An intein-split transactivator for intersectional neural imaging and optogenetic manipulation

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The cell-type-specific recording and manipulation is instrumental to disentangle causal neural mechanisms in physiology and behavior and increasingly requires intersectional control; however, current approaches are largely limited by the number of intersectional features, incompatibility of common effectors and insufficient gene expression. Here, we utilized the protein-splicing technique mediated by intervening sequences (intein) and devised an intein-based intersectional synthesis of transactivator (IBIST) to selectively control gene expression of common effectors in multiple-feature defined cell types in mice. We validated the specificity and sufficiency of IBIST to control fluorophores, optogenetic opsins and Ca2+ indicators in various intersectional conditions. The IBIST-based Ca2+ imaging showed that the IBIST can intersect five features and that hippocampal neurons tune differently to distinct emotional stimuli depending on the pattern of projection targets. Collectively, the IBIST multiplexes the capability to intersect cell-type features and controls common effectors to effectively regulate gene expression, monitor and manipulate neural activities.
The cell-type diversity is a hallmark of the brain circuitry that enables us to process sensory stimuli in the environment, generate appropriate behaviors and emotions and gain the knowledge from learned experiences. The properties of neural cell types play pivotal roles in governing the neural functions of circuit networks across brain regions1-3, thus it becomes increasingly important for functional studies to specifically label a homogenous cell type depending on multiple features such as spatial locations, molecular markers and neuronal connections4-6. Genetic mouse lines offer great tools to intersectionally label specific neuronal population7,8, but are often hindered by the requirement of high-level gene expression for optogenetic opsins and Ca2+ indicators6,8. The breeding and crossing between multiple mouse lines can be laborious and time consuming, and region-specific control is also challenging. Combining the adeno-associated virus (AAV) vectors with transgenic mice brings powerful and convenient strategies to label a defined population in an intersectional manner and drives sufficient expression of opsins and Ca2+ indicators for optogenetic manipulation and neural recording, respectively9. Pioneering works have constructed AAV effectors for fluorophore and Channelrhodopsin-2 (ChR2) that selectively respond to specific combinations of controllers such as Cre and Flpo4,10,11. While more intersectional effectors are made available for optogenetic opsins and Ca2+ indicators12, the number of intersectional controllers/features is still limited. These effectors for opsins and Ca2+ indicators, which demand high level of expressions, may exhibit varying expression levels depending on experimental conditions and hence require laborious functional validations. Thus, it is desirable to fulfill both the dependency on more controllers/features and the compatibility with common effectors that is widely applicable across brain regions and animal species.

To this end, we took a strategy of reconstituting a single controller to achieve the intersectional control7-13. This strategy supports multi-feature dependent reconstitution of the single controller and in turn enables the compatibility with common effectors, which could be well verified to have reliable and sufficient gene expressions for recording or manipulation. Because most existing cell-type-specific driver lines are controlled by Cre14,15, we sought to reconstitute non-Cre controllers which can be complementary for Cre, and then exploited the application with AAV and rabies vectors which are broadly used in neural circuit studies. The tetracycline-controlled transactivator (tTA) is such a widely used controller and has additional features with temporal and reversible regulation. The tTA is formed by fusing tetracycline repressor protein with an activation domain of virion protein 16 (VP16) from herpes simplex virus16,17. We reasoned that splitting tTA into these two parts would achieve separable expressions in distinct constructs, which most likely result in the reconstitution of fully functional tTA. We leveraged the intervening sequences (intein) mediated protein splicing technique to ligate the split parts of tTA and achieve the efficient reconstitution of tTA. The intein has been shown to facilitate successful reconstitution of two parts together at the protein level in Caenorhabditis elegans18, neural stem cell19, cell line20 and mouse in vivo7. Compared to other reconstitution approaches such as leucine zipper-mediated dimerization and adapter pair-based covalent bonding, the intein approach introduces minimal heterologous sequence and exhibits better kinetics and higher activity recovery18,21-23.

In summary, we have developed a system with intein-based intersectional synthesis of transactivator (IBIST) to achieve the intersectional expression of a single controller. It in turn fulfills the compatibility with common effectors and multiplexes the intersectional features based on other existing controllers. This system supports: (1) flexible design of modular expression of split tTA in various conditions, (2) compatibility with common effectors dependent on tetracycline-responsive promoter element (TRE), and (3) versatile use with the viral vectors and rich resources of increasing mouse driver lines3,14-15,24. To demonstrate the capability of IBIST to intersectionally control opsins and Ca2+ indicators, we have created various AAV and rabies vectors to express tTA fragments controlled by site-specific recombinase, promoters or circuit tracing. We have validated the specificity and efficiency of IBIST tools both in vitro and in vivo, and then performed electrophysiological and photometric recording to demonstrate the successful application of IBIST tools for optogenetics and Ca2+ imaging in various neuronal populations with multiple features.

Results

The design of IBIST and the validation in vitro and in vivo. We split the tTA into N-terminal (Tet repressor, TetR) and C-terminal (three tandem minimal VP16 activation domains) parts from its structure junction17. The N-terminal part was further tagged with a blue fluorescence protein (BFP, Fig. 1a). To facilitate the tTA reconstitution from two split parts, we attached the N- and C- fragment of intein sequences of Gp41-118,21 to the N- and C-terminal parts of tTA because these intein sequences were reported to be more efficient and faster for protein splicing than other intein sequences25,26. This pair of ready-to-reconstitute subunits are referred as tTAN and tTAC respectively and are designed to form functional tTA after intein-mediated splicing (Fig. 1a-c).

