{60,61} This light was used to activate the iC++ opsin, thereby photoinhibiting the VGLUT2-negative neurons in the CLA projecting to the mPFC. The ANY-maze software (Stoelting Co., USA) was used to track the position of the mice throughout the maze. The 470 nm light was delivered randomly in 30% of the trials within each daily session of 10 trials. For photoinhibition during the sample and transition periods, light delivery began when the mice entered the open sample arm (Event 4; see Figure S2 and 'Behavioral analysis' below) and ended when they returned to the starting zone (Event 6). For photoinhibition during the delay period, light delivery started when the mice re-entered the starting zone (Event 6) and continued until they exited it (Event 7). For photoinhibition during the decision period, light delivery was initiated when the mice entered the mid-arm by leaving the starting zone (Event 7) and ended when they entered the return arm by leaving the reward zone (Event 10). Each photoinhibition condition was tested across 10 sessions, with different task-period photoinhibitions performed in separate daily sessions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image acquisition and processing

To visualize the axonal innervations of mPFC-projecting CLA neurons in fixed brains, the axons and cell bodies of these neurons were detected and quantified using methods previously described.^{62,63} To localize the mPFC-projecting VGLUT2-negative neurons relative to the PV-rich core in the CLA, fluorescence from oScarlet, PV, and DAPI was examined under a 20X objective on a confocal microscope (Zeiss LSM 800). Fluorescent images were also acquired using a 20X objective on a slide scanner (ZEISS Axio Scan Z1, USA). To ensure accurate comparisons across sections, all images were captured with consistent light intensity, camera exposure time, and gain. The scanned images of the brain sections were stitched together using ZEN Blue 3.1 acquisition software (Carl Zeiss Microscopy GmbH). Background subtraction was applied to the stitched brain sections to correct for autofluorescence and enhance the definition of cell bodies and axons.

Atlas-based registration and quantification of axonal projections

For axonal projection density quantification, we used the QUINT workflow,^{34,37} which integrates imaging data with spatial registration for high-resolution analysis of projection patterns. Experimental sections were first registered to the Allen Mouse Brain Atlas⁶⁴ using the QuickNII component. This step involved anchoring selected key sections by superimposing the reference atlas onto section images, adjusting cutting angles through affine transformations, and then propagating spatial information across the entire image series. Following atlas registration, fluorescence-labeled sections were segmented using the ilastik pixel classification workflow, which distinguished axonal labeling from background based on intensity, edge, and texture features. The segmented images were then processed with the Nutil Quantifier, which employs a recursive pixel-filling algorithm to detect objects and assign them to atlas-defined regions generated by QuickNII. Axonal projection density was quantified for each brain region as the proportion of axon-labeled pixels relative to the total number of pixels within that region. We then normalized these regional densities by the total axonal densities within each animal to account for inter-animal variability in fluorescence intensity.

Laminar boundary delineation in the mPFC

Fluorescent images were binarized and thresholded in ImageJ. We applied the FeatureJ Hessian filter (“Largest eigenvalue of Hessian tensor,” “Absolute eigenvalue comparison,” smoothing scale = 0.5), converted the images to 8-bit, and used ImageJ’s default threshold to isolate axonal signals. The laminar boundaries were defined by aligning pia-to-white matter distances in our experimental images with the proportional layer ratios from the Allen Mouse Brain Atlas. This approach ensured that each cortical layer was scaled according to its relative thickness and position, as in the previous study.²⁴ Axonal projection density within each layer was quantified as the percentage of total fiber density contained in that laminar region. This approach normalizes for differences in layer thickness and section area, enabling direct comparison across layers and subjects.

Spatial distribution analysis of mPFC-projecting CLA neurons

To define the anatomical distribution of retrogradely labeled mPFC-projecting neurons, we performed a systematic spatial registration analysis relative to an established claustrum landmark. The parvalbumin (PV)-dense core was used as a reliable anatomical reference for CLA.^{5,18,28,34,35,65} For each section, the centroid of the PV-dense CLA core was identified, and the positions of all labeled neurons were registered in a common coordinate framework relative to this centroid.³⁵ Using these PV-core-centered coordinates, we computed two-dimensional cell density maps (cells per $30 \times 30 \mu\text{m}^2$ bin) separately for VGLUT2-negative and VGLUT2-positive labeled populations, averaging across sections. To further quantify spatial biases, we calculated marginal distributions of cell density (cells/ μm^2) along the dorsoventral and mediolateral axes relative to the PV-core centroid. For each section, spatial distributions were smoothed using a Gaussian kernel ($\sigma = 2 \mu\text{m}$) prior to averaging across sections.

Behavioral analysis

Custom MATLAB code was used for the behavioral analysis, combining output from the video camera tracking of mouse behavior with the fiber photometric calcium measurements of neuronal activity. The behavior of the mice during the T-maze task was separated into the following events (Figures 2C and S2B):

Encoding phase:

- (1) Start zone before the start of each trial (Start).
- (2) Entry into the mid-arm (Mid) before arriving at the junction.
- (3) Entry into the open sample arm (Arm, left or right), while the opposite arm remained gated.
- (4) Entry into the Reward zone (Rew) to obtain food reward.
- (5) Entry into the Return arm (Ret) leading back to the starting zone.

Maintenance phase:

- (6) Re-entry back to the start zone (Start).

Decision phase:

- (7) Entry into the mid-arm again (Mid).
- (8) Entry into the Left or Right Arm (Arm).
- (9) Entry into the Reward zone (Rew).
- (10) Entry into Return arm (Ret) to return to the start zone.

