Capturing and Manipulating Activated Neuronal Ensembles with CANE Delineates a Hypothalamic Social-Fear Circuit

**Highlights**

- CANE is a new technology for capturing activated (Fos+) neuronal ensembles
- CANE tags Fos+ neurons with high specificity, temporal precision, and efficiency
- Hypothalamic neurons eliciting social-fear behaviors are identified using CANE
- CANE-based mapping reveals a highly distributed and recurrent social-fear circuit

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**In Brief**

Sakurai et al. developed a new technology (CANE) to capture and manipulate recently activated (Fos+) neurons in the mouse brain. CANE is applied to delineate the causal functions and connectivity of hypothalamic neurons activated by a social-fear experience.
Capturing and Manipulating Activated Neuronal Ensembles with CANE Delineates a Hypothalamic Social-Fear Circuit

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SUMMARY

We developed a technology (capturing activated neuronal ensembles [CANE]) to label, manipulate, and transsynaptically trace neural circuits that are transiently activated in behavioral contexts with high efficiency and temporal precision. CANE consists of a knockin mouse and engineered viruses designed to specifically infect activated neurons. Using CANE, we selectively labeled neurons that were activated by either fearful or aggressive social encounters in a hypothalamic subnucleus previously known as a locus for aggression, and discovered that social-fear and aggression neurons are intermixed but largely distinct. Optogenetic stimulation of CANE-captured social-fear neurons (SFNs) is sufficient to evoke fear-like behaviors in normal social contexts, whereas silencing SFNs resulted in reduced social avoidance. CANE-based mapping of axonal projections and presynaptic inputs to SFNs further revealed a highly distributed and recurrent neural network. CANE is a broadly applicable technology for dissecting causality and connectivity of spatially intermingled but functionally distinct ensembles.

INTRODUCTION

The immediately early gene (IEG) Fos has been widely used as a reliable marker for activated neurons in response to various sensory stimuli or in different behaviors (Morgan and Curran, 1989). Previous studies also identified brain regions in which brief behaviors of opposite valences activated spatially intermingled populations of Fos+ neurons (Johnson et al., 2010; Kollack-Walker and Newman, 1995; Kovalc, 1998; Lin et al., 2011; Veening et al., 2005; Wu et al., 2014; Xu et al., 2014). Thus, tools that can determine whether the same or different ensembles of neurons are activated and express Fos in different contexts or behaviors, and can manipulate the activated neurons to determine their causal functions are highly desired.

Different Fos-based methods have been used to express transgenes in active neurons, including Fos-GFP (Barth et al., 2004), Fos-tTA transgenic mouse (Liu et al., 2012; Reijmers et al., 2007), and the Fos-CreERT2 mice (Guenthner et al., 2013). Fos-GFP can be used to visualize Fos+ neurons but cannot be used for functional manipulations. The Fos-tTA mouse is a transgenic but not a knockin line; therefore, it cannot fully recapitulate endogenous Fos expression patterns. Furthermore, the Fos-tTA method has high background expressions (Glazewski et al., 2001) and depends on 1–2 days of doxycycline withdrawal, followed by tagging neurons and then reapplication of doxycycline, and thus has a long time window for labeling Fos+ neurons, which include neurons non-specifically activated during the period (Liu et al., 2012; Reijmers et al., 2007). Fos-CreERT2 is a null allele that disrupts the endogenous Fos gene. It was reported that Fos<sup>−/−</sup> heterozygous mice have haploinsufficiency neural phenotypes (Deng et al., 1999; Watanabe et al., 1996). Additionally, tamoxifen or 4-hydroxy tamoxifen is required to activate CreER (Denny et al., 2014; Guenthner et al., 2013). Once inside the cell, tamoxifen can remain active for 24 hr such that the permissive temporal window for tamoxifen-induction-based labeling is longer than the behaviors under investigation. Furthermore, a single tamoxifen injection typically only activates a fraction of CreER enzymes; thus, the Fos-CreERT2 strategy may result in stochastic labeling of small subsets of activated neurons.

We therefore set out to develop a new technology to capture Fos+ neurons with the following critical features. First, the method must be temporally precise to effectively capture Fos+ neurons that are induced by transient bouts of neuronal activity following, for example, a brief behavioral encounter. Second, the method must be highly specific (low background) and efficient. Third, the method can drive gene expression in captured Fos+ neurons, enabling one to manipulate their activities to establish their causal functions. Finally, the method should allow connectivity mapping, including transsynaptic tracing of inputs onto captured Fos+ neurons. We came up with a strategy that included a knockin mouse, pseudotyped lentiviruses (LVs), and pseudotyped rabies viruses (RVs). We named this technology “CANE” for capturing activated neuronal ensembles with engineered mice and viruses. Below, we describe the design and validation of CANE, and its application in delineating a hypothalamic social-fear circuit.
RESULTS

The Design of the CANE Technology

To achieve both anatomical and temporal specificity in capturing behaviorally activated Fos+ neurons, we conceived a lock-and-key strategy requiring an engineered mouse and pseudotyped viruses (Figure 1). We developed a knockin mouse (Fos-2A-dsTVA) in which activity-induced Fos expression triggers co-expression of a destabilized TVA (dsTVA) receptor (Figure 1). TVA is an avian-specific receptor that is not present in the mouse genome (Barnard et al., 2006; Barnard and Young, 2003), and 2A is a self-processing peptide that enables Fos and dsTVA to be co-translated (de Felipe et al., 2006). To construct dsTVA, the C terminus of the TVA receptor is fused with a "PEST" sequence that targets the protein for degradation (Li et al., 1998). See Figure S1 and Experimental Procedures for a detailed description. This knockin line is designed with the expectation that in the freely behaving Fos-2A-dsTVA mouse (hereafter referred to as FosTVA), activated neurons will transiently express dsTVA with a time course similar to that of Fos itself. TVA is an exquisitely specific receptor for the envelope protein of the avian sarcoma and leukosis virus (EnvA) (Barnard and Young, 2003). Therefore, EnvA-coated (pseudotyped) viruses can be introduced into the brain of FosTVA mice within a short time window that parallels the expression of dsTVA/Fos to specifically infect those recently activated neurons (Figure 1). More specifically, LVs and deficient monosynaptic RVs can be pseudotyped with the EnvA envelope protein (EnvA-LV or EnvA-RV, respectively) to exploit this transient susceptibility of activated neurons to viral infection (Figure 1). Because LVs are non-toxic and enable stable transgene expression, they are ideal for introducing and persistently expressing desired transgenes in transiently Fos+ neurons (Figure 1), whereas the monosynaptic RVs (Callaway, 2008; Wickersham et al., 2007) can be used for transsynaptic tracing of presynaptic inputs onto Fos+ neurons. We named this technology CANE for capturing activated neuronal ensembles.

Rationale to Use the Hypothalamic Neurons Activated by the Resident-Intruder Behavior as a Platform for Testing the CANE

CANE is designed to use engineered viruses to infect activated neurons in the FosTVA mice. We need to demonstrate that this method is specific and efficient. To do this, we will need to use CANE to capture neurons activated by the first behavior and drive expression of a transgene such as a fluorescent protein in these neurons. After allowing more than 10 days to 3 weeks for sufficient expression of the transgene, mice will be asked to perform the behavior a second time to induce Fos expression (Figure 2A, scheme). Showing that captured neurons following one behavioral epoch overlap highly with Fos+ neurons following a second behavior would provide a stringent validation of this technology’s efficiency and specificity.

However, rarely had any previous studies examined whether the same populations of neurons that express Fos following a brief, one-time behavior were reactivated and reexpress Fos weeks later by the “same” behavior. Furthermore, no behaviors performed at different times can be identical. Thus, to minimize the inevitable behavioral variability, we tested CANE using innate behaviors that are thought to involve genetically hardwired hypothalamic neurons and are therefore more stereotyped (Dubic and Kimchi, 2007; Manoli et al., 2013). In the rodent male-male resident-intruder assay, an aggressive male resident attacks an intruder evoking a highly stereotyped behavior in the intruder, which includes retreating to a corner and avoiding the resident (see Movie S1).

Previous studies showed the presence of Fos+ neurons in the ventromedial hypothalamus ventrolateral division, VMHvl, in both the intruder and the resident after a brief encounter (Kolack-Walker et al., 1997; Lin et al., 2011; Silva et al., 2013). We also observed that brief (10 min or less) male-male encounters, in which the intruder was attacked 10–15 times by the resident, consistently induced Fos expression in similar numbers of neurons in all intruders examined (262 ± 20 Fos+ neurons; n = 9 intruders). We called these neurons social-fear neurons (SFNs) (Figure S2). Similarly, the encounters consistently induced Fos expression in 138 ± 14 VMHvl neurons in resident mice (n = 9 residents, aggression neurons) (Figure S2). The consistent numbers of Fos+ SFNs and aggression neurons induced by a highly stereotyped behavior suggest that these neurons are likely reactivated by repeated social-fear or aggression behavior, and therefore ideal for testing the CANE technology.

dsTVA Tracks a Fos Expression Pattern in FosTVA Mice

For CANE to work, the expression of dsTVA needs to mimic that of Fos. We used male FosTVA mice as intruders, where they were attacked 10–15 times over 10 min or less. The mice were returned to their home cage and then killed either immediately (0 hr) or 1, 2, or 6 hr later (Figures 2B and 2C). Immediately following the social-fear experience (0 hr), minimal levels of
Fos+ or TVA+ signals were observed in ventromedial hypothalamus (VMH) of the intruder, indicating that background expression of TVA is very low (Figure 2C). At 1 hr after the encounter, moderate Fos and TVA expression was detected in a column of cells in VMHvl, as well as in a few cells scattered in the dorsomedial and central subdivision of VMH (VMHdm,c) (Figure 2C). Both Fos and TVA expression reached peak levels 2 hr after the encounter (68% ± 2% Fos+ neurons express TVA; n = 4). The fewer number of TVA+ cells compared to Fos+ neurons was likely due to the detection limitation of TVA antibody. By 6 hr, both Fos and TVA expression levels were diminished. Most importantly, TVA staining always co-localized with strong Fos-expressing neurons and the anti-TVA signal in the cell membrane surrounding the Fos+ nucleus (Figure 2C, inset).

Our analyses establish that TVA expression mimics the spatio-temporal pattern of Fos expression in FosTVA mice, which is a critical criterion necessary for selectively tagging neurons that transiently express Fos.