To validate whether the tTA could be successfully reconstructed in mammalian cells, we expressed tTAN and tTAC as well as a TRE-controlled GFP reporter27 in cultured HEK293T' cells (see methods; TRE-dependent expression of histone-GFP, TVA and rabies glycoprotein, termed TRE-HTG). The fluorescence and protein level of TRE-dependent reporter became prominent after transfection, which was in stark contrast to that in control groups (Fig. 1d and f and Supplementary Fig. 1a). These results confirmed that split tTA are specifically rescued in the targeted cells with the help of intein-mediated splicing. All GFP-positive cells also expressed the fluorescence tag of tTAN (Fig. 1e and g), suggesting that the tTA reconstitution is highly specific.

To test whether similar strategy is applicable for reverse tTA (rtTA), we engineered the constructs for split rtTA with same intein sequences (Supplementary Fig. 1b). We first transfected the HEK293T' cells with plasmids for C-terminal part of rtTA (rtTAC) and reporter TRE-mCherry. To test whether the rtTA fragments can be expressed by viral vectors for neural tracing, we then inoculated the cells with a rabies variant expressing the N-terminal part of rtTA and the GFP tag (termed RV-rtTAN), which could be a retrograde transsynaptic tracer28. In the presence of doxycycline, the fluorescence and protein level of TRE-mCherry became prominent after transfection, but non-detectable in the control group (Supplementary Fig. 1a, c and e). Nearly all mCherry positive cells were co-labeled by the RV-rtTAN (Supplementary Fig. 1d and f), suggesting that the rtTA reconstitution is highly specific. Taken together, the fragments of tTA and rtTA are both able to efficiently and specifically reconstitute tTA and rtTA in the mammalian cells with the help of intein sequences, respectively.

To validate whether the split tTA could form functional reconstitution in vivo, we produced the AAV vectors and injected into the hippocampus with the AAVs of TRE-HTG and CaMKIIa dependent tTAC and BFP tagged tTAN. We observed strong fluorescence expression of reporter AAV-TRE-HTG in the hippocampus, whereas little fluorescence was seen in the control animals (Fig. 1h and j). Majority of HTG-positive...
cells co-expressed endogenous fluorescence tag of tTAN (Fig. 1i and k). Together, these results suggest that intein-split tTA/rTTA fragments are capable of forming specific reconstitution in an intersectional manner.

The function of IBIST-controlled optogenetic opsins and Ca$^{2+}$ indicators. Combining intersectional approaches with optogenetic manipulations is instrumental to delineate neural circuit functions$^9$. Although many intersectional labeling strategies exist for fluorophore labeling, it remains to be tested whether these strategies are applicable for optogenetic opsins as high expression of opsin is required for optogenetic manipulations in functional studies. We sought to test this for the IBIST tools by injecting the hippocampus with AAVs expressing TRE-oChIEF-mCherry$^{29}$ and CaMKIIα-dependent tTAN and tTAC, respectively (Fig. 2a).

As expected, potent mCherry fluorescence were only seen in...
animals with all three AAVs injected compared to control animals with AAV-tTAN or AAV-tTAC omitted (Fig. 2b and c). To further validate the faithfulness of reporter expression, we carried out immunostaining to amplify the endogenous fluorescence. We found that nearly all TRE-oChiEF positive cells were also co-labeled by tTAN tag (Supplementary Fig. 2a and b).

Using patch-clamp recording with optogenetic stimuli in acute brain slice (see methods), we found that the blue LED light robustly evoked spikes in hippocampal neurons from the AAV infected brain slices (200 ms constant light stimuli, Fig. 2d and e; 2 ms light pulses at 25 Hz, Supplementary Fig. 2c). These light-evoked spikes are absent in TRE-oChiEF negative cells (Supplementary Fig. 2d–f). In control animals injected with AAVs of tTAN or tTAC omitted, no spikes were evoked by the blue LED light (Fig. 2d and e). We continued to record some of those cells with light stimuli at the maximal power of the LED device (17 mW at the 60X objective), and still observed no spikes in hippocampal cells from control animals (Fig. 2e).

The viral vectors serve as indispensable tools in primate research as transgenic primate animals are very limited. Thus, we further tested whether the same viral vectors were able to reconstitute tTA in the primate brain. We injected same AAVs into primate visual cortex and performed patch-clamp recording with optogenetic stimuli (Fig. 2f). The fluorescence tag of effectors (shown in green) was only seen in brain slices with all three AAVs transfected. Arrows: co-labeled cells. f Summary of green fluorescence intensity (arbitrary units, arb. units) of HEK293T cells (Supplementary Fig. 3a) and PERL2–tTAN (Supplementary Fig. 4a). Amino acid sequence of intein-fused tTA fragments. g Percentage summary of cells labeled by HTG and tTAN in e (100 ± 0% co-labeled vs. 0 ± 0% reporter only; Mann-Whitney U test, \( p = 0.029, n = 4 \) FOV). h Examples showing fluorescence (AAV-TRE-HTG) after its injection into hippocampus and co-injections of AAVs (CaMKIIα promoter) for iTAN, iTAC, both or neither, respectively. i Examples showing fluorescence of iTAN (shown in magenta) and HTG reporter (shown in green) in hippocampal cells. Open arrows: reporter only. Filled arrows: co-labeled. j Summary of green fluorescence intensities in hippocampal neurons (Each group, \( n = 6 \) FOV, N = 3 animals). One-way ANOVA between groups: \( F_{(3,20)} = 20.57, P = 2.52 \times 10^{-6} \), Turkey's multiple comparisons: TRE-HTG + iTAC–tTAN group vs. others, \( ***P < 0.0001 \). k Percentage summary of cells labeled by reporter and iTAN (88.4 ± 1.3% co-labeled vs. 11.6 ± 1.3% reporter only; paired t-test, \( ***P = 7.95 \times 10^{-7}, n = 6 \) FOV, N = 3 animals). Scale bars: 200 μm (h), 20 μm (d, i), 5 μm (e). Data summary: mean ± SEM. Statistical tests: two sided. Source data are provided as a Source Data file.

