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Fear Memory Engram and its Plasticity in the Hypothalamic Oxytocin System

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SUMMARY

Oxytocin (OT) release by axonal terminals onto the central nucleus of the amygdala exerts anxiolysis. To investigate which subpopulation of OT neurons contributes to this effect, we developed a novel method: virus-delivered **Genetic Activity-induced Tagging of cell Ensembles** (vGATE). With the vGATE method, we have identified and permanently tagged a small subpopulation of OT cells, which, by optogenetic stimulation, strongly attenuated contextual fear-induced freezing **and** pharmacogenetic silencing of tagged OT neurons impaired context-specific fear extinction demonstrating that the tagged OT neurons are sufficient and necessary, **respectively**, in controlling contextual fear. Intriguingly, OT cell terminals of fear-experienced rats displayed enhanced glutamate release in the amygdala. Furthermore, rats exposed to another round of fear conditioning displayed five-fold more activated magnocellular OT neurons in a novel environment than a familiar one, possibly for a generalized fear response. Thus, our results provide first evidence that hypothalamic OT neurons represent a fear memory engram.

INTRODUCTION

Emotional memory representations (also called memory engrams), such as for fear, are pivotal for animal survival. Fear-associated behaviors have evolved over millions of years in living systems, from lower to higher animals, so that they can sense, evaluate, respond and adapt to adequately deal with dangerous situations (Mobbs et al., 2015). Fear-related disorders, such as specific phobias and posttraumatic stress disorder (PTSD), are among the most prevalent human psychiatric conditions that pose debilitating health burdens to affected individuals and immense costs to society (Kessler and Bromet, 2013). Understanding the neural basis of fear learning, expression and extinction is of paramount importance for PTSD treatment, for example, by targeted circuit-specific therapeutics.

The hypothalamus is an evolutionary old and deeply located brain structure that relays fear-related emotional behavior in mammals. Fear memory engrams are thought to be distributed between the different brain regions in the nervous system, for example, the amygdala, hippocampus and the medial prefrontal cortex (mPFC) (Kitamura et al., 2017). Notably, the hypothalamus is reciprocally connected to different brain regions including the amygdala (Knobloch et al., 2012) and the mPFC (Heidbreder and Groenewegen, 2003), which in turn interact with various other brain regions in organizing fear memories (Tovote et al., 2015). These findings support the view that brain networks, composed of distributed cell assemblies, generate behavior, including the formation of memory representations or engrams. We thus hypothesized that the hypothalamus, being the oldest brain structure, which evolved over time alongside the newer higher brain structures, might be equipped with mechanisms to encode for engram-like features and capable of plasticity.

The central nucleus (CeA), a subregion of the amygdala, participates in the acquisition, consolidation, storage, expression and extinction of fear memories (Ciocchi et al., 2010; Herry et al., 2010; Letzkus et al., 2011) and the subsequent physiological response: the freezing behavior (Viviani et al., 2011). Hypothalamic oxytocin (OT) neurons precisely project to the lateral part of the CeA (CeL), where axonal OT release activates CeL neurons (Knobloch et al., 2012), which in turn inhibit GABAergic neurons in the medial part of the CeA (CeM), thereby attenuating the fear-related freezing response in rodents. However, the precise role of OT neurons and their projections to the CeA during fear learning is still not well understood. It is unknown which fraction(s) of OT neurons contributes to the anxiolytic effect, how OT neurons are recruited during different fear episodes and whether the hypothalamus-amygdala circuit is subject to fear-dependent plasticity and contextual specificity.

To tackle these questions, we developed a novel virus-delivered genetic method, named virus-delivered **Genetic Activity-induced Tagging of cell Ensembles** (vGATE) to tag fear experience-activated OT neurons in rats during contextual fear conditioning (FC) (Ehrlich et al., 2009; LeDoux, 2007). Here, we provide first evidence that establishes the role of OT neuronal ensembles in fear expression and extinction. We discovered that optogenetic activation of a small subset of fear exposure activated OT neurons drastically reversed freezing behavior (“unfreezing”) and their silencing impaired context-specific fear extinction. Importantly, the fear exposure tagged OT neurons display enormous capacity for experience-dependent plasticity by enhancing glutamatergic over OT-ergic transmission in the hypothalamic-amygdalar circuitry. These results clearly demonstrate that the fear exposure tagged OT neurons are both sufficient and necessary for attenuation of fear expression. Altogether, these results satisfy the key criteria of the synaptic plasticity memory hypothesis (Martin et al., 2000) in identifying

and validating a fear memory engram in the hypothalamic OT circuits. Thus, we conclude that memory engrams are not only restricted to higher brain regions, such as the hippocampus and cortex, but are also present in a lower brain region, such as the hypothalamus. This study is a shift in paradigm revealing the anatomical and functional connectivity organization of fear memory engrams as network-wide distributed cell assemblies, which include both higher and lower brain regions.