Developing a Pseudotyped Lentivirus—CANE-LV-Cre—that Selectively Infects Fos+ Neurons

To efficiently and selectively infect neurons that express Fos/TVA, we generated a lenti-hSyn-Cre (synapsin promoter driving Cre recombinase expression) construct and packaged pseudotyped LVs with either the wild-type EnvA coat protein, or with several mutated forms of the EnvA (EnvA<sup>M4</sup>, EnvA<sup>M5</sup>, and EnvA<sup>M21</sup>) protein that have different degrees of reduced binding affinities to TVA (Rong et al., 1997) (see Experimental Procedures). We generated multiple mutated-EnvA proteins for pseudotyping LVs because the extremely high affinity of the wild-type EnvA protein for the TVA receptor may result in background infection of neurons even if they express Fos/TVA at very low levels, whereas mutated-EnvA with reduced affinity will result in the infection of only those neurons with high-level TVA/Fos.

To test these different pseudotyped viruses, we crossed FosTVA mice with Rosa-flox-STOP-tdTomato (Ai14) reporter mice (Madisen et al., 2010). Fos<sup>TVA</sup>; Ai14 mice were subjected to a brief intruder social-fear experience (experimental group) or placed in a novel cage (control group), and then injected 1–2 hr later with 0.5 μL of pseudotyped LV-Cre into VMHvl. Ten to 14 days later, we examined Cre-induced tomato expression. Notably, only the intruder mice injected with the mutated EnvA<sup>M21</sup>-coated LVs exhibited highly selective infection of VMHvl neurons (putative SFNs), with no background infection in the control mice (Figure S3) (for more details, see later). In contrast, the wild-type EnvA, as well as EnvA<sup>M4</sup>- and EnvA<sup>M5</sup>-coated LVs all exhibited varying degrees of background infection even in control mice likely due to high-affinity binding of the viral coat to neurons with low basal levels of Fos/TVA expression (Figure S3). Therefore, all subsequent experiments were performed with EnvA<sup>M21</sup>-coated LVs. We named the EnvA<sup>M21</sup>-coated LV “CANE-LV.”

CANE-LV-Cre Selectively Captures Hypothalamic SFNs

We next determined whether CANE-captured SFNs in VMHvl overlapped with Fos+ SFNs induced by a second intruder experience. We injected CANE-LV-Cre after the first intruder experience in Fos<sup>TVA</sup>; Ai14 mice, and 10–14 days later, the intruder...
mice were attacked by the same aggressive resident for inducing Fos expression (Figure 3A). Despite the fact that no two resident-intruder encounters could be perfectly identical, we found extensive overlap between CANE+ (red) and Fos+ neurons (green) (Figure 3C). Again, there was minimal background infection in control mice (Figure 3B). With injection of 0.5 $\mu$L of CANE-LV-Cre (5 $\times$ 10^6 infectious units [ifu]/ml), we captured 131 ± 8 neurons (CANE+, about 50% of the number of Fos+ neurons), and 63% ± 3% of captured neurons were Fos+ (n = 9; Figure 3G). Thus, the second encounter reactivated many (>60%) of the same cells excited by the first social-fear behavior. Concentrated higher titer CANE-LVs (1 $\times$ 10^8 ifu/ml) could capture more neurons (200–240 neurons; see later result in Figure 7B). Additionally, we tested the capturing efficiency at different time points (Figure S4). The best capturing result was obtained with injection at 1.5–2.0 hr after the behavior, whereas injections at 30 min (earlier) or 6.5 hr (later) had significantly lower capturing efficiency. These results further confirm that timed injection of CANE-viruses can be used to selectively, permanently, and relatively efficiently capture neurons that transiently express Fos after a brief behavior.

in our animal housing conditions, the total number of Fos+ AGNs (average, 138 ± 14 per animal in VMHvl; n = 9) was considerably fewer than that of Fos+ SFNs (262 ± 20; n = 9). We co-injected CANE-LV-Cre together with a Cre-dependent AAV-Flex-GFP into the VMHvl of FosTVA aggressive resident mice after they attacked the intruder mice. Then, 10–14 days later, the resident mice were exposed to another intruder in their home cage, which elicited another round of attacks by the resident. Here, co-injection of CANE-LV-Cre and AAV-Flex-GFP was used to label AGNs following the first encounter, and anti-Fos staining was used to visualize SFNs following the second encounter. Only 19% ± 2% of CANE+ AGNs co-expressed Fos (SFNs) (n = 5; Figures 3F and 3G). We also performed the CANE Capturing Reveals that Social Fear and Social Aggression Activate Largely Distinct Ensembles in VMHvl

The CANE method allowed us to resolve whether SFNs are similar or distinct from the aggression-activated VMHvl neurons in the same animal. To this end, we first tested whether CANE-LV can also be used to efficiently and selectively capture aggression-activated neurons (AGNs) in VMHvl. As mentioned above, in our animal housing conditions, the total number of Fos+ AGNs (average, 138 ± 14 per animal in VMHvl; n = 9) was considerably fewer than that of Fos+ SFNs (262 ± 20; n = 9). We co-injected CANE-LV-Cre together with a Cre-dependent AAV-Flex-GFP into the VMHvl of FosTVA aggressive resident mice after they attacked the intruder mice. Then, 10–14 days later, the resident mice were exposed to another intruder in their home cage, which elicited another round of attacks by the resident. Here, co-injection of CANE-LV-Cre and AAV-Flex-GFP was used to label AGNs following the first encounter, and anti-Fos staining was used to visualize SFNs following the second encounter. Only 19% ± 2% of CANE+ AGNs co-expressed Fos (SFNs) (n = 5; Figures 3F and 3G). We also performed the CANE Capturing Reveals that Social Fear and Social Aggression Activate Largely Distinct Ensembles in VMHvl

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converse paradigm: first social fear-second aggression. Again, the CANE+ SFNs were largely non-overlapping with Fos+ AGNs (10% ± 1% CANE+ neurons were Fos+; n = 9) (Figures 3D and 3G). In summary, we show repeated bouts of social aggression or repeated bouts of social fear weeks apart reactivate many of the same VMHvl neurons, which indicates that CANE is both efficient and selective. However, we see limited overlap after either a bout of social aggression followed by social fear, or a bout of social fear followed by social aggression, indicating that these opposite behaviors activate largely distinct populations of neurons in the VMHvl.

We also examined a couple molecular markers known to be expressed in VMH neurons, namely estrogen receptor 1 (Esr1) (a presumed marker for AGNs in VMHvl), and steroidogenic factor 1 (SF1) (a marker for predator fear neurons in the dorsomedial part of VMH, VMHdm,c) (Kunwar et al., 2015; Lee et al., 2014; Silva et al., 2013; Wang et al., 2015). Using two-color immunofluorescence or two-color in situ hybridization, we found that 65% ± 2% of Fos+ AGNs co-expressed Esr1 (n = 4), whereas only 39% ± 4% Fos+ SFNs co-expressed Esr1 (n = 4; Figures S5A and S5B). In both cases, about 43% of Esr1+ neurons (n = 4;Figure S5C). Finally, all Fos+ SFNs express vGlut2 and firming that SFNs are largely distinct from predator fear neurons (Figures S5A and S5B). Only 4% ± 1% of Fos+ SFNs co-expressed SF1, confirming that SFNs are largely distinct from predator fear neurons (n = 4; Figure S5D). Finally, all Fos+ SFNs express vGlut2 and therefore are likely to be excitatory neurons (n = 4; Figure SSD).

Optogenetic Activation of SFNs Using CANE Induces Social-Fear Behaviors in Resident Mice

All previous electrical or optogenetic stimulation experiments in various mammalian species showed that activation of VMHvl elicits aggressive behavior (Fuchs et al., 1985; Kruk et al., 1983; Lammers et al., 1988; Lee et al., 2014; Lin et al., 2011). Here, we found that less than 3% of VMHvl neurons (9,442 ± 1983 neurons total; n = 4) are Fos+ SFNs. We therefore wondered whether activating this small number of CANE-captured SFNs would be sufficient to evoke fear-like behaviors in non-fearful situations. To test this idea, we first co-injected CANE-LV-Cre with Cre-dependent AAV-Flex-ChR2-EYFP (Zhang et al., 2010) unilaterally into the VMHvl of Fos\textsuperscript{TVA} mice after a brief social-fear experience (Figure 4A), resulting in the expression of ChR2 selectively in SFNs in VMHvl (Figure 4E). Photostimulation-dependent activation of ChR2-expressing SFNs was confirmed using whole-cell patch-clamp recording in acute hypothalamic slices in vitro (Figures 4B–4D; 100% spike fidelity is achieved up to 10-Hz, 2- or 20-ms light pulses). Mice unilaterally co-injected with CANE-LV-Cre and AAV-Flex-GFP were used as controls for in vivo optogenetic experiments. All mice were tested in their home cages under infrared light and were exposed to either an intruder male or an intruder female on different days, each for ~20 min. Animals were photostimulated five to six times (10-Hz, 20-ms pulse width; 2–3 mW/mm\textsuperscript{2}; 30-s duration for each stimulation).

In male-male interactions, the resident male normally investigated and sometimes attacked the intruder male, both of which are considered as forms of “active social contact” here. Unilateral photoactivation of ChR2-expressing SFNs caused the resident male to cease ongoing interactions or attacks, and retreat to a corner or against a wall of the cage where they remained largely stationary (i.e., stationary cornering behavior) (Movie S2). By contrast, illuminating GFP-expressing SFNs in control mice had negligible effects on the resident’s ongoing investigatory or aggressive behaviors (Movie S3; Figure 4N). Post hoc histology revealed that the average duration of stationary cornering behavior positively correlated with the number of ChR2-captured SFNs (R\textsuperscript{2} = 0.83) (Figure 4H), whereas the time spent in active social contact inversely correlated with the number of ChR2 cells (R\textsuperscript{2} = 0.62) (Figure 4J). The population size effect on fear-like behavior in male-male interactions could also be modeled by a sigmoid fit (R\textsuperscript{2} = 0.88) (Figure 4L), suggesting a possible threshold effect. We performed cluster analysis to separate the experimental mice into two groups: a Lo-ChR2 group (n ≤ 137 of ChR2+ neurons, n = 6 mice) and a Hi-ChR2 group (n > 137 of ChR2+ neurons, n = 6 mice). The light-induced stationary cornering behavior and reduced social interactions were displayed by the Hi-ChR2 but not the Lo-ChR2 group (Figures 4M and 4N; 14.47 ± 1.32-, 2.38 ± 0.75-, or 1.29 ± 0.43-s stationary time; and 5.09 ± 0.82-, 8.89 ± 1.22-, or 9.86 ± 1.57-s interaction time for Hi-ChR2, Lo-ChR2, and GFP groups, respectively). These results indicate that activating ~140 or more VMHvl SFNs (on one side of the brain) is sufficient to elicit fear-like behaviors in resident male mice presented with a male intruder.