IBIST-based cell types defined by genetic marker and neuronal connectivity. The genetic marker, neuronal connectivity and neuronal location constitute the major features to define the cell type. While neuronal locations are conveniently controlled by stereotaxic injections of viral vectors, we sought to test the compatibility of IBISIT-based tools when defining the other two types of features in various means. We first tested whether IBIST tools were able to combine features of anterograde labeling and promoter-based viral labeling. The hippocampal CA3 cells send collaterals to both pyramidal cells and interneurons in the CA1 area. We designed a strategy to combine AAV-serotype-1-mediated anterograde transsynaptic tracing with pyramidal-cell-specific fluorophore labeling in ventral CA1 (vCA1), aiming to specifically label pyramidal cells with direct inputs from dorsal CA3 (dCA3). We injected AAV1-EF1α-tTAN into the dorsal hippocampus and AAV-CaMKIIα–tTAC and reporter AAV-TRE-HTG into vCA1 (Supplementary Fig. 5a). Although AAV1-EF1α-tTAN travelled in both retrograde and anterograde directions and in turn labeled cells in DG, CA1 and subiculum, we observed specific labelling of CA3-targeted CA1 pyramidal cells owing to the local infection of AAV-CaMKIIα–tTAC in vCA1 (Supplementary Fig. 5b and c). As comparison, no GFP expression was detected in CA1 from the control animals. We then tested whether IBIST tools were able to combine features of anterograde labeling and Cre-dependent labeling in transgenic mice. Using AAV1-Flopo as an anterograde tracer, we specifically labeled Parvalbumin expressing (PV+) cells with direct inputs from dCA3 and combined injections of AAV-FRT-BFP–tTAN, AAV-DIO-tTAC and AAV-TRE-GCaMP6s in PV-ires-Cre mice (Supplementary Fig. 5d). As a result, we observed a specific labeling of CA3-targeted PV+ cells in vCA1 (Supplementary Fig. 5e and f). We further tested whether IBIST tools are able to combine features of retrograde rabies tracing and...
promoter-based viral labeling. We engineered tTAN-expressing rabies vectors and specifically labeled dCA3 neurons with TRE reporter (Supplementary Fig. 5g–i). We continued to test whether IBIST tools are able to combine features of both anterograde and retrograde connections. The nucleus of the reuniens (RE) is known to be a critical relay to transfer information from the medial prefrontal cortex (mPFC) to the hippocampus38. By local injections of anterograde AAV1-EF1α-tTAN into mPFC, retrograde AAVretro-CaMKIIα-tTAC into vCA1 and AAV-TRE-oChiEF-mCherry into RE respectively, we specifically labeled RE cells with direct inputs from mPFC and direct outputs to vCA1 (Supplementary Fig. 5j and k). No cells were labeled if either tTAN or tTAC component was omitted from injections (Supplementary Fig. 5k). These results demonstrated that IBIST-based tools are capable of defining cell types with the genetic marker and neural connectivity by various means using viral vectors and transgenic animals.

Fig. 2 The IBIST-based optogenetic excitation. a Scheme depicting AAV injections into hippocampus. b Examples showing mCherry fluorescence (shown in green) in hippocampus injected with AAV-TRE-oChiEF and AAVs (CaMKIIα promoter) of tTAN, tTAC or both, respectively. Scale bars: 200 µm. c Summary of fluorescent intensity in b. One-way ANOVA between groups (F(2,21) = 25.26, ****P = 2.58 × 10^-6). Turkey’s multiple comparisons revealed that the fluorescence is significantly higher in tTAN+tTAC group (tTAN vs. tTAN+tTAC, ****P = 3.07 × 10^-6; tTAC vs. tTAN+tTAC, ***P = 0.0002). d Examples showing whole-cell current-clamp recording from hippocampal cells in brain slices with blue LED light stimulation (blue line). All animals were injected with AAV-TRE-oChiEF and co-injected with AAVs (CaMKIIα promoter) of either tTAN, tTAC or both. e Summary of spike probabilities (prob.) in hippocampal cells evoked by blue LED light (3 mW or 17 mW) from animals injected with AAV-TRE-oChiEF and other CaMKIIα-specific AAVs: none, N = 1 animal; tTAN, N = 6 animals; tTAC, N = 2 animals; tTAN+tTAC, N = 4 animals. The recordings were from local neurons at the injected site. f Scheme depicting AAV injections into V1 of Macaca fascicularis. g, Examples showing mCherry fluorescence (shown in green) in V1 after injected with AAV-TRE-oChiEF-mCherry and CaMKIIα-specific AAVs of tTAN, tTAC or both. Scale bars: 10 µm. h, Summary of fluorescent intensity in g. Each group consists of brain slices obtained from corresponding injection site (n = 3 FOV/group). One-way ANOVA between groups (F(2,6) = 58.19, ***P = 0.0001) and Turkey’s multiple comparisons revealed that the fluorescence is significantly higher in tTAN+tTAC group (***P < 0.001 for all). i Examples showing whole-cell current-clamp recording in brain slices with blue LED light stimulation (blue line). All groups were injected with AAV-TRE-oChiEF and co-injected with CaMKIIα-specific AAVs of either tTAN and tTAC, or both. j Summary of spike probabilities evoked by wide-field blue LED light (3 mW) from animals injected with AAV-TRE-oChiEF and other AAVs. Data summary: mean ± SEM. Statistical tests: two sided. Source data are provided as a Source Data file.
Recent studies showed that different subgroups of vCA1 neurons exhibited distinct physiological functions. Thus, it is important to control cell-type specific fluorescence for Ca2+ imaging and optogenetic manipulation.

**IBIST-based Ca2+ imaging for cells with multiple projections.** To exploit the application of IBIST tools to label cells with multiple projections, we turned to ventral hippocampus where neuronal functions diversify by their downstream targets. As an example, the NAc projector contributed to drug-induced place preference and the lateral septum (LS) projector contributed to feeding. Interestingly, mPFC and amygdala projection cells exhibited distinct functions in fear extinction compared to single-projection cells. In line with this, we found that mPFC-amygdala-projectors exhibited different biophysical properties from the single-projectors. Recent studies showed that different subgroups of vCA1 neurons connected with distinct patterns of downstream areas and exhibited distinct physiological functions. Thus, it is important to control fluorescence in these CA1 cells with multi-projections by various means, achieve cell-type specific fluorescence and suffice for Ca2+ imaging and optogenetic manipulation.