RESULTS

Activity-Dependent Tagging of OT neurons

To specifically label activated OT neurons, we developed and describe here a genetic method for virus-delivered Genetic Activity-induced Tagging of cell Ensembles (vGATE) (Fig. 1A). In the vGATE system, a c-fos promoter (P_{fos}) fragment (Schilling et al., 1991) drives the expression of the reverse tetracycline sensitive (tet) transactivator (rtTA) (Dogbevia et al., 2015; Dogbevia et al., 2016). In activated neurons, stimulation of the c-fos promoter rapidly induces rtTA expression. To obtain sustained rtTA expression for permanent tagging of the c-fos activated neurons, we designed an autoregulatory expression loop by introducing rtTA binding DNA sequences (tet operator sequences or $(tetO)_7$) upstream of the c-fos promoter (P_{fos}) to drive rtTA expression (the full genetic module is $(tetO)_7$ - P_{fos} -rtTA). In the presence of doxycycline (Dox), transient P_{fos} activity drives rtTA expression, which upon binding to $(tetO)_7$ takes over the transient c-fos promoter activity by establishing a Dox-controlled rtTA-dependent and self-sustaining autoregulatory loop (Fig. 1A) for persistent rtTA expression. A second virus (virus 2) is equipped with a bidirectional tet promoter (P_{tetbi}) that drives the expression of genes encoding for the Cre recombinase and a fluorescent proteins (Fig. 1A). Finally, a third virus (virus 3) is equipped with a cell type-specific promoter (OT in our case) that drives Cre-dependent expression of any gene(s) (Fig. 1A).

vGATE-assisted Dox-dependent rtTA expression in vivo

As a proof-of-principle, we first validated the vGATE method *in vitro* (Fig. S1A-D) and, subsequently, *in vivo*. For *in vivo* validation, we injected the vGATE construct, rAAV- $(tetO)_7$ - P_{fos} -rtTA, into the supraoptic nucleus (SON) of the hypothalamus. Three weeks later, rats were treated with four different conditions; +/- Dox and +/- salt loading (SL), which induced robust c-fos expression in OT neurons (Kato et al., 2010) (Fig. 1B-C, Table S1). Only under the +Dox/+SL condition, robust rtTA expression under a synthetic c-fos promoter was detected (red) within 24 hours in roughly all of the OT neurons (Hamamura et al., 1991) (Fig. 1C). This result shows that the vGATE method is compatible with the endogenous c-fos expression. With rAAV- P_{OT} -Venus as a tracer, it was possible to verify proper virus targeting and spread of infection after injection.

In a different set of experiments, we injected a cocktail of three viruses called here OT^{vGATE}: (virus 1 = rAAV- $(tetO)_7$ - P_{fos} -rtTA, virus 2 = rAAV- P_{tetbi} -Cre/YC3.60, virus 3 = rAAV- P_{OT} -FLEX-hChR2-mCherry) and analyzed for YC3.60 (virus 2) and hChR2-mCherry (virus 3). Under +SL/+Dox robust YC3.60 (P_{tetbi} -Cre/YC3.60) and hChR2-mCherry (Cre-dependent: P_{OT} -FLEX-hChR2-mCherry) immunosignals were co-localized in the majority of OT neurons (96.30% \pm 1.88 SON, 97.22% \pm 2.66 PVN, Fig. 1D). In these animals, we found that 2.4 \pm 1.1% (48 out of 2011) of the YC3.60/hChR2-mCherry expressing cells in the paraventricular nucleus (PVN) and 5.7 \pm 1.4% (132 out of 2320) of cells in the SON were non-OTergic (n=6 animals). In the +Dox /-SL (Fig. 1D) condition, we found that 7.3 \pm 2.2% YC3.60/hChR2-mCherry

positive cells around the SON were non-OTergic (179/2450 neurons) and $0.8 \pm 0.5\%$ YC3.60/hChR2-mCherry positive cells around the PVN were non-OTergic (22/2450 neurons, $n=6$; n =animal number, 6 sections per animal). These unidentified YC3.60/hChR2-mCherry-positive cells were within a radius of 400 μm from the border of the respective nucleus. Under -SL/-Dox conditions (**Fig. S1E**), we observed virtually no YC3.60 and mCherry labeling ($0.1 \pm 0.03\%$, 5 out of 540 for PVN, $0.3 \pm 0.09\%$, 22 out of 722 for SON, $n=6$, 6 sections per animal), a result of minimal leakiness of the $\text{P}_{\text{tet}^{\text{bi}}}$. Furthermore, we observed more than 93% ($94.74\% \pm 1.75$ for SON and $93.56\% \pm 2.46$ for PVN, **Table S1**) co-localization of endogenous c-fos and mCherry in vGATE animals subjected to SL for 5 consecutive days (**Fig. 1E**).