In male-female interactions, the resident male normally spent most of its time chasing and investigating the female. Photoactivation of SFNs also resulted in a population size-dependent stationary cornering behavior in males presented with females (Figure 4L;Movie S4; linear-regression analysis, R\textsuperscript{2} = 0.84). Again, the Hi-ChR2 group showed a longer duration of cornering behavior during photostimulation than either the Lo-ChR2 or GFP control groups (9.42 ± 1.27-, 1.91 ± 0.80-, or 1.84 ± 0.67-s stationary time for Hi-ChR2, Lo-ChR2, and GFP groups, respectively; Figure 4M). However, photostimulation only moderately reduced the active contact with females (Figures 4K and 4N). Thus, unilateral activation of SFNs in male mice elicited milder fear-like behaviors toward females than toward males.

We also measured the latency from light stimulation to the onset of stationary cornering behavior in both behavior paradigms, and the Hi-ChR2 group showed significantly shorter latency to exhibit cornering behavior in male-male encounters compared with Lo-ChR2 and GFP-expressing controls (11.03 ± 1.32-, 25.74 ± 1.15-, or 26.69 ± 1.39-s latency for Hi-ChR2, Lo-ChR2, and GFP groups, respectively; Figure 4O) and a moderate latency to cornering when interacting with females (15.20 ± 1.51-, 24.12 ± 1.52-, or 25.63 ± 1.71-s latency for Hi-ChR2, Lo-ChR2, and GFP groups, respectively;Figure 4O). Finally, optogenetic activation of SFNs never elicited any aggressive behavior in any situations, further indicating that SFNs are distinct from neurons eliciting aggression in the VMHvl.

Optogenetic Activation of SFNs Using CANE Induces Social Avoidance in a Three-Chamber Social-Preference Test

To further demonstrate that CANE-mediated activation of SFNs have a causal effect on social-interaction behaviors, we
Figure 4. Behavioral Effects of Optogenetic Activation of Captured SFNs in the Home Cage

(A) Strategy of expressing channelrhodopsin (ChR2) in VMHvl SFNs using CANE.

(B–D) Whole-cell patch-clamp recording from ChR2-expressing SFNs (B) in acute brain slices. Photostimulation-evoked representative spiking (C) and the spike fidelity (D) are shown (open circles, 2-ms light pulse width, n = 8; filled circles, 20-ms light pulse width, n = 8). Data are mean ± SEM.

(E) Representative low- and high-magnification images of captured ChR2-EYFP-expressing SFNs in VMHvl. The square dashed line indicates where the fiber optic was implanted.

(F and G) Representative images of ChR2-EYFP captured SFNs in Lo-ChR2 (F) and Hi-ChR2 (G) groups. Photostimulation (after all behaviors were completed) was used to induce Fos expression in ChR2-EYFP+ SFNs to facilitate counting of the numbers of captured neurons in each animal.

(H and I) Linear-regression analysis of the duration of stationary cornering behavior during photostimulation (fear-like behavior) versus the numbers of ChR2-EYFP-activated SFNs. (H) Male-male; (I) male-female interaction. Data are mean ± SEM over multiple trials (n = 12 mice). R² value is shown on each graph. p < 0.001 (H), p < 0.001 (I).

(J and K) Linear-regression analysis of the duration of active social contact during photostimulation versus the numbers of ChR2-EYFP-activated SFNs. (J) Male-male intruder; (K) male-female. Data are mean ± SEM over multiple trials (n = 12 mice). R² value is shown on each graph. p = 0.002 (J), p = 0.43 (K).

(L) A sigmoidal fit for describing the relationship between the duration of stationary cornering behavior and the number of ChR2-EYFP-activated SFNs (toward a male intruder). Data are mean ± SEM over multiple trials (n = 12 mice).

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(legend continued on next page)
employed the three-chamber social-interaction test, which is a widely used assay to examine social preference or avoidance (Figures 5A and 5B). We unilaterally injected ChR2-YFP or GFP (controls) in SFNs using CANE. Behavioral results from mice with more than 140 CANE-captured SFNs, determined by post hoc analyses, were quantified. In the three-chamber test, a non-aggressive male or a female mouse was contained in an inverted wire cup in the "social" chamber (Figure 5A). The wire containment reduces the potential for territorial disputes in the male-male interactions. Representative spatial heat maps showed the locations of the free-moving experimental mice in these different conditions (Figures 5C and S6A). When the contained mouse was a male, optical stimulation of SFNs expressing ChR2, but not GFP, caused the animal to avoid the social chamber and stay in the non-social chamber (Figure 5C). Quantitative analysis showed a drastic reduction in the time spent in the social zone or in social contact with the male (Figures 5D and 5E). Milder reductions in social interactions were observed when a female mouse was placed in the social chamber (Figures S6B and S6C). Thus, the three-chamber tests independently confirmed that activating SFNs in the VMHvl induces fear-like social avoidance toward a non-aggressive male.

The intruder mice often exhibit social-withdrawal behaviors (Silva et al., 2013), a sign of fear for conspecifics. We therefore subjected control mice or mice with TeLC expressed in SFNs (SFNs-silenced group) to a very brief social-fear experience during which they were attacked three to five times, after which they were returned to their home cage (Figure 6C). Three minutes later, a non-aggressive male mouse was introduced to the home cage (Figure 6C). Control mice avoided the intruder nearly every time the intruder approached, and often maintained a constant distance from the intruder (Figures 6D–6F). By contrast, the SFNs-silenced mice displayed normal resident behaviors, including close investigations of the intruder through sniffing the intruder’s snout and anogenital regions (Figures 6G–6I). Two SFNs-silenced mice even attacked the intruder. Thus, silencing SFNs reduced the expression of social withdrawal in mice after they had recently been attacked by an aggressive male, or reduced the generalization of social fear toward a non-aggressive conspecific male.

CANE-Mediated Bilateral Silencing of SFNs Reduces Social Fear

We next asked whether silencing SFNs using CANE would reduce social fear. We expressed the neuronal silencer, tetanus toxin light chain (TeLC), in SFNs by co-injecting CANE-LV-Cre and AAV-Flex-TeLC-GFP (or AAV-Flex-GFP as controls) bilaterally into the VMHvl after a social-fear experience (Figures 6A and 6B; a total of 397 ± 28 neurons from both sides were infected; n = 8 mice). It was shown previously that immediately after being defeated by aggressive residents, the intruder mice often exhibit social-withdrawal behaviors (Silva et al., 2013), a sign of fear for conspecifics. We therefore subjected control mice or mice with TeLC expressed in SFNs (SFNs-silenced group) to a very brief social-fear experience during which they were attacked three to five times, after which they were returned to their home cage (Figure 6C). Three minutes later, a non-aggressive male mouse was introduced to the home cage (Figure 6C). Control mice avoided the intruder nearly every time the intruder approached, and often maintained a constant distance from the intruder (Figures 6D–6F). By contrast, the SFNs-silenced mice displayed normal resident behaviors, including close investigations of the intruder through sniffing the intruder’s snout and anogenital regions (Figures 6G–6I). Two SFNs-silenced mice even attacked the intruder. Thus, silencing SFNs reduced the expression of social withdrawal in mice after they had recently been attacked by an aggressive male, or reduced the generalization of social fear toward a non-aggressive conspecific male.

Figure 5. Behavioral Effects of Optogenetic Activation of SFNs in the Three-Chamber Social-Interaction Test

(A) Schematic illustration of three-chamber social-preference test.
(B) Experimental time course used for the three-chamber test.
(C) Representative spatial heat maps showing the locations of the experimental mouse in the presence of a male mouse.
(D) Quantification of time spent in the social chamber in the presence of a male mouse.
(E) Quantification of duration of active contact in the presence of a male mouse.

Data are mean ± SEM (GFP group, n = 7; ChR2, n = 6 to male). **p < 0.01, ***p < 0.001. Unpaired t test.

Mapping the Axonal Projection Targets of CANE-Labeled VMHvl Social-Fear Neurons

The activation and silencing results described above indicated that CANE can be used to establish the causal function of
captured neurons. To understand how such a small number of SFNs (~260 on one side of VMHvl) is both necessary and sufficient to regulate social fear, we decided to elucidate their functional connectivity. We first used CANE to identify the output targets of SFNs. High-titer CANE-LV-Cre (1 × 10^8 ifu/ml) and AAV-Flex-GFP were co-injected into the VMHvl of FosTVA mice to label SFNs with GFP (Figure 7A). Three weeks later, the mice were subjected to another social-fear experience to reinduce Fos expression (61% ± 4% of CANE-labeled VMHvl neurons were also Fos+, n = 3, again confirming specificity of labeling) (Figures 7B–7D). These neurons projected axons widely to regions of the limbic system (Figures 7E–7R). To measure SFN projection patterns in each region, we quantified both the “total axon projections” (averaged and normalized total pixel numbers of GFP-labeled axons; n = 3) and the “density of innervation” (total pixel numbers of GFP-labeled axons divided by the area of the nuclei and averaged across animals; n = 3) using a method similar to that described previously (Oh et al., 2014). This analysis identified 18 brain regions that are targets of VMHvl SFNs, including bed nucleus of the stria terminalis (BNST), medial and lateral preoptic area/nucleus (MPA and LPO), anterior hypothalamic nucleus (AHA) and paraventricular hypothalamic nucleus (PVH), limbic cortex regions (PrL, IL, and Cg1), nucleus accumbens (Nac), lateral septum (LS), paraventricular thalamic nucleus (PVT), central and medial amygdala (CeA and MeA), the dorsal and ventral hypothalamic premammillary nucleus (PMd and PMv), dorsomedial and lateral periaqueductal gray (PAGDM and PAGL), and in the most lateral region of the midbrain reticular formation (MRNL)(Figures 7E–7R). The majority of projections were ipsilateral with small numbers of axons innervating the contralateral side (Figures 7P–7R). A schematic summary of the SFN projection pattern is shown in Figure 8T (green lines with arrows). Note that all of the SFN target also contain Fos+ neurons induced by the same intruder experience (Figure 7, red signals), indicating that SFNs project to regions that are also activated by social fear.
Developing CANE-Rabies Virus for Transsynaptic Tracing of Presynaptic Inputs for VMHvl Social-Fear Neurons

We next wanted to establish CANE-based method to map presynaptic inputs onto captured neurons in combination with the monosynaptic RV strategy (Wickersham et al., 2007). To achieve this, we first needed to produce a pseudotyped deficient RV expressing mCherry in place of endogenous glycoprotein (rabies-G) with the mutated low-affinity EnvAM21 envelope (CANE-RV-mCherry), and to demonstrate that it can specifically infect Fos+ neurons. To test viral specificity, we injected CANE-RV-mCherry into the VMHvl of FosTVA mice after a social-fear experience, and 5 days later, the mice were again used as intruders to induce Fos (Figure 8A). In total, 61% ± 6% of CANE-RV-mCherry-captured neurons were also Fos+ (Figures 8B–8E). Furthermore, no long-range infection outside of VMHvl was detected, indicating that dsTVA was not sufficiently expressed in axon terminals from neurons projecting into VMHvl to allow for CANE-RV infection (Figure 8B; data not shown). These results showed that CANE-RV selectively infects Fos+ neurons only at cell bodies. As a control experiment, we injected CANE-RV-mCherry into the VMHvl of FosTVA mice housed in their home cage. Only minimal background infection was observed without social behavior (Figure S7A).