**Fig. 3 The IBIST-based optogenetic inhibition.** a Scheme depicting AAV injections into hippocampus. b Examples showing GFP fluorescence in hippocampus after injected with AAV-TRE-NpHR and AAVs (CaMKIIα promoter) of tTAN, tTAC or both, respectively. Scale bars: 500 µm. c Summary of fluorescent intensity in b. One-way ANOVA revealed significant fluorescence differences between groups (F[2,23] = 123.1, ****P = 2.52 × 10⁻¹²) and Turkey’s multiple comparisons revealed that the fluorescence is significantly higher in tTAN+tTAC group than in others (****P < 0.0001 for all). N = 2 animals for each group. d Examples showing whole-cell current-clamp recording from hippocampal cells in brain slices upon current injections with 40 pA steps (first and second sweeps with prominent spikes; yellow with light; black without light). e Frequency of spikes in hippocampal cells in the absence (black) or presence (yellow) of 589 nm laser light (18 mW) from animals injected with AAV-TRE-NpHR and other CaMKIIα AAVs. The tTAN+tTAC animals showed a significant decrease in frequency of spikes after light stimulation (Wilcoxon matched-pairs signed rank test; first sweep, 8.3 ± 1.1 Hz vs. 1.8 ± 1.0 Hz with light; **P = 0.001; second sweep, 17.6 ± 2.3 Hz vs. 7.1 ± 2.5 Hz, ***P = 0.001; n = 12 cells, N = 2 animals). The tTAN animals, paired t-test; first sweep, 4.5 ± 0.7 Hz vs. 4.0 ± 1.1 Hz with light, P = 0.5414; second sweep, 10.8 ± 1.6 Hz vs. 9.5 ± 2.0 Hz with light; P = 0.21; n = 10 cells, N = 2 animals. The tTAC animals, paired t-test; first sweep, 7.7 ± 1.0 Hz vs. 6.5 ± 1.2 Hz with light, *P = 0.04; second sweep, 14.8 ± 1.3 Hz vs. 14.1 ± 1.5 Hz with light, P = 0.25; n = 11 cells, N = 3 animals. Data summary: mean ± SEM. Statistical analysis: two sided. Source data are provided as a Source Data file.
and interesting to investigate how different subgroups of vCA1 projection cells respond to external stimuli with distinct emotional valences. To address this question, we used IBIST tools to label vCA1 cells with triple features defined by two projection targets and pyramidal cell-specific CaMKIIα promoter. We injected AAVretro-CaMKIIα-tTAN and AAVretro-CaMKIIα-tTAC into one or two vCA1 downstream targets including amygdala, nucleus accumbens (NAC) and mPFC respectively, and injected AAV-TRE-GCaMP6s into vCA1 (Fig. 5a). We found that the double-projection cells were successfully labeled, which were not seen in control experiments with either AAVretro-CaMKIIα-tTAN or AAVretro-CaMKIIα-tTAC omitted (Fig. 5c). Using photometric recording from the vCA1 when animals underwent conditioned place preference (CPP) training and two foot-shock sessions (Fig. 4).

**Fig. 4 The IBIST-based Ca^{2+} recording for cells defined by genetic marker and neural connectivity.** a Scheme illustrating AAV injections to define cells by two features: anterograde transsynaptic tracing and CaMKIIα-specific fluorophore labeling. b Examples showing fluorescence of the ventral hippocampus infected by AAVs of Flpo, ITAN (magenta), tTAC and TRE-GCaMP6s (GCa., green) and co-injected with blue beads (magenta). Scale bars: 500 µm. c Summary of fluorescent intensity of GCaMP6s in b. One-way ANOVA between groups: F(2,33) = 66.19, ****P = 2.82 × 10^{-12}. Turkey’s multiple comparisons: tTAN vs. tTAN + tTAC, ****P = 4.91 × 10^{-11}; tTAC vs. tTAN+tTAC, ****P = 3.88 × 10^{-11}. N = 3 animals/group. d Scheme illustrating the open field test. e Box plot of the percentage time in the center zone and surround. Center vs. surround, 8.2 ± 1.0% vs. 91.8 ± 1.0%, paired t-test, ****P = 2.08 × 10^{-18}; N = 17 animals. f Examples of averaged traces of Ca^{2+} signals and animal speeds upon center entry (green) and exit (black) in the open field test. g Summary of normalized Ca^{2+} responses upon center entry and exit. One-way ANOVA between groups (ITAN, 0.3 ± 0.2%, N = 6 animals; tTAC, 0.4 ± 0.2%, N = 7 animals; tTAN+tTAC, 1.7 ± 0.8%, N = 4 animals; F(2,14) = 4.275, *P = 0.0356; Turkey’s multiple comparisons, *P < 0.05). h Scheme illustrating the foot shock test. i Examples of heatmaps and averaged traces of Ca^{2+} signals upon foot shocks (between dash lines.). j Summary of Ca^{2+} responses. One-way ANOVA between groups in first session (ITAN, 0.1 ± 0.2%, N = 6 animals; tTAC, 0.01 ± 0.01%, N = 7 animals; tTAN+tTAC, 3.9 ± 1.1%, N = 4 animals; F(2,14) = 21.17, ***P < 0.001) and second session (tTAN, 0.1 ± 0.1%, N = 6 animals; tTAC, 0.5 ± 0.2%, N = 7 animals; tTAN+tTAC, 4.3 ± 0.2%, N = 4 animals; F(2,14) = 141.3, ****P < 0.0001). Data summary: mean ± SEM. Statistical analysis: two sided. Box plots: whiskers (min/max), middle line (median), plus (mean), box (25/75 percentile). Source data are provided as a Source Data file.
(Fig. 5b, Supplementary Fig. 3c, Supplementary Fig. 8), we observed that distinct Ca$^{2+}$ activity was evoked in different types of vCA1 projection cells by the water reward, sucrose reward and foot shock (Fig. 5d–g). No detectable Ca$^{2+}$ signals were recorded in the control animals (Fig. 5d–g). For the sucrose reward, the NAc-projecting and NAc-Amy-projecting vCA1 cells responded strongly and all types of vCA1 cells with mPFC connections responded weakly, if not none (Fig. 5h and i). Notably, water reward elicited specific activations of NAc-projecting and NAc-Amy-projecting vCA1 cells. Interestingly, the omission of water...
Fig. 5 The IBIST-based Ca\(^{2+}\) recording for cells defined by multiple projections. a Scheme illustrating AAV injections to define cells by three features: dual retrograde tracings and pyramidal-cell-specific (CaMKII\(\alpha\) promoter) Ca\(^{2+}\) recording. b Schematic showing the behavioral design of water CPP and shock sessions. c Examples of fluorescence images in vCA1 (top, injected with AAV-TRE-GCaMP6s, shown in green) and downstream regions (bottom). AAVretro-tTAN (shown in magenta) and AAVretro-tTAC were injected into two downstream regions, co-injected into one region, or omitted. All the AAVs were co-injected with blue beads (shown in magenta). The arrows indicate optical fiber tracks. mPFC: medial prefrontal cortex. NAc: nucleus accumbens. Amy: amygdala. Scale bars: 500 µm. d–g Examples of heatmaps and averaged traces of Ca\(^{2+}\) signals upon water reward (d, blue), sucrose reward (e, yellow), water omission (f, black) and foot shock (g, red). h Summary of Ca\(^{2+}\) responses in vCA1 depending on targeted areas. The significance for Ca\(^{2+}\) response in each group is determined by a single sample t-test (HO: \(\mu = 0\)), \(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\). i Polar Plots of Ca\(^{2+}\) responses to foot shocks and water and sucrose rewards normalized by the max response in each type of emotional stimuli. Data summary: mean ± SEM, details in Supplementary Table 2. Statistical analysis: two sided, details in Supplementary Table 3. Source data are provided as a Source Data file.