Optogenetic Stimulation of Tagged OT Neurons Reverses Freezing Behavior in Fear-Conditioned Rats

We investigated what fraction of the entire OT population actually contributes to the anxiolytic effect and how OT neurons are recruited during the expression of fear using contextual FC in rats (**Fig. 2A**). We found that only a small number of OT neurons in the SON and PVN expressed c-fos during fear exposure (**Fig. S2A, B**). Next, we generated OT^{vGATE} rats with bilateral injections of the vGATE viral cocktail to permanently tag activated OT neurons in the SON and PVN for activity manipulation by humanized channel rhodopsin (hChR2) (virus 1 = rAAV-(tetO)₇- P_{fos} -rtTA, virus 2 = rAAV- $\text{P}_{\text{tet}^{\text{bi}}}$ -Cre/YC3.60, virus 3 = rAAV- P_{OT} -FLEX-hChR2-mCherry) (OT^{vGATE}). We specifically chose day 3 (d3) for the Dox injection, since we wanted to exclusively label fear-activated OT neurons, but not pain-sensitive OT neurons. As a control, we generated rats that constitutively expressed rAAV-delivered hChR2 in all OT neurons ($\text{OT}^{\text{Constitutive}}$) in the SON and PVN. While the $\text{OT}^{\text{Constitutive}}$ group displayed viral expression in virtually all OT neurons ($99.4 \pm 0.8\%$, $n=4$, **Fig. 2B1**), in the OT^{vGATE} group only a small fraction of the OT neurons was tagged during fear expression (341/2470, $13.8 \pm 0.7\%$ of OT neurons in the SON and 331/2666, $12.4 \pm 1.6\%$ of OT neurons in the PVN) out of ~ 6600 cells comprising the PVN and SON in rats (Althammer and Grinevich, 2017, **Fig. 2B2; Table S2**). We found that only 1.2% of non-OTergic cells were labelled non-specifically (57 cells out of 2051 cells in total labelled via vGATE, PVN/SON combined, $n=4$, 6 sections per animal) in the fear conditioned animals (**Fig. S2C**). To determine if OT^{vGATE} neurons project axons terminating within the CeA subregion of the amygdala, we analyzed brain slices containing the CeL and found mCherry-positive fibers in CeL, which were also OT-immuno-positive (**Fig. 2B2**). Optogenetic stimulation of these OT axons in the CeL with blue light (BL) induced a prominent decrease in the freezing response (unfreezing) in both groups. Surprisingly, the BL-evoked unfreezing effect in the OT^{vGATE} group was much stronger (Freezing time: $6.9 \pm 1.1\text{s}$ vs. $38.1 \pm 7.6\text{s}$, $p<0.0001$, **Fig. 2C2**) and occurred faster (Onset: $3.8 \pm 0.7\text{s}$ vs. $25.9 \pm 10.8\text{s}$, $p<0.0001$, **Fig. 2D**) than in the $\text{OT}^{\text{Constitutive}}$ group (**Fig. 2C1, 2D**). To rule out that the observed behavioral changes were mediated by brain regions other than the CeA, we injected 4 animals with rAAV-(tetO)₇- P_{fos} -rtTA, rAAV- $\text{P}_{\text{tet}^{\text{bi}}}$ -Cre/YC3.60 and rAAV- P_{OT} -FLEX-GFP. Four animals injected with rAAV- P_{OT} -Venus served as a control. We analyzed the brains of the vGATE-injected animals and did not find any GFP-positive fibers outside of the CeA, whereas control animals showed prominent labeling of OT fibers in various brain regions, as previously reported (**Fig. S2D**, Knobloch et al., 2012). This indicates that fear-activated, vGATE-labeled OT neurons project exclusively to the CeA. In addition, we did not find a single mCherry-positive fiber in the CeA that was negative for OT-immunoreactivity ($n=6$ animals, 6 sections per animal,

in which >15mm of total axon length was analyzed) indicating that axonal projections from vGATE-labeled axons exclusively represent OT fibers. Conversely, we found no difference between OT^{Constitutive} (98.2 ± 1.4%, n=6) and OT^{vGATE} (96.9 ± 2.1%, n=6) groups regarding the labeling of OT-positive fibers in the CeL, indicating that all OT neurons projecting to the CeL are fear-sensitive. Next, to provide additional proof that OT^{vGATE} neurons precisely project to the CeA, we injected green retrobeadsTM into the CeA of rats with OT^{vGATE} neurons labeled by mCherry. The anatomical analysis revealed that 94.6 ± 3.1% of mCherry-positive OT neurons (1337 OT^{vGATE} neurons, PVN/SON combined, n=4) contained green retrobeadsTM (**Fig. S2E**), indicating that the vast majority of OT^{vGATE} neurons project to the CeA.