Having demonstrated the specificity of CANE-RV-mCherry to infect Fos+ neurons, we next performed experiments to trace sources of synaptic inputs onto SFNs. We first co-injected CANE-LV-Cre and the helper virus AAV-SynP-DIO-TVA-EGFP-RG (Kohara et al., 2014) into the VMHvl after a social-fear experience, which enabled Cre-dependent expression of the TVA receptor, the rabies-G, and GFP in SFNs (Figures 8F and 8G). Three weeks later, CANE-RV-mCherry was injected into the VMHvl. GFP+/mCherry+ double-positive neurons represented the “starter” SFNs that expressed the rabies-G and could complement the deficient CANE-RV to enable its transsynaptic spreading (Figures 8F–8I). Such starter neurons were observed only in VMHvl (Figure 8I).

Neurons that were only mCherry+ outside of the VMHvl, representing putative monosynaptic afferents to SFNs, were observed in multiple structures within the ventral forebrain including nucleus accumbens (Nac), lateral septum (LS), medial and lateral preoptic area (MPA/MPN, LPO), the bed nucleus of the stria terminalis (BNST), as well as in several hypothalamic nuclei including anterior (AHA), paraventricular (PVH), paraventricular thalamic nucleus (PVT), and the dorsal and ventral premammillary (PMD, PMv) nuclei (Figures 8J–8Q). The labeling results (normalized by dividing numbers of labeled presynaptic neurons by the number of starter neurons and averaged across animals; n = 3) (Figure 8S) are schematically summarized in Figure 8T (red lines; regions with >0.25 average labeled neurons per starter neuron are shown). Interestingly, all regions that provide input onto SFNs also receive input from SFNs, as revealed in the projection studies described above (Figure 8T). These findings indicate that SFNs in VMHvl are reciprocally connected with multiple brain regions (orange-colored regions in Figure 8T) that together drive social-fear behaviors (see Discussion). Notably, no transsynaptically labeled neurons were observed in medial amygdala (MeA) (Figure 8R), which is known to contain neurons that are selectively activated by male or female mice, or by predators (Bergan et al., 2014; Choi et al., 2005; Gross and Canteras, 2012; Pérez-Gómez et al., 2015; Petrovich et al., 2001; Unger et al., 2015) and that project axons to VMHvl. This result suggests that inputs from MeA to VMHvl terminate on neurons other than SFNs, although we cannot rule out the possibility that the lack of MeA labeling was caused by differences in viral neurotropism.

As a further control experiment, we injected CANE-LV-Cre and AAV-SynP-DIO-TVA-EGFP-RG into VMHvl of FosTVA mice housed in their home cage without social behavior. Three weeks later, CANE-RV-mCherry was injected into the same region (with only home cage experience). Again, only minimal background infection of both viruses was observed in the VMHvl in these controls (Figure S7B).

CANE Is a Generally Applicable Tool to Label Activated/Fos+ Neurons

To further demonstrate the specificity and general applicability of this technology, we used CANE to capture activated neurons in two other behavioral paradigms. First, we used CANE to label barrel cortical neurons activated by whisker-dependent tactile exploration. FosTVA; Ai14 mice with only a single whisker (C2 whisker) remaining, or with all-but-C2 whiskers intact, were introduced in a novel environment in the dark. Two hours after the exploration, CANE-LV-Cre was injected into the barrel cortex in these mice to infect activated neurons (Figure 9A). Ten days later, mice were subjected to another 2-hr environmental exploration to reinduce Fos expression. As expected, in the single C2 whisker-only mice, the majority of Fos+ neurons were observed...
Figure 8. Visualization of Transsynaptically Labeled Presynaptic Neurons for the VMHvl SFNs

(A) Strategy for capturing SFNs in VMHvl using CANE-RV-mCherry.

(B–D) Representative images of CANE-RV-mCherry-captured SFNs. Red, captured SFNs activated by the first social-fear behavior (B); green, Fos expression induced by a second social-fear behavior (C). (D) is the overlay of (B) and (C).

(E) In total, 61% ± 6% of CANE-RV-mCherry+ -infected neurons with Fos. Data are mean ± SEM (n = 4).

(legend continued on next page)
in layer 4 of the C2 barrel column, as were CANE+ neurons (Figure 9B). Importantly, 80% ± 5% of CANE+ neurons reexpressed Fos (n = 3). In the all-but-C2 mice, C2 column had the least number of CANE+ (or Fos+) neurons with the majority of CANE+ neurons observed in layer 4 of neighboring barrel columns surrounding C2 (Figure 9B). Note that CANE-LV is a lentiviral vector that has a diffusion range of approximately three to four barrel columns. These results strongly suggested that sensory-experience-activated cortical neurons can be selectively captured by CANE.

Next, we performed experiments that allowed us to directly compare CANE labeling with Fos-tTA-based labeling. Tayler et al. (2013) used Fos-tTA system to induce the stable H2B-GFP expression in neurons activated by contextual fear conditioning in basolateral amygdala, dentate gyrus, and other regions. Two weeks later, they reexposed labeled mice to the same context again to examine what percentage of H2B-GFP+ neurons were reactivated (using anti-Fos staining) by the remote memory. They discovered that Fos-tTA/H2B-GFP labeled ~2.5% of basolateral amygdala neurons, and ~4% dentate gyrus neurons upon fear conditioning training. Importantly, only ~10% of H2B-GFP-labeled amygdala neurons reexpressed Fos+, and only ~2% of labeled dentate neurons were Fos+ upon reexposure to the context. Results from this previous study indicate that remote memory only reactivate a very small number of neurons that were active during memory encoding. We followed the contextual fear conditioning paradigm (Maren, 2001; Maren et al., 2013)(Figure S8A) but used CANE to capture activated neurons. Two weeks later, mice were placed back to the conditioning box to induce Fos. We found that CANE labeled 6.9% ± 0.9% of amygdala, and 8.7% ± 0.7% of dentate neurons (Figures S8B–S8D), and that 17.6% ± 1.1% of CANE-captured amygdala, and 3.3% ± 0.4% of captured dentate neurons reexpressed Fos by the remote memory retrieval task (Figures S8B, S8C, and S8E). Thus, CANE captured moderately higher numbers of neurons and revealed moderately higher number of reactivated neurons, but overall our results are comparable to those obtained by Tayler et al. (2013), further validating the general applicability of CANE technology.

DISCUSSION

In summary, we developed a novel technology called CANE that has achieved our goals: to be able to selectively tag Fos+ neurons with high efficiency and high spatial and temporal specificity; to be able to manipulate tagged neurons, and to be able to trace efferent and afferent connections (i.e., functional connectivity) of Fos+ neurons. Using CANE, we characterized a subset of neurons activated by fearful social encounters in the VMHvl, a nucleus previously implicated mainly in aggressive or sexual behaviors (Lee et al., 2014; Lin et al., 2011; Swanson, 2000; Yang et al., 2013). Mapping the outputs and inputs to these SFNs revealed a large distributed and recurrent circuit in which they are embedded.

The Aggression Locus Also Contains a Largely Separable Circuit Regulating Social Fear

Prior conclusions that VMHvl is a locus for aggression were based on the fact that ablating progesterone receptor-expressing neurons in VMHvl significantly reduced male aggression...
togenetically stimulating Esr1+ VMHvl neurons elicits aggression (Yang et al., 2013), whereas electrically activating VMHvl or optogenetically stimulating VMHvl neurons elicits aggression or mounting behaviors (Fuchs et al., 1985; Kruk et al., 1983; Lammers et al., 1988; Lee et al., 2014; Lin et al., 2011). Here, using CANE-capturing and Fos staining in four different behavioral combinations (fear-fear, aggression-aggression, fear-aggression, aggression-fear), we showed that the social-aggression and social-fear ensembles in VMHvl are composed of largely non-overlapping pools of neurons. However, there is a 10–20% overlap between social-fear and aggression VMHvl neurons. We speculate that the small overlap may represent shared neurons between the two behaviors that encode social cues; or alternatively, these shared neurons might be a potential substrate for fear-induced aggression.

Although the numbers of SFNs in VMHvl labeled with CANE was relatively small (~200 Fos+ neurons), they exerted pronounced behavioral effects when synchronously activated with light. Our connectivity mapping study revealed that many of the regions targeted by VMHvl SFNs provide inputs back onto SFNs. It is likely that synchronously activating a small number of ChR2-expressing SFNs in the presence of otherwise non-threatening social cues may recruit fear-associated neurons in multiple downstream targets of SFNs, some of which could in turn recurrently excite both tagged and untagged SFNs in the VMHvl. Through these recurrent interconnections, activity in SFNs may be sufficient to recruit and activate many parts of the social-fear circuitry, exceeding the threshold required to trigger the behavioral expression of fear. The input-output map of VMHvl SFNs provides a blueprint for future studies that seek to understand how each of the nodes in the distributed recurrently connected circuit contributes to social fear. Future experiments are needed to test whether the identified hypothalamic SFNs are also involved in other types of anxiety and fear behaviors.

CANE as a General Tool for Studying Spatially Intermixed, but Functionally Distinct, Ensembles

Similar to VMHvl, many brain regions contain Fos+ neurons that encode distinct or even opposite functions (Xiu et al., 2014). The CANE method is likely to be especially well suited to dissect such intertwined functional sub-circuits, especially when other molecular markers for distinguishing these circuits are unavailable (see Table S1 for detailed comparison of CANE with other immediate early genes-based methods). First, the FosTVA mouse for the CANE method is a knockin line that maintains endogenous Fos expression; thus, this method can in principle be applied to any brain regions with sufficient Fos expression. Second, CANE has a better temporal precision for labeling recently activated neurons without the ambiguity caused by the slow metabolism of doxycycline or tamoxifen. The CANE virus is injected into the desired brain region at the time when Fos expression reaches peak levels; therefore, the window of infection is precisely controlled by the experimenter and can completely follow endogenous Fos expression. Third, the precise tagging allows for specific transsynaptic tracing of presynaptic inputs to functionally defined neurons in a heterogeneous brain region. Fourth, CANE is highly versatile. It can be used to label neurons in the same nucleus but activated by different stimuli or behavioral conditions by sequential labeling with CANE viruses expressing different fluorescent proteins or transgenes (an example of two-color sequential labeling of aggression versus fear neurons is shown in Figure S9). Of course CANE also has its own limitations: it cannot be used for systematic capturing of activated neurons across the entire brain because it requires stereotaxic surgical injection of the CANE viruses. Furthermore, because anesthesia is needed for surgery, using CANE to capture neurons involved in processes affected by anesthesia requires additional steps: a cannula needs to be implanted into the desired brain region first, followed by the behavior episode to induce Fos, and followed by CANE virus delivery without additional surgery and anesthesia. A full comparison of advantages and limitations between different activity-dependent tagging methods is listed in Table S1.