reward elicited noticeable inhibitory signals in NAc-projecting and Amy-projecting vCA1 cells (Fig. 5h and i). For aversive foot shocks, vCA1 cells were activated in a target-dependent manner (Fig. 5h and i). The foot shock-evoked Ca\(^{2+}\) activity was strong in Amy-projecting and NAc-projecting cells, weak in mPFC-projecting cells and moderate in the double-projecting cells (see detailed summary and statistics in Supplementary Tables 2 and 3).

Among the double-projecting vCA1 cells, mPFC-NAc-projectors preferentially responded to foot shocks but not sucrose rewards, whereas NAc-Amy-projectors strongly responded to both (Fig. 5i). These results promoted us to examine their causal roles in the emotion processing. To address this, we employed the same strategies of AAV injections and selectively expressed optogenetic opsins in mPFC-NAc-projecting and NAc-Amy-projecting vCA1 cells, respectively. Using a real-time place preference paradigm, we performed bidirectional manipulations of those vCA1 cells when animals were exploring in the context. We found that activating mPFC-NAc-projectors led to a negative preference for context paired with light stimulation, while inhibiting mPFC-NAc-projectors led to a positive preference for stimulated context (Supplementary Fig. 3d and e and Supplementary Fig. 9). In contrast, there were no significant behavioral effects by manipulating NAc-Amy-projectors (Supplementary Fig. 9). These optogenetic results, together with photometric indicators12, but new combinations of controllers would still require sophisticated construct design, which may suffer from insufficient gene expression in some conditions. The IBIST tools could help to resolve these concerns by intersectional synthesis of controller (tTA) which does not need that high expression level as the effector does. Accordingly, it is more straightforward to design constructs for IBIST effectors and utilize a large library of ready-to-use common effectors, and more feasible to achieve sufficient gene expression for opsins and Ca\(^{2+}\) indicators. In our work, we have validated TRE-based viral effectors of oChIEF (ChR2 variant)29, NpHR30, GCaMP6s31 and HTG (histoneGFP-TVA-Glycoprotein)32, which can be broadly utilized in various conditions with viral serotypes, promoters and transgenic animals. As the design of splitting tTA is also applicable for splitting tTA (Supplementary Fig. 1b–f), there could be more adaptable options for intersectional control in various experimental purposes.

Finally, we tested whether the IBITS tools could label specific types of hippocampal cells based on more downstream targets. We injected AAV-TRE-GCaMP6s into vCA1 and AAVs (AAVretro) of EF1α-FRT-tTAN, EF1α-Flpo, EF1α-DIO-tTAC, CaMKIIα-Cre into NAc, mPFC, LS and amygdala, respectively. As expected, we observed prominent fluorescence of GCaMP6s in vCA1, which was absent in controls where either tTAN or tTAC was omitted from injections (Fig. 6a and b, Supplementary Fig. 3f). Accordingly, we recorded robust Ca\(^{2+}\) signals from vCA1 upon foot shock (Fig. 6c and d). Interestingly, these Ca\(^{2+}\) signals exhibited adaption over trials (first session, first 3 trials vs. last 3 trials, 2.1 ± 0.7% vs. 1.2 ± 0.4%; paired t-test, \(P = 0.0349\)). Therefore, the IBIST-based Ca\(^{2+}\) imaging is applicable for cell types defined by five features including one promoter and four different projection targets. Taken together, the selective recording of Ca\(^{2+}\) activity in various types of projection cells in vCA1 revealed distinct activity profiles in response to aversive and appetitive stimuli, highlighting the importance to define cell types based on multiple projection targets.