Events (4) and (5) correspond to the 'Sample' and 'Transition' periods, respectively, while Event (6) represents the 'Delay' period, and Events (7) to (9) correspond to the 'Decision' period. Fiber photometric signal analysis was carried out based on these event markers. These event markers were extracted from the video by using a custom-written Bonsai code. The video was analyzed frame by frame, tracking mice by comparing the contrast in intensity between the dark-colored mice and the white T-maze. Events were logged when mice entered the region of interest (ROI) for the different events. Reaction time was defined as the interval between entry into the mid-arm (Event 7) and entry into the left or right choice arm (Event 8) during the decision phase.

Fiber photometry calcium signal preprocessing

Raw fluorescent signals were resampled to 50 Hz and binned into 200 ms segments. The alternating GCaMP6m and isosbestic signals were fitted and linearized using linear regression between the calcium-dependent signal (470 nm) and the isosbestic control signal (405 nm). This procedure also corrected for motion artifacts and photobleaching across both channels. The resulting fit served as the baseline for normalizing the calcium signal.

This interleaved signal could be separated into its two components, which could be differentiated by differences in their signal amplitude. The change in fluorescence over time ($\Delta F/F$) was calculated using the formula: $\Delta F/F = (F(t) - F_m) / F_m$, where $F(t)$ represents the fluorescence intensity at each time point, and F_m is the mean fluorescence intensity during the entire session.

To normalize fluorescence across mice and trials, signals were z-scored by subtracting the mean activity during the sample period of each trial and dividing the result by the standard deviation of that period. Because the duration of each task segment varied across trials, depending on when the mouse transitioned to the next segment in the T-maze, we temporally resampled the z-scored signals within each segment. Specifically, for each event segment, we resampled the signals to the median segment duration across trials, ensuring temporal alignment of the data for downstream analysis.⁴¹

General Linear Modeling (GLM) of fiber photometry signal

To assess how fiber photometry signals were influenced by task-related and behavioral variables, we modeled the neural activity of each session using a General Linear Model (GLM). Specifically, the fluorescence signal was expressed as a weighted linear combination of multiple regressors (Figure S2D), similar to previous work.⁴³ The model included variables capturing different aspects of the task and behavior: task phase (sample, transition, delay, and decision periods), choice (left or right trial), trial outcome (correct or error), reward expectation (assigned a value of 1 for positions with the left/right arms during the decision phase (i.e., Event 8), 0 otherwise), running direction (outbound or inbound), speed, and acceleration. Although speed and acceleration were entered as separate regressors, we jointly referred to their influence as "kinematics" by averaging the output parameters obtained from GLM. Modeling was performed on data from the entire task period of each trial, beginning with the sample period and ending at the decision period. For each imaging session, 80% of the time points were randomly selected to fit the model (using the 'fitglm' function in MATLAB). The remaining 20% of the data were held out for cross-validation. Model performance was evaluated by computing the squared Pearson correlation coefficient (r^2) between the predicted and actual neural signals.

To quantify the individual contribution of each variable, we first fit a full model using all regressors. We then constructed reduced models by omitting each variable of interest from the model and calculated the reduction in model performance (Δr^2) relative to the full model. This reduction (termed real Δr^2) reflects the variance uniquely explained by the omitted variable. To evaluate statistical significance, we created shifted models by temporally misaligning each regressor, thereby disrupting its alignment with the neural data. For each shifted model, we again computed the Δr^2 after removing the misaligned variable (random Δr^2). This process was repeated 1000 times per variable to generate a null distribution of Δr^2 values expected by chance. A session was deemed significantly modulated by a variable if its real Δr^2 exceeded the 95th percentile of the corresponding random Δr^2 distribution.

To quantify the relative contribution of each predictor across imaging sessions, we calculated the percentage reduction in r^2 after removing each variable from the full model (Figure S2E). Additionally, we computed the percentage of imaging sessions in which each variable significantly modulated neural activity (Figure S2F).

Statistical analysis

Statistical analyses are described in the main text and the figure legends. Statistical analyses were performed using MATLAB and GraphPad (GraphPad PRISM v.9). All statistical tests were two-sided unless otherwise stated. No statistical method was used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications.^{60,61,66}

To determine whether VGLUT2-negative and VGLUT2-positive projection neurons differentially innervate cortical layers of the mPFC, we fit a linear mixed-effects model ('fitlme' in MATLAB). We then performed ANOVA ('anova' in MATLAB) to analyze axonal fiber density percentages across mPFC layers, with Layer (1, 2/3, 5, 6) and Cell Type (VGLUT2-negative, VGLUT2-positive) included as fixed effects, and histological sections treated as a random effect. This analysis served as a two-way mixed-effects ANOVA.

For fiber photometry analysis, neural activity was z-scored relative to the sample period within each trial. A one-way mixed-effects ANOVA was conducted to compare activity across task periods (transition, delay, and decision periods), with task period as a fixed effect and trial and subject as random effects. To further examine whether neuronal activity was influenced by additional factors, we performed a two-way ANOVA with Laterality (contralateral vs. ipsilateral, relative to the recording hemisphere) and Trial Outcome (correct vs. error) as fixed factors.

In optogenetics experiments, the Wilcoxon signed-rank test was used to compare the performance between the laser stimulation and non-stimulation conditions across animals. To assess the consistency of the laser stimulation effect on task performance across sessions, we performed a two-way mixed-effects ANOVA with Photoinhibition (ON vs. OFF) and Session (sessions 1–10) as fixed factors, and Mouse (nested within group) as a random factor.