In conclusion, CANE is a widely applicable and powerful tool to study specific functions and connectivity of transiently activated neuronal ensembles in many brain regions and in diverse behaviors and sensory processes.

EXPERIMENTAL PROCEDURES

Generating FosTVA Knockin Mice

FosTVA knockin mice were generated by inserting a 2A sequence, followed by a destabilized nuclear CFP (nCFP fusing a PEST sequence), the second 2A sequence, followed by destabilized TVA (TVA fusing a PEST sequence), bGH polyA, and a Frt-flanked PGK-neomycin resistance cassette immediately before the stop codon of Fos coding sequence by homologous recombination. All experiments were conducted according to protocols approved by the Duke University Institutional Animal Care and Use Committee. The FosTVA mice are available from The Jackson Laboratory (stock No. 027831).

Production of CANE-LV and CANE-RV

EnvA^{R21} has three (R213A, R223A, R224A) mutations in the extracellular domain of the EnvA protein. CANE-LV-Cre viruses are produced and packaged using HEK293T cells by co-transfecting a plasmid encoding the EnvA^{R21}-VSVG fusion envelope protein, the pLenti-hSyn-Cre-WPRE plasmid, and the psPAX2 plasmid into these cells. CANE-RV-mCherry viruses are produced by pseudotyping the SAD-Delta9-mCherry rabies virus (Ojikado and Callaway, 2013) using cell lines that produce the EnvA^{R21} (extracellular and transmembrane)-rabies G (cytoplasmic region of SAD-B19 rabies glycoprotein) fusion envelope.

Virus Injection for Capturing SFNs

Adult male FosTVA mice at ages more than 8 weeks were singly housed for at least 1 day, and then subjected to the intruder social-fear experience. Briefly, for capturing SFNs using CANE-LV or CANE-RV, they were introduced into a singly housed aggressive ICR male mouse for up to 10 min to be attacked 10–15 times, and returned into their home cage. One hour later, mice were anesthetized and underwent stereotaxic surgery for virus injection.

Optogenetic Activation of Captured SFNs in the VMHvl

SFNs in the VMHvl were captured using virus mixture of CANE-LV-Cre and AAV-EF1a-Flex-ChR2-EYFP or AAV-CAG-Flex-GFP in FosTVA mice. After more than 7 days of recovery periods, virus-injected mice were implanted with a custom-made optic. Three to 4 weeks later, the animals were subjected to behavioral testing in their home cage. Blue (473 nm) light was delivered with 20-ms pulses at 10 Hz, and the final output powers ranged from 2 to 3 mW/mm².

Behavioral Effects of Silencing SFNs

FosTVA mice in which either TeLC or GFP were expressed bilaterally in SFNs were subjected resident-intruder test for 2 min and were attacked three to four times.
approximately five times by an aggressive resident. Then mice were returned to their own home cage and waited for 3 min, followed by social-interaction test by introducing a non-aggressive adult male C57BL6 mouse for 5 min. For more detailed full description of all experimental procedures, please refer to Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the CANE-LV viral envelope gene sequence reported in this paper is GenBank: KX990266. The accession number for the CANE-RV viral envelope gene sequence reported in this paper is GenBank: KX990267.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, nine figures, one table, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.10.015.

AUTHOR CONTRIBUTIONS

F.W. conceived the idea. F.W. and K.S. designed the experiments. K.S. performed the majority of the experiments and data analysis. S.Z. helped optimize the method to package viruses, produced some of the CANE-LV, and produced the CANE-RV. J.T. and J.L. quantified axon projection and many behavior test results, E.R. performed slice physiology recording. A.D.L. generated the anti-TVA antibody. M.F. performed subsets of viral injection experiments in barrel cortex. B.-X.H. took care of mouse husbandry and genotyping. F.W. and K.S. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Vincent Prevosto for help with analyzing the behavior results, Dr. Ian Wickersham for pAV-Synp-DIO-TVA-EGFP-RG construct, and Drs. Richard Mooney, Stephen Lisberger, and Kevin Franks for their comments on this manuscript. This work was supported by NIH Grants DP1MH103908 and NS077986, as well as Simons Foundation Autism Research Initiative Pilot Grant 275163 to F.W. J.T was supported by a JSPS fellowship.

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Supplemental Information

Capturing and Manipulating Activated Neuronal Ensembles with CANE Delineates a Hypothalamic Social-Fear Circuit

Katsuyasu Sakurai, Shengli Zhao, Jun Takatoh, Erica Rodriguez, Jinghao Lu, Andrew D. Leavitt, Min Fu, Bao-Xia Han, and Fan Wang
Capturing and Manipulating Activated Neuronal Ensembles with CANE Delineates A Hypothalamic Social Fear Circuit

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Supplemental Information

Figures S1-S9
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Figure S1. Generation of the FosTVA knock-in mouse (Related to Figure 1)
(A) Strategy for generating FosTVA knock-in mice. E, exon; 2A, 2A-sequences that mediate translational skip; nCFP, nuclear localized CFP; ds, destabilized signal sequence (PEST sequence); TVA, the avian receptor for EnvA-coated virus, pA, bGH polyA signal sequence.
(B) Southern blot analysis of BglII digested genomic DNA from WT (wild type) and correctly targeted FosTVA+Neo ES cells. The 12.7 and 8.6 kb fragments indicate mutant (targeted) and WT allele, respectively.
(C) Genomic PCR analysis of the genotypes. WT and mutant alleles produce 416 and 634 bp fragments, respectively.
Figure S2. Fos expression patterns in VMHvl after social fear or aggressive behavior (Related to Figures 2 and 3)

Anti-Fos staining was performed 2 hours after each behavior. White dash circle indicates structure of VMH. The white arrow indicates Fos expressing neurons in the VMHdm. Blue; DAPI stain. Scale bar, 100 µm.
Figure S3. Testing the wild type or mutated version of EnvA pseudotyped lentivirus for their selectivity in capturing SFNs (Related to Figures 1 and 3)

EnvA-LV-Cre (EnvA<sup>WT</sup>) infected non-specifically in Fos<sup>TV</sup>A mice subjected to either a novel cage experience (control) or a social fear experience including many glial cells. The EnvA<sup>M4</sup> and EnvA<sup>M5</sup> coated lentiviruses infected neurons in VMHvl both after a novel cage experience and after a social fear experienced in Fos<sup>TV</sup>A mice. By contrast, EnvA<sup>M21</sup> pseudotyped lentivirus (CANE-LV) specifically infected VMHvl neurons only after social fear experienced in Fos<sup>TV</sup>A mice. White dash circles indicate the structure of VMH. Blue; DAPI or fluorescent Nissl stain. Scale bar, 100 µm.
Figure S4. Testing the capturing efficiency at the different time points following social fear experience (Related to Figure 3)
CANE-LV-Cre and AAV-Flex-GFP were co-injected into VMHvl at different time point (0.5-1.0h, 1.5-2.0h or 6.5-7.0h) following a brief (10 min) social fear behavior. The ratios of CANE+ neurons to Fos+ neurons in VMHvl are: 19 ± 2% (0.5-1.0h), 64 ± 5% (1.5-2.0h), and 33 ± 6% (6.5-7.0h). Green, CANE+ neurons captured after the 1st social fear behavior; Red, Fos expression at 2 hours following a 2nd social fear behavior. White dash lines circle the boundary of VMH. Blue, fluorescent Nissl stain. Scale bar, 100 µm. Data are Mean ± S.E.M. (n = 4)
Figure S5. Molecular characterization of aggression and social fear neurons in VMHvl (Related to Figure 3)
(A) Aggression-activated (anti-Fos, red) and Esr1-expressing neurons (anti-Esr1, green) in VMH. 65 ± 2% of aggression induced Fos+ neurons co-expressed Esr1. The fraction of Esr1+ neurons that co-expressed Fos is 43 ± 4%. Note that most of Esr1+ neurons locate in VMHvl, however, scattered Esr1+ neurons are observed in VMHdm (white arrow).

(B) Social fear-activated neurons (anti-Fos, red) and Esr1-expressing neurons (anti-Esr1, green) in VMH. 39 ± 4% of social fear induced Fos+ neurons co-expressed Esr1. The fraction of Esr1+ neurons that co-expressed Fos is 43 ± 5%.

(C) Two-color fluorescent in situ hybridization showing social fear activated (Fos+, green) and SF1-expressing neurons (red). 4± 1% of social fear-activated VMHvl neurons co-expressed SF1.

(D) Two-color fluorescent in situ hybridization showing social fear activated (Fos+, green) and vGlut2+ neurons (red). Almost all social fear-activated VMHvl neurons co-expressed vGlut2.

White dash lines circle the boundary of VMH. Blue, DAPI stain. Scale bar, 100 µm. Data are Mean ± S.E.M. (n = 4)

(E) Venn Diagram showing the relationships between Esr1+ and Fos+-SFNs, and between Esr1+ and Fos+-AGNs.
Figure S6. Behavioral effects of optogenetic activation of SFNs in males when a female mouse was presented in the three-chamber social interaction test (Related to Figure 5)

(A) Representative spatial heat maps showing the locations of the experimental male mice in the presence of a female mouse.
(B) Quantification of time spent in the social chamber containing a female mouse.
(C) Quantification of duration of active contact.
Data are Mean ± S.E.M. (GFP group, n = 7; ChR2, n = 5). Unpaired T-Test.
Figure S7. Testing the background labeling of CANE-RV-mCherry in the home cage Fos\textsuperscript{TVA} mice (Related to Figure 8)

(A) Control experiments in which CANE-RV-mCherry was injected into VMHvl of Fos\textsuperscript{TVA} mice housed in their home cage revealed minimal background infection.

(B) Control experiments in which CANE-LV-Cre and AAV-SynP-DIO-TVA-EGFP-RG were co-injected into VMHvl of Fos\textsuperscript{TVA} mice housed in their home cage, followed 2 weeks later by injection of CANE-RV-mCherry into the same region. Green, EGFP/TVA/rabies-G labeled cells; Red, CANE-RV-mCherry labeled cells. Blue, fluorescent Nissl stain. Scale bar, 100 µm.
Figure S8. Application of CANE technology to capture activated neurons in contextual fear conditioning behaviors (Related to Figure 9)

(A) Strategy for capturing contextual fear conditioning activated neurons in basolateral amygdala (BLA) and dentate gyrus (DG) in FosTVA mice using CANE-LV-Cre and AAV-Flex-GFP, followed by Fos induction through remote memory retrieval two weeks later.