Discussion

Our work demonstrated that the IBIST can be efficiently constituted in various ways depending on promoters, brain regions, neuronal connections and site-specific recombinases. The IBIST system allows for more intersectional features than prior tools by multiplexing the number of fragmented tTA (Supplementary Fig. 10) and enables technological features such as ease to design the IBIST construct, reconstitution of tTA at the protein level, common TRE-effectors for various experimental strategies, reversible control by doxycycline, compatibility with Cre and Flp transgenic animals and compatibility with rabies-based transsynaptic tracing.

While intersectionally labeling neurons by fluorophores is widely used, it is still challenging to achieve the specificity and sufficiency for optogenetic manipulations and Ca\(^{2+}\) imaging in vivo. Pioneering works developed intersectional effectors for opsins and Ca\(^{2+}\) indicators12, but new combinations of controllers would still require sophisticated construct design, which may suffer from insufficient gene expression in some conditions. The IBIST tools could help to resolve these concerns by intersectional synthesis of controller (tTA) which does not need that high expression level as the effector does. Accordingly, it is more straightforward to design constructs for IBIST effectors and utilize a large library of ready-to-use common effectors, and more feasible to achieve sufficient gene expression for opsins and Ca\(^{2+}\) indicators. In our work, we have validated TRE-based viral effectors of oChIEF (ChR2 variant)29, NpHR30, GCaMP6s31 and HTG (histoneGFP-TVA-Glycoprotein)32, which can be broadly utilized in various conditions with viral serotypes, promoters and transgenic animals. As the design of splitting tTA is also applicable for splitting tTA (Supplementary Fig. 1b–f), there could be more applicable options for intersectional control in various experimental purposes.
that the NAc projectors in vCA1 responded to both sucrose rewards and aversive foot shocks (Fig. 5h and i). This is consistent with the notion that the NAc contributes to both appetitive and fearful motivations51,52. Interestingly, the NAc-mPFC-projectors preferentially responded to foot shocks (Fig. 5h and i) and causally regulated real-time place preference (Supplementary Fig. 9). As a comparison, the NAc-Amy-projectors may function as salience detectors by responding to all rewards and aversive shocks (Fig. 5i).

Thus, our study using the IBIST system outlines an emerging picture for the projection-pattern-dependent emotional processing in hippocampal neurons (Fig. 5). Future studies with IBIST tools could further reveal specific physiological functions of more types of multi-area projectors46.

Our IBIST system is not only compatible with increasing mouse driver lines with Cre and Flp for most major cell types in the brain4,12, but also complementary for the existing intersectional tools such as Cre- and Flp-dependent constructs created by pioneering works4,12. Intersectional control with more than five features (Supplementary Fig. 10) is possible if the split tTA fragments are built upon those Cre- and Flp-dependent constructs12, antero- and retrograde tracing or other promoter and enhancer-dependent constructs53,54. Intersectional ON and
OFF is also possible by the IBIST tools if one of the tTA fragments is flanked by palindromic sites recognized by site-specific recombines (e.g. Cre-loxp site-specific recombination system). As the intein-split strategy could be adapted for other controllers such as Cre, it would be possible to devise two orthogonal strategies in the same animal by independent and parallel synthesis of two single controllers in intersectional manners, respectively. Given that the IBIST tools can be entirely implemented in viral vectors including AAV and rabies vectors, our system not only offers experimental conveniences but also holds great promise as intersectional tools for animal species with limited transgenic resources such as primates.

Methods

Plasmids. All vectors in this study were constructed using EasyGeno Assembly Cloning kit (TIANGEN, Beijing) according to the manual, except for the specially noted plasmids. Briefly, the backbone plasmid was linearized and the insert fragments were amplified through normal polymerase chain reactions. All DNA products were separated in agarose gel to confirm sizes and following purified using TIANgel Midi Purification Kit (TIANGEN). The mixture of 0.01 μM of backbone and 0.03 μM of each insert fragments were delivered for transformation in Stable3 competent bacteria after 30 minutes incubation in 50 °C. All the plasmids are verified by sequencing. The intein sequence for N-terminal part in this study is: accgcagcagcactcagcaacccggctactgcctggacctgaagacccaggtgcagaccccccagggcatgaaggagatcagcaacatc-.

Reconstitution of tTA in vitro. The HEK293T cells were divided into four groups and transfected with plasmids by Lipofectamine 2000 (11668-027, Invitrogen). The four groups (tTAN, tTAC, both or neither) of plasmids for transfection consist of reporter pAAV-TRE-HTG and intein-split tTA plasmids with EF1a promoter. The fluorescence images for HEK293T cells were acquired at 48 h and 72 h after the transfection.

Reconstitution of rTα in vitro. The HEK293T cells were divided into three groups and transfected with plasmids of pAAV-TRE-HTG and pAAV-EF1a-rtTα by Lipofectamine 2000 (11668-027, Invitrogen). The culture medium was replaced with doxycycline-containing medium (100 ng/ml, MedChemExpress, HY-N0565B) 6 h after the transfection. Eighteen hours later, the culture medium was replaced with RV-rTα-containing medium (rabies-containing supernatant from B7GG cells) for one group of cells and incubated for 6 h, and then replaced with fresh medium again (with doxycycline 100 ng/ml). For the control groups, pAAV-EF1a-rtTα plasmid or RV-rTα-containing medium was omitted, respectively. The fluorescence images for HEK293T cells were acquired at 48 h and 72 h after the transfection.

Quantification for fluorescence and co-labeling in vitro. The fluorescence images for HEK293T cells were acquired by Olympus microscope (CX5X3 with X-CITE LED, 10X objective, same exposure settings for the same type of experiment). One FOV image per culture plate was taken for quantification (green for TRE-HTG and red for TRE-mCherry and blue for TAN-BFP). The mean fluorescence intensity in the whole FOV was measured and normalized by the area of FOV in ImageJ. The reporter cells and co-labeled cells were counted in Imaris (v7.4.2, Bitplane).