(B) Representative images of CANE-captured neurons (Green) versus context-A re-activated Fos+ neurons (red) in BLA. White dash lines circle the structure of BLA. The white arrow indicates co-labeled neurons.

(C) Representative images of CANE-captured neurons (Green) versus context-A re-activated Fos+ neurons (red) in DG. The white arrow indicates co-labeled neurons.

(D) The percentage of captured neurons (CANE+) among Nissl+ neurons in BLA or DG. Data are Mean ± S.E.M. (BLA, n = 7; DG, n = 8)

(E) The percentage of captured (CANE) and Fos double positive neurons among captured neurons in BLA or DG. Data are Mean ± S.E.M. (BLA, n = 7; DG, n = 8)

Blue, fluorescent Nissl stain (B and C). Scale bars, 500 µm (B and C).
Figure S9. Sequential capturing of aggression and social fear neurons in the same VMHvl using two different CANE viruses (Related to Figure 3)

(A) Schematic for capturing AGNs and SFNs sequentially with different CANE viruses in FosTVA mice. First, the FosTVA was subjected to an aggression experience and AGNs were captured with CANE-LV-Cre together with AAV-CAG-Flex-GFP in VMHvl. More than 2 weeks later, the same animal was subjected to a social fear experience and SFNs were captured with CANE-RV-mCherry in VMHvl.

(B) Representative images of captured AGNs (Green) and SFNs (Red) in VMHvl. The lower panels indicate high magnification. AGNs and SFNs are largely non-overlapping. White dash lines circle the structure of VMH. Blue, fluorescent Nissl stain. Scale bars, 100 μm.
<table>
<thead>
<tr>
<th>Method</th>
<th>Utilized IEG</th>
<th>Details of the mouse line used to capture/label activated cells</th>
<th>Facilitator/Vehicle used to capture/label activated cells</th>
<th>Effector gene application</th>
<th>Applied effectors in publications</th>
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<td>CANE</td>
<td>Fos</td>
<td>Fos&lt;sup&gt;lox&lt;/sup&gt; mouse (knock-in) (note: Fos and dsTVA are co-expressed through the use of the 2A sequence.)</td>
<td>• Require injection of CANE viruses (CANE-LV-GeneX) (CANE-RV-GeneX)</td>
<td>• Cre-dependent AAV/Cre-dependent mice (e.g. AI14, AI32)</td>
<td>• A14 mice • AAV-Flx-GFP • AAV-Flx-ChR2 • AAV-Flx-Chr2 • AAV-DIO-TVA-EGFP-IRG</td>
<td>Similar to the window of Fos expression (note: dsTVA mimics the kinetics of endogenous Fos expression)</td>
<td>• Capable of tagging 2 or more distinct ensembles in the same brain region using different CANE-viruses (e.g. different XFP, or Cre versus Flp) • Have a good temporal resolution and specificity for labeling behaviorally relevant neurons • Capable of efficiently tagging the majority of activated neurons in a brain region • Tag neurons with desired transgene permanently • Enable transsynaptic tracing of inputs to activated neurons</td>
<td>• Cannot be used for systematic labeling, because stereotoxic injection is needed • Virus injection must be performed carefully in order to minimize brain injury induced Fos expression • Requires time to achieve high-level transgene expression and therefore not suitable for experiments requiring manipulation of neuronal activities within a few days after tagging • Need to be cautious of processes that are sensitive to anesthesia and surgery after behavior</td>
<td>Sakurai et al. (this paper)</td>
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<td>TRAP</td>
<td>Fos/Arc</td>
<td>FosCreER&lt;sup&gt;lox&lt;/sup&gt;/ArcCreER&lt;sup&gt;lox&lt;/sup&gt; mouse (knock-in) (note: CreER&lt;sup&gt;lox&lt;/sup&gt; is inserted right after the 5'-translational start site of Fos or Arc gene, therefore creating a null allele of Fos or Arc.)</td>
<td>• Require tamoxifen (TM) or 4-OH tamoxifen (4-OHT) injection</td>
<td>• Cre-dependent AAV/Cre-dependent mice (e.g. AI14)</td>
<td>• A14 mice</td>
<td>From 0–6 hours after 4-OHT or TM injection till the drug is metabolized (up to 24 hours after injection)</td>
<td>• Enable systematic tagging of activated neurons without surgical injection, compared to focal capturing with CANE • Tag neurons with desired transgene permanently</td>
<td>• Endogenous Fos/Arc expression is disrupted by CreER&lt;sup&gt;lox&lt;/sup&gt; • Relatively long labeling window if TM is used • Inefficient and stochastic labeling due to transient expression of CreER&lt;sup&gt;lox&lt;/sup&gt; and the mosaic activation of CreER&lt;sup&gt;lox&lt;/sup&gt; • There are background labeling (spontaneous activation of CreER&lt;sup&gt;lox&lt;/sup&gt;)</td>
<td>Guenthner et al. (2013) Ye et al. (2016)</td>
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<td>Tet-Tag</td>
<td>Fos</td>
<td>Transgenic mouse generated by co-injection of Fos-promoter-tTA and Fos-promoter-shEGFP (transgenic)</td>
<td>• Require doxycyclin (Dox) administration, removal, then re-administration</td>
<td>• tTA-dependent mice (TetO-promoter driven transgenic mice) • tTA-dependent AAV</td>
<td>• TetO-tauLacZ • TetO-GFP-GluR1 • TetO-hM3Dq • TetO-CreER&lt;sup&gt;lox&lt;/sup&gt;-Tomato • AAV-TRE-ChR2 • AAV-TRE-EYFP • AAV-TRE-mCherry • AAV-TRE-ArchT</td>
<td>From the removal of Dox (usually 1-2 days before experiments) till a few hours after re-administration of Dox</td>
<td>• Effector gene rapidly expresses and therefore can be used to manipulate neuronal functions within few days after Dox removal • Potentially can be used for reversible tagging and manipulations of captured neurons with the use of Dox • Labelling efficiency is high</td>
<td>• Relatively high background labeling • Relatively poor temporal resolution due to slow metabolism and clearing of Dox • Cannot be used for permanently tagging transiently activated neurons • Fos-tTA transgene does not fully recapitulate endogenous Fos expression • There is shEGFP expression from the second transgene</td>
<td>Reijmers et al. (2007) Matsuo et al. (2008) Liu et al. (2012) Garner et al. (2012) Ramírez et al. (2013) Redondo et al. (2014) Cowan et al. (2014) Ramírez et al. (2015) Kitamura et al. (2015)</td>
</tr>
<tr>
<td>Arc-CreER&lt;sup&gt;lox&lt;/sup&gt;</td>
<td>Arc</td>
<td>Arc-CreER&lt;sup&gt;lox&lt;/sup&gt; (BAC transgenic)</td>
<td>• Require tamoxifen (TM) injection</td>
<td>• Cre-dependent mice (e.g. AI14) • Cre-dependent AAV/ • R26R-STOP floxed EYFP mice • AI35 mice • AI39 mice • AAV-Flx-ChR2</td>
<td>From 0–6 hours after 4-OHT or TM injection till the drug is metabolized (up to 24 hours after injection)</td>
<td>• Enable systematic tagging of activated neurons without surgical injection, compared to focal capturing with CANE • Tag neurons with transgenes permanently</td>
<td>• Relatively long labeling window if TM is used • Transgene may not recapitulate endogenous Arc expression • May have background labeling in certain brain regions</td>
<td>Denny et al. (2014) Root et al. (2014)</td>
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**Movie S1 (Related to Figure 4)**

**Cornering and escaping behaviors displayed by the intruder during the intruder-resident encounter**
The dark-colored mouse is an intruder placed into the cage of an aggressive resident (white-color ICR mouse). The intruder flees from the resident when it is being attacked. When it is not under attack, the intruder stays at the corner.

**Movie S2 (Related to Figure 4)**

**Optogenetic activation of VMHvl SFNs (n=205) in a resident male exposed to a male intruder**
In this mouse, channelrhodopsin is expressed in 205 SFNs using CANE, and photo stimulation of SFNs terminates social interactions and induces a prolonged stationary cornering behavior.

**Movie S3 (Related to Figure 4)**

**Photo stimulation of GFP expressing VMHvl SFNs in a resident male exposed to a male intruder**
Photo stimulation of GFP-expressing SFNs has minimal effects on the behaviors of the resident.

**Movie S4 (Related to Figure 4)**

**Optogenetic activation of VMHvl SFNs in a resident male exposed to a female mouse**
Photo activation of channelrhodospin-expressing SFNs in a resident male mouse interrupts its interaction with the female and induces stationary cornering behaviors.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals

Generating FosTVA knock-in mice
We used Fos bacterial artificial chromosome (BAC) clone (RP23-196C17) and employed BAC recombinering to make targeting vector. We designed the targeting vector by inserting a 2A sequence, followed by a destabilized nuclear CFP (nCFP fusing a PEST sequence), the second 2A sequence, followed by destabilized TVA (TVA fusing a PEST sequence). bGH polyA, and a Frt-flanked PGK-neomycin resistance cassette immediately before the stop codon of Fos coding sequence by homologous recombination. The PEST sequences used (SHGFPPPEVEQDDDGLPLMSCAQESGMDRHPAACASARINV) are supposed to target the fusion protein for rapid degradations. Targeted ES cells were generated and confirmed by Southern blotting. To detect the FosTVA mutant allele by PCR, PCR primers were designed as follows: ks238, 5’-CTGCACAGCAATCTCTGATGG-3’; ks239, 5’-AGGACTGGAGGCCACAGTGATGGATGC-3’; ks240, 5’-TACCCCTCGGACACGGCCTGGTGCTGG-3’. The wild type allele produces a 416 bp fragment with ks238 and ks239 primers, whereas the mutant allele is detected by a 634 bp fragment with ks238 and ks240 primers. The Frt-flanked PGK-neomycin resistance cassette was removed by crossing with transgenic mice expressing flippase in all cells (Jackson Laboratory, Stock No. 007844). We found that the direct nCFP fluoresence was faint and invisible under the fluorescent microscope in FosTVA mice brain slices. However, when we used anti-GFP antibody to stain for nCFP, we found wide-spread immunofluorescence signals in the nucleus in many neurons. This is likely due to the partially degraded fragment of nCFP protein persisting in nucleus and can be stained by anti-GFP antibody (Tayler et al., 2013). Therefore we did not use either the CFP fluorescence or anti-GFP staining in this study.

The FosTVA mice are available from Jackson Laboratory, stock No. 027831.

Ai14 mice and FosTVA; Ai14 mice
The Cre-dependent tomato reporter mice Ai14 were obtained from Jackson Laboratory (Stock No. 007908) and crossed with FosTVA mice to generate FosTVA; Ai14 mice.

All experiments were conducted according to protocols approved by The Duke University Institutional Animal Care and Use Committee.

Immunohistochemistry
Animals were deeply anaesthetized with isoflurane, and then transcardially perfused with 10% sucrose in Milli-Q water, followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4% PFA). Dissected brains were post-fixed for overnight in 4% PFA at 4 °C and transferred to 30% sucrose in 0.1 M phosphate buffer saline, pH7.4 (PBS) at 4 °C. After overnight incubation, brains were frozen in Tissue-Tek O.C.T. Compound (Sakura) and stored at -80 °C until sectioning. Coronal brain sections were sliced at 80 mm using a cryostat (Leica Biosystems). The serial brain sections were collected in a 24 well plate and washed with PBS for 3 times. The sections were incubated in 1% Triton in PBS at room temperature for 3 h. Then, sections were blocked with 10% Blocking One (nacalai tesque) in PBS with 0.3% Triton X-100 (Blocking solution) at room temperature for 1 h. The sections were treated with 1st antibody in Blocking solution at 4 °C for overnight. The sections were washed 3 times followed by secondary antibody treatment at 4 °C for overnight. Sections were counter stained with NeuroTrace fluorescent Nissl stain (fluorescent Nissl stain) (Invitrogen, N-21479) or 4’, 6-diamidino-2-phenylindole (DAPI) (Sigma, D9564). After this incubation, sectioned were washed, mounted and coverslipped.

The primary antibodies used in this study are: goat anti-Fos (Santa Cruz Biotechnology, sc52-g, 1:300), rabbit anti-Fos (Calbiochem, PC3, 1:5000), rabbit anti-Esr1 (Santa Cruz Biotechnology, sc542, 1:1500) and rabbit anti-TVA (1:300) (Murphy and Leavitt, 1999). The secondary antibodies are: Alexa Fluor 488 donkey anti-goat (Jackson immunoresearch, 705-547-147 1:1,000), Cy3 donkey anti-goat (Jackson immunoresearch, 705-165-147, 1:1,000), Alexa Fluor 488 donkey anti-rabbit (Jackson immunoresearch, 703-545-155, 1:1,000) and Cy3 donkey anti-rabbit (Jackson immunoresearch, 711-165-152, 1:1,000).

In situ hybridization
Fluorescent floating two-color in situ hybridization was performed according to standard methods. The mouse cDNA fragments of the Fos, SF1 and vGlut2 were amplified by PCR with the antisense primers containing the T7 promoter sequence. In vitro transcription was then performed from the PCR-amplified template using T7 RNA polymerase (Roche, Indianapolis, IN) for the synthesis of the antisense probes.
**Plasmid construction**

The sequences of EnvA-VSVG fusion glycoproteins coding extracellular and transmembrane domains of EnvA fused with the cytoplasmic domains of VSVG were subcloned into pCAGGS/ES vector and generated pCAGGS/ES-EnvA-VSVG (EnvA-VSVG) fusion envelope plasmid. To generate several mutated versions of EnvA (EnvAM4, EnvAM5 and EnvAM21)-VSVG, the mutated region of coding sequences were synthesized (GenScript) and subcloned into EnvA-VSVG. EnvAM4 has single mutation (K227A), EnvAM5 has three mutations (R223I, R224I, K227I), EnvAM21 has three (R213A, R223A, R224A) mutations (Rong et al., 1997).

**Production and purification of lentivirus**

All pseudotyped lentiviruses were packaged and produced in HEK293T cells. In brief, HEK293T cells were plated at a density of 1.2 x 10^7 per one 15 cm culture dish the day before transfection. Next day, HEK293T cells of one 15 cm culture dish were transiently transfected with the mixture of the pLenti-hSyn-Cre-WPRE (10.5 µg), psPAX2 (8 µg) and the envelope plasmid (8 µg) using polyethyleneimine “MAX” (PEI MAX) (Polysciences, Inc. 24765). Cells were washed with 15 ml of culture medium twice after six hours of transfection, and were cultured in 16-20 ml of UltraCULTURE medium (Lonza, 12-725F) with 2% fetal bovine serum for another 48 hours. The cell culture media were collected, centrifuged at 3,000 rpm for 15 min, and the supernatant was filtered with 0.45 um filter unit (Millipore, SCHVU01RE). The filtered virus-containing supernatant was loaded into the centrifuge tubes with 3 ml of 20% sucrose cushion at the bottom, spun at 7,000 rpm with Beckman SW-28 rotor for 20 hours at 4 °C. The pellet was resuspended in an appropriate volume of phosphate buffered saline without calcium and magnesium (PBS, -Ca2+, -Mg2+). Virus solution was further washed with 4 ml of PBS twice, and concentrated to an appropriate volume with the Amicon Ultra-4 centrifugal filter unit (100 KDa) (Millipore, UFC810024). Usually starting from twelve 15 cm culture dishes, virus solution was concentrated to 50 µl, to give a titer about 5 X 10^6 ifu/ml, 2 µl aliquots were stored at -80 °C.

**Production and purification of EnvAM21-pseudotyped rabies virus (CANE-RV)**

Pseudotyped rabies viruses were produced and purified following a standard protocol (Osakada and Callaway, 2013). To generate low affinity TVA binding EnvA (EnvAM21) coated pseudotyped rabies virus, we made a pLenti-CMV-H2B-GFP-2A-EnvAM21-RabiesG-WPRE-bGH-PolyA construct, which contains a nuclear localized GFP (nGFP) and a hybrid glycoprotein composed of EnvAM21 extracellular domain, transmembrane domain and the C-terminus of rabiesG glycoprotein. We packaged it into VSVG-coated lentivirus and used that lentivirus to infect low density HEK293T cells in 6 well culture dish to ensure 100% infection. The EnvAM21-RabiesG glycoprotein expressing cells were further expanded to 10 cm culture dish, then 15 cm culture dish. Expanded cells were aliquoted and stored as stable cell line that expresses EnvAM21-RabiesG glycoprotein. To package the EnvAM21-RabiesG coated CANE-RV-mCherry, SAD-DeltaG-mCherry rabies viruses were first expanded in B7GG cells (Osakada and Callaway, 2013), and subsequently, the virus containing culture supernatant was concentrated by centrifugation at 25,000 rpm with Beckman SW-28 rotor for 2 hours. The pellet was resuspended and used to infect the EnvAM21-RabiesG expressing stable cell line. After a series of culture media collection, the virus containing media were concentrated as above, resuspended in an appropriate volume, aliquoted and stored at -80 °C. The titer of CANE-RV is about 5 X 10^8 ifu/ml, and we use 1:100 dilution for injection in transsynaptic tracing experiments.

**AAV viral vectors**

pAAV-SynP-DIO-TVA-EGFP-RG (pAAV-SynP-DIO-sTpEpB)(Kohara et al., 2014) was a gift from Dr. Ian Wickersham and packaged in serotype AAV2/rh8 in the University of Pennsylvania Vector Core (4 X 10^12 genomic copies per ml). AAV2/1-EF1α-CAG-Flex-ChR2 (H134R)-EYFP(Zhang et al., 2010) (7.86 X 10^12 genomic copies per ml) was purchased from the University of Pennsylvania Vector Core. AAV2/1-CAG-Flex-GFP (2 X 10^12 genomic copies per ml) was purchased from the University of North Carolina Gene Therapy Department. AAV2/8-hSyn-Flex-TeLC-P2A-GFP was described previously (Zhang et al., 2015).

**Surgery**

Animals were lightly anaesthetized with isoflurane and then deeply anaesthetized with intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The anaesthetized animals were placed in a stereotaxic frame (David Kopf Instruments) and small craniotomies were made over the target area. Stereotaxic coordinates of virus injection were based on Paxinos and Franklin mouse brain atlas (VMHvl, AP -1.0, ML ±0.7, DV -5.65; barrel cortex, AP -1.5, ML ±3.4, DV -0.4; basolateral amygdala, AP -1.3, ML ±3.25, DV -4.4; dentate gyrus, AP -1.3, ML ±1.0, DV -2.0 from Bregma). Virus was delivered into the target site at a flow rate of 100 nl per min using a pulled thin glass capillary (Warner Instruments) connected to an UltraMicroPump controlled by a SYS-Micro4 Controller.
The thin glass capillary was slowly lowered to the target site to minimize the brain injury. For transsynaptically labeling experiment, virus (CANE-LV-Cre and AAV-SynP-DIO-TVA-EGFP-RG) injected animals were singly housed during 3 weeks recovery period, and then subjected to a brief social fear behavior followed by virus injection (CANE-RV-mCherry).

The injected viruses and the waiting period for virus transgene expression for the different experiments are: for experiments in Figure 3, CANE-LV-Cre (500 nl) or CANE-LV-Cre (300 nl) together with AAV-CAG-Flex-GFP (300 nl), >10 days; for experiments in Figures 4 and 5, CANE-LV-Cre (500 nl) together with either AAV-EF1α-Flex-ChR2-EYFP (500 nl) or with AAV-CAG-Flex-GFP (250 nl), > 4 weeks; for experiments in Figure 6, CANE-LV-Cre (400 nl) together with either AAV- hSyn-Flex-TeLC-P2A-GFP (400 nl) or with AAV-CAG-Flex-GFP (250 nl), > 3 weeks; for experiments in Figure 7, CANE-LV-Cre (300 nl) together with AAV-SynP-DIO-TVA-EGFP-RG (200 nl), > 3 weeks; for experiments in Figure 8, CANE-LV-Cre (300 nl) together AAV-SynP-DIO-TVA-EGFP-RG (200 nl), 3 weeks, then CANE-RV-mCherry (300 nl), additional 1 week; for experiment in Figure 9, CANE-LV-Cre (500 nl), > 10 days.

**Behavioral experiments for Fos and TVA immunostaining**

Adult male C57BL6 or FosTVA mice at ages more than 8 weeks were singly housed at least one day before the brief resident-intruder encounter. For visualizing Fos expression induced by social fear (in intruder), mice were introduced into the singly housed aggressive ICR male mouse cage for up to 10 min during which period the intruder mice were attacked 10~15 times (mice were taken out after being attacked 15 times), and then returned to their home cage. 2 hours later, the intruder mice were perfused (as described in the method for immunostaining above). For Fos and TVA double immunostaining, sampling was either perfused immediately (0 hour), or at 1, 2, or 6 hours after the social fear experience. For visualizing Fos expression elicited by attacking (aggressive) behavior, the resident male mice were exposed to male C57BL6 mice and allowed to interact for 30 minutes, and 2 hours later, mice were perfused and stained for Fos.