Animals. Animals were housed under a 12 h light/dark cycle with temperature controlled between 21 and 23 °C and humidity-controlled between 40–70%. Animals were provided with food and water ad libitum in the Institute of Neuroscience animal facility (mice and cynomolgus monkey). All animal procedures were performed in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Neuroscience (CAS Center for Excellence in Brain Science and Intelligence Technology), Chinese Academy of Sciences. Wild-type C57BL/6J (Slac Laboratory Animal, Shanghai), PV-ires-Cre10 and SOM-ires-Cre11 mice were used. All the experiments aden used in the study were done with male mice (C57BL/6J) or female cynomolgus monkey (Macaca fascicularis, 4.2 kg, 13 years old) was obtained from nonhuman primate facility of Institute of Neuroscience after its full use in reproductive research.

Stereotactic injections. Mice were anesthetized with isoflurane (induction 5%, maintenance 2%, RWD510P, China) and fixed in a stereotactic frame (RWD, China). Before the surgery, local anesthetics (Lidocaine, H37022147, Shandong, Huala Pharmaceutical) were administered. A feedback-controlled heating pad (FHC) was used to maintain the body temperature at 35 °C. Glass pipettes (tip diameter 10–20 μm) connected to a Picospritzer III (Parker Hannifin Corporation) were filled with virus solutions and injected at following coordinates (posterior to Bregma, AP; lateral to the midline, LAT; below the brain surface, DV; in mm): mpFC: AP +1.98, LAT ± 0.6, DV -1.62, angle 10°; Amygdala: AP -1.2, LAT ± 1.0, DV -4.33; NAc: AP +1.0, LAT ± 1.0, DV -4.2; RE: AP -0.3, LAT ± 1.3, DV -4.5, angle 18°; dCA3: AP -1.5, LAT ± 2.1, DV -1.7; vCA1: AP -3.2, LAT ± 3.4, DV -1.5; angle 10°. To identify the injection site, the virus solution was typically mixed at 1:500 with blue beads (fluorescent polymer microspheres, Thermo Scientific).

Local injections in hippocampus. A mixture (300 nL) of AAV2/9-TRE-HTG, AAV2/9-CaMKIIa-tTAC and AAV2/9-CaMKIIa-tTAC-BFP (ratio 1:1:1) were injected into dCA3 (AP -1.5, LAT ± 2.1, DV -1.7). In control groups, one of these three viruses (AAV2/9-TRE-HTG, AAV2/9-CaMKIIa-tTAC and AAV2/9-CaMKIIa-tTAC-BFP) was omitted for injections (ratio 1:1 for two AAVs). In negative control group, only AAV2/9-TRE-HTG (300 nL) was injected into dCA3.

For electrophysiological experiments combined with optogenetics, same AAVs were injected into vCA1 (AP -3.2, LAT ± 3.4, DV -1.5), except that AAV2/9-TRE-oChiEF-mCherry or AAV2/9-TRE-NpHR-EGFP was used instead of AAV2/9-TRE-HTG.

Cell culture. HEK293T (SCSP-502; Cell bank, Shanghai) and BG77 cells (provided by E.M. Callaway) were cultured with 10% (v/v) FBS/DMEM at 5% CO2 and 37 °C.
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Images from cell cultures were captured using a Nikon Eclipse 80i fluorescence microscope. Images from brain slices were acquired by an Olympus VS120 virtual microscopy system (VS120 camera with VS1000 computer) and Genietic software. All setup parameters were consistent with the acquisition settings for one experiment. For analysis of endogenous fluorescence intensities, hippocampal contours were drawn in Fiji. Mean fluorescence value (arbitrary units) of hippocampal slices were measured after background subtraction and normalized by the area size. Typically, 2–10 slices (SOV) per brain were analyzed. For co-labeling analysis, confocal images were imported into ImageJ (v7.4.2, Biopta) for cell counting.

Western blot. The transfected HEK293T cells in 24-well plates were lysated with 200 μL protein lysis buffer (50 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 0.1% Bromophenol blue, 10% glycerol, 1% β-Mercaptoethanol), denatured for 10 min at 95 °C before loading. Using a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel to separate proteins, samples were transferred to a PVDF membrane (Millipore, Spectrum IPVH00101). Blots were blocked with blocking solution (1% BSA, 0.1% Tween-20 in PBS) for 1 h at room temperature and then incubated in the corresponding primary antibodies (via horseradish peroxidase-conjugated 1:5000, Sigma). All membranes were washed three times in PBS. The membranes were incubated in peroxidase-conjugated secondary antibody (1:10,000, Zymed, A0545) for 1 h at room temperature. Development was performed with ECL chemiluminescence reagents (GE Healthcare). Bands were quantified with ImageJ software.

Slices were cut from an injection site and immediately transferred to ice-cold NMDG-based ACSF. For optogenetic stimuli, the blue light pulse (480 nm) from a LED engine (Sola SE5-20) was delivered through optical fibers positioned above the slice brain tissue. The power at tip of fiber was 18 mW with ~1 mm distance to the recording site. For the cells expressing TRE-Effectors, we only recorded fluorescent cells expressing effectors target unless otherwise stated. The slice data were acquired by pCLAMP10 (Molecular Device) and analyzed by Clampfit (v10.7).

To obtain the primate brain slices, the cynomolgus monkey was maintained under general anesthesia. The brain was excised after perfusion with 0.9% saline and 4% paraformaldehyde, respectively. The brain was stored in 30% sucrose overnight at 4 °C and then cut into 50 μm thick coronal slices using a vibratome (Leica). Slices were stored in 4°C PBS with 10% sucrose.