**Behavioral experiments for capturing social fear neurons (SFNs) with CANE virus**

Adult male FosTVA mice at ages more than 8 weeks were singly housed for at least one day, and then subjected to the social fear experience. Briefly, for capturing SFNs using CANE-LV or CANE-RV, they were introduced into a singly housed aggressive ICR male mouse cage for up to 10 min to be attacked 10~15 times, and returned into their home cage. One hour later, mice were anesthetized and undergo stereotaxic surgery for virus injection. There is no noticeable difference in the capturing efficiency when virus is injected between 1-2.5 hours after the behavior.

**Behavioral experiments for capturing aggression neurons with CANE virus**

Adult male FosTVA mice at ages more than 8 weeks were singly housed for at least two days, and then subjected to the aggression. Briefly, for capturing aggression neurons using CANE-LV, a non-aggressive adult male C57BL6 mouse was introduced into a singly housed FosTVA male mouse for 30 min during which period the aggressive resident attacked the intruder 20~25 times. (The attacks happen mostly in the first 10 minutes, subsequently, the intruder usually stays at a corner and the resident comes to check on the intruder to make sure it does not move around). One hour later, FosTVA mice were anesthetized and undergo stereotaxic surgery for virus injection.

**Behavioral experiments for capturing barrel cortex neurons activated by whisker-dependent tactile exploration with CANE virus**

Adult male FosTVA; Ai14 mice of more than 8 weeks old were anaesthetized and the desired whiskers were removed under the dissection microscope. After whisker removal, mice were singly housed for two days, and then were introduced into a novel environment with nesting materials in the dark for whisker dependent exploration. After 2 hours, the animals were deeply anaesthetized and the stereotaxic surgery was performed to inject CANE-LV-Cre.

**Behavioral experiments for capturing contextual fear conditioning activated neurons with CANE virus**

Adult male FosTVA mice of more than 8 weeks old were singly housed for at least two days, and then subjected to the contextual fear conditioning (Maren, 2001; Maren et al., 2013; Tayler et al., 2013). Briefly, individual FosTVA mouse was introduced into the conditioning chamber in context A for 500s. A foot shock (2s, 0.8 mA) was given at 200s, 280s, 360s, and 440s. Then the mouse was returned to its home cage. One hour later, the fear conditioned mouse was anesthetized and undergone stereotaxic surgery for CANE-LV-Cre and AAV-Flex-GFP virus co-injection.

**Quantification of the number of CANE captured neurons or Fos expressing neurons**
Fos stained sections were imaged on Zeiss 700 or 710 laser scanning microscopy (x10 or x20 objective lens, 10 mm optical thickness). The captured neurons and Fos expressing neurons were manually counted.

**Optogenetic activation of captured SFNs in the VMHvl in the home cage**

SFNs in the VMHvl were captured (as described above) using virus mixture of CANE-LV-Cre and AAV-EF1α-Flex-ChR2-EYFP or AAV-CAG-Flex-GFP in FosTVa mice. After more than 7 days of recovery periods, virus injected mice were implanted with a custom-made optic fiber (200 nm core diameter, Thorlabs) which was placed at 850 mm above the virus injection site and fixed on the skull with dental cement (Parkell). 3 to 4 weeks later, the animals were subjected to behavioral testing in their home cage. Before optogenetic stimulation, the animals were lightly anaesthetized with isoflurane and a ferrule patch cord (Doric Lenses) was connected to the ferrule optic fiber implant using mating sleeve (Thorlabs). Mice were allowed to recover from anesthetic for more than 30 min in their home cage placed in the behavior box which has infrared lights and 2 web-cameras (top and side). The optical fiber was connected to a laser (473 nm; Opto Engine LLC) via an optical commutator (Doric Lenses). Laser pulses were controlled through Master-8 pulse generator (A.M.P.I). Blue (473 nm) light was delivered with 20 ms pulses at 10 Hz, and the final output powers ranging from 2 to 3 mW/mm². The blue light was delivered when the virus injected mice showed the active contact behavior toward a male, or a female intruder. The duration and the interval of each light stimulation were 30 s and > 2 min, respectively. The average number of light stimulation for each mouse in each behavior test was 5-6.

After all behavior tests were completed, the mice were given a train of light (15 s on and 15 s off, 20 ms pulses, 10 Hz, repeated 30 times) to elicit ChR2- or photostimulation-dependent Fos expression in their home cage. Subsequently, animals were perfused at 2 hours after the final stimulation and processed for Fos immunostaining and ChR2-EYFP fluorescence imaging. The number of ChR2+, Fos+ double positive neurons was counted for each experimental animal.

**Optogenetic activation of captured SFNs in the VMHvl in the three-chamber social interaction test**

Capturing of SFNs, optic fiber implantation and blue light delivery were performed the same as described above for the home cage experiments. The three-chamber social interaction test was performed according to standard method (Moy et al., 2004). The size of each chamber is 30.5 x 19.5 x 31.0 cm, made with clear acrylic Plexiglas. The experimental mouse (connected to optogenetic setup) was placed in the center chamber and allowed free exploration in all chambers for 3 min. Subsequently, an unfamiliar adult male or female C57BL6 inside an inverted wire cup was placed in one of the side chamber (social chamber), while an empty wire cup was placed in the other side chamber (non-social chamber). The experimental mouse was allowed to explore all three chambers without blue light stimulation (OFF) for 3 min followed by blue light stimulation (10 Hz, 20 ms pulse-width, 2-3 mw/mm²) for 3 min.

Heat maps showing the location of experimental mouse were generated using a custom-written MATLAB code (Mathworks). Each frame of the video was first processed with a series of 2D filters to remove the background. Subsequently, for each pixel in the video, we computed its standard deviation in the temporal domain to approximate the time the mouse spent on the position. The results were also checked manually by another investigator and verified.

**Behavioral effects of silencing SFNs**

FosTVa mice in which either TeLC or GFP were expressed bilaterally in SFNs were subjected resident-intruder test for 2 min and were attacked 3–5 times by an aggressive resident. Then mice were returned to their own home cage and waited for 3 min, followed by social interaction test by introducing a non-aggressive adult male C57BL6 mouse for 5 min.

Mice were tracked using a custom-written MATLAB code (Mathworks). Each frame of the video was processed with a series of 2D filters to extract the body of the mice. The position of a mouse was defined as the regional max of the body after filtering. To compute the distance between the mice during the test, we measured the distance between the positions of the mice within a session, and normalized the distance according to the diagonal distance of the cage in the video of each session. The extracted mouse-to-mouse distance over the 5 min period was plotted (after applying a 1D Gaussian low-pass filter).

**Behavioral annotations**

Behavioral testing experiments were recorded from the top and side of the cage using web-cameras (Logitech and TekNet) at a frame rate 15-30 fps, simultaneously. Behavioral annotations were performed manually by an
investigator blind to the genotypes of injected virus using ANVIL annotation software (Kipp, 2001). The result of annotation was quantified and analyzed for each behavioral episode.

Electrophysiological recording in acute hypothalamic slices
SFNs in the VMHvl were captured using virus mixture of CANE-LV-Cre and AAV-EF1α-Flex-ChR2-EYFP in FosTVA mice. After a 3-4 weeks incubation period, mice were anesthetized with isofluorane, and transcardially perfused in ice-cold NMDG artificial cerebrospinal fluid (NMDG-ACSF; containing 92 mM NMDG, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 2 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 10 mM MgSO4, 0.5 mM CaCl2), and bubbled with 5% CO2/95% O2. The brain was then extracted and sectioned into 300 µm thick coronal slices using a vibratome (VT-1000S, Leica Microsystems) containing ice-cold oxygenated NMDG-ACSF. Coronal sections including the VMH were then bubbled in same solution at 37 °C for 8 min, and transferred to bubbled, modified-HEPES ACSF at room temperature (20-25 °C; 92 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 2 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 2 mM MgSO4, 2 mM CaCl2) for at least 1 h before recording. Recordings were performed in a submerged chamber, superfused with continuously bubbled ACSF (125 mM NaCl, 2.5 mM KCl, 4 mM MgCl2, 20 mM HEPES, 4 mM Na2ATP, 0.4 mM NaGTP, 0.5 mM EGTA). Light-activation of channelrhodopsin was delivered by TTL control of 473 nm LED (CoolLED, pE4000). Light intensity was set to be 100% for generation of spikes in ventral-medial hypothalamic ChR2+ neurons and pulse length was 2 ms and 20 ms at a various frequencies (5 Hz, 10 Hz, 20 Hz, 40 Hz). All electrophysiology data were analyzed off-line using Neuromatic package (Think Random) in Igor Pro software (WaveMetrics). The spike fidelity in captured SFNs was measured by counting the number of successfully evoked action potentials upon 473 nm photostimulation at the various frequencies.

Quantification of Axonal projection
To quantify the axonal projection from captured SFNs, we adopted a similar method used by Allen brain atlas (Oh et al., 2014). Briefly, 80 mm of coronal serial sections covering the whole brain were stained with fluorescent Nissl. Every other section was imaged using a Zeiss 700 laser scanning confocal microscopy (x10 objective lens, 20 µm optical thickness, 0.625 µm/pixel). Intensity of GFP signals were first normalized and then binarized by a custom written MATLAB (Mathworks) code. Region of interests (ROI) were manually outlined, and total GFP-positive pixels and total number of pixels in each ROI were counted. The data was normalized between animals by their own values in the AHA (anterior hypothalamic area).

Quantification of the number of transsynaptically labeled neurons
To quantify the number of transsynaptically labeled neurons from captured SFNs, coronal sections (80 µm) covering the whole brain were stained with fluorescent Nissl and imaged using a Zeiss 700 laser scanning confocal microscopy (x10 objective lens, 20 µm optical thickness). Intensity of mCherry signals was normalized by a custom written MATLAB code. Numbers of labeled cells in each ROI were manually counted. The data was normalized between animals by dividing with the number of starter neurons (GFP and mCherry double positive neurons in the VMHvl) in each animal.

Statistics
The analysis of linear regression and nonlinear regression was carried out using MATLAB (MathWorks). For the other statistical analysis, unpaired t test was performed using Prism 6 (GraphPad).
Linear regression: We used linear equation to fit the data for all conditions, with 95% confidence of the fitted parameters. The $R^2$ value represents the square of correlation coefficient, which quantifies the compatibility of the data with the regression model.
For nonlinear regression: We used general sigmoid function:
\[ f = \frac{a}{b + e^{-c(x-d)}} + e \]

to fit the duration of stationary behavior toward male intruder, with 95% confidence of the fitted parameters.

Clustering: k-medoids algorithm was used to cluster the behavioral data. We used plugin function “kmedoids” in Matlab, and chose two as the number of clusters.
Supplemental References