For slice physiology, slices were placed on a slice warmer with a temperature of 32 °C and monitored with a headstage connected to a multiclamp 700B amplifier and a Digidata 1440A A/D converter (Molecular Devices). Voltage-clamp recording was conducted at holding potential of −70 mV. Current-clamp recording was conducted at membrane potential −70 mV. For optogenetic stimuli, the blue light pulse (480 nm) from a LED engine (Sola SE5-20) was delivered through optical fibers positioned above the slice brain tissue. The power at tip of fiber was 18 mW with ~1 mm distance to the recording site. For the cells expressing TRE-Effectors, we only recorded fluorescent cells expressing effectors target unless otherwise stated. The slice data were acquired by pCLAMP10 (Molecular Device) and analyzed by Clampfit (v10.7).

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open field test, animals were placed in an open arena (40 x 40 x 45 cm) and allowed to explore for 15 min.

For conditioned place preference (CPP), the apparatus consists of two square contexts (25 x 25 x 30 cm, connected via a bridge 7 x 10 x 30 cm) with identical pump port on the opposite site but distinct walls and odors. Animals were water restricted and maintained at 90% body weight two days before experiments. During habituation, mice were habituated to the two connected contexts for 15 min without water reward. During the training sessions (day 2–4), mice were first confined in the rewarding context for 10 min (2 µl drop upon licking, one drop per 5 s), and then confined in nonrewarding context for another 10 min (no water). On day 5, animals were allowed to freely explore the two contexts for 15 min without reward (CPP memory retrieval) and then for another 15 min with water reward available. On day 6, animals went through the same procedure as day 5 except that setup orientation was reversed and the reward was changed to sucrose. The CPP score is calculated as (A–B)/(A + B) where A and B are the time that the animal has stayed in the two contexts respectively. For foot shock sessions, animals were simultaneously by a multi-emission signals and behavioral videos (640 × 480 pixels at 30 Hz) were captured. The Ca2+ signal is calculated as (A + B)/(A + B + C) where A and B are the time that the animal entered and stayed in the stimulation context and was turned off once animal left the stimulation context. The laser intensity was 19–20 mW at the tip of the optic fiber and was controlled by a master-8 (A.M.P.L. Israel). The preference score for the stimulation context is calculated as same as CPP score.

For photometry in behaving animals, the optic fibers were connected with a 665 nm red LED light source (Cinelex, Plexon Inc). The excitation light at the tip of fiber was adjusted to be 14–16 µW (setting 10–12 in the LED controller). Emission signals and behavioral videos (640 × 480 pixels at 30 Hz) were captured simultaneously by a multifiber photometry recording system (Cinelex, Plexon Inc.). The animal trajectories were analyzed and plotted by the Cinelex GUI. Raw data of emission signals in photometry were acquired and directly extracted for analysis in MATLAB (MathWorks) without any filtering. The area (mean) of the Ca2+ signals in a defined window were quantified for the event-related responses. The baseline and response periods were divided into equal numbers of bins. The Ca2+ signals in each bin were averaged. For non-significant Ca2+ response (Mann Whitney U Test), the amplitude was set as zero. As the maximal water/sucrose reward was one drop per 5 s, the shortest trial for water/sucrose reward is 5 s long. Thus, the 0.5 s period before water/sucrose reward onset was computed as the baseline. Based on the decay of Ca2+ signals, the 3 s period after sucrose delivery and the 2 s period after water delivery were computed as the response. For the foot shocks, the 2 s before foot shock onset was computed as the baseline and the 10 s period after stimulus onset was computed as the response, except 5 s response period in Fig. 6 which showed faster decay. For polar plot, the averaged response for each type of projection cells was computed in each animal. Then the responses were averaged across animals for each projection type and normalized by the maximum response in all projection types. To analyze photometric signals when the animal is moving to the center of the open field (the locomotion is continuous), the filtered Ca2+ signal from the Cinelex was used (smoothed with an exponentially weighted moving average, τau = 0.2 s). The center area is defined as a rectangle with 40% of width and length of the open arena. In each center entry trial, the 4 s before the center entry was computed as the baseline and 2 s after the entry was computed as the signal changes related to the center entry. The same time setting also applies for each exit trial. Short trials (<4 s) were not included for analysis.

Statistics and reproducibility. Mean values are accompanied by the standard error (mean ± SEM). The null hypothesis of normal distribution was rejected, non-parametric statistical tests were used. Mann-Whitney U Test, Wilcoxon matched-paired signed rank test, two-sided unpaired or paired t-test, and one-way ANOVA test were used to test for statistical significance. Statistical parameters including the exact value of n, precision measures (mean ± SEM) and statistical significance are reported in the text and in the figure legends (as additional sections). The significance threshold was placed at α = 0.05 (n.s., P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001). *P ≤ 0.05 was selected as a significance level.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Source data are provided with this paper and publicly available at the following repository: https://github.com/XuChunLab/IBIST. The plasmids created in this study are available on Addgene (#172119–172127). Source data are provided with this paper.

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Code availability. The codes used for analysis in this manuscript are available from the following repository: https://github.com/XuChunLab/IBIST.

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**Author contributions**

H.-S.C., X.-L.Z., R.-R.Y., G.L. and C.X. conceived the project. G.L. and C.X. supervised the work in the study. X.-L.Z. designed and tested DNA constructs of split controller, performed Western blot and analyzed the results. R.-R.Y. generated the rabies vectors, performed rabies-related experiments and analyzed the results. H.-S.C., G.-L.W., R.-R.Y., N.Z. and X.-Y.Z. injected the AAV vectors, performed the histology and confocal imaging and analyzed the results. G.-L.W. performed the electrophysiological recording from acute brain slices and analyzed the results. H.-S.C. and S.Q. performed the photometric recording and analyzed the results. Y.-F.X., L.-J.Z. and Z.-M.S. performed the surgery in the primate. D.-Y.W. and X.-H.X. performed AAV injection and the histology and analyzed the results. H.-S.C. and C.X. wrote the manuscript with inputs from all authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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