Because our FC paradigm comprised an additional shock session (d14, memory reinforcement) to achieve higher basal freezing rates, we wanted to rule out the possibility that this session leads to the recruitment of new OT cells. Therefore, we performed another experiment in which animals were injected with rAAV-(tetO)₇-P_{fos}-rtTA and rAAV-P_{tet}bi-Cre/YC3.60 and subjected to our FC paradigm (d3 Dox injection, d4 shock, **Fig. S2F**). On d14, animals received an injection of rAAV-P_{OT}-FLEX-hChr2-mCherry and were subjected to another round of FC two weeks later. Then, one group received another Dox injection and the control group was injected with saline one day prior to the additional shock session on d29 (**Fig. S2F**). While the group that was injected with Dox twice displayed normal labelling (224/2033, 11 ± 1.4% for PVN; 260/1940, 13.4 ± 1.7% for SON, n=5, **Fig. S2G**) of OT neurons, we only found a total of 11 mCherry-positive cells in the PVN (11/2020, 0.5%, n=5) and 17 mCherry-positive cells in the SON (17/2177, 0.8%, n=5) in the group receiving saline in the first round (**Fig. S2G**). Thus, this experiment demonstrates that, once Dox has been cleared from the brain, only few additional OT cells get labeled. To verify that the comparable labeling of cells also held true on the axonal level, we compared OT-positive fibers within the CeA of animals perfused after 14 days with animals perfused after 43 days (2 weeks after the d29 shock session, **Fig. S2F**) and found no difference in vGATE-mediated labeling (d14: 96.9 ± 2.1%, n=6 vs d43: 95.3 ± 3.1%, n=6).

Since a recent study highlighted the existence of freezing (somatostatin-ergic) and flight (corticotropin releasing hormone-ergic) promoting neurons (Fadok et al., 2017), we sought to find out which cell types within the CeL get activated upon BL stimulation. Therefore, we injected rats with our vGATE viruses (rAAV-(tetO)₇-P_{fos}-rtTA and rAAV-P_{tet}bi-Cre/YC3.60) and either rAAV-P_{OT}-FLEX-Venus (n=4) or rAAV-P_{OT}-FLEX-hChr2-mCherry (n=4) and subjected them to our FC protocol (**Fig. 2A**). On day 15 we illuminated the CeL for 2 minutes and sacrificed animals 90 minutes later. We found that fear exposure itself did not cause c-fos expression in the CeL, while BL stimulation robustly induced c-fos in this structure. In the BL-stimulated animals, we counted 888 CRH-positive neurons and 290 SOM-positive neurons, while 104 neurons expressed both markers. 84 ± 3.6% of exclusively SOM-positive neurons, 11 ± 0.9% of the exclusively CRH-positive neurons and 58 ± 4.3% of the mixed population expressed c-fos (**Fig. S2H**). Thus, these findings suggest that OT release in the CeL predominantly activates SOM-positive interneurons.

Fear Experience-Dependent Anatomical and Functional Plasticity of the Hypothalamic OT Neurons Projecting to the CeA

To investigate potential anatomical and molecular changes following fear experience, we generated

OT^{vGATE} rats (virus 3: rAAV-**P**_{OT}-FLEX-GFP) and quantitatively analyzed OT axons within the CeL. In parallel, we analyzed fear-naïve rats expressing **P**_{OT}-Venus for constitutive labelling of all OT neurons (OT^{Constitutive}, **Fig. 3A1**). Although the total length of axonal segments was similar in both groups (**Fig. 3A3**), there was a substantial increase (~3-fold) in the numbers of GFP-positive axonal varicosities containing immunosignals of the vesicular glutamate transporter 2 (vGluT2) in the OT^{vGATE} group (**Fig. 3A3, Table S3**). Furthermore, we found that the detectable OT levels in vGATE fibers were at least 3-fold lower compared to the fear-naïve animals injected with **P**_{OT}-Venus, potentially resulting from the enhanced glutamate expression (Fear naïve: $58.0 \pm 8.4\%$ vs. Fear experienced: $15.3 \pm 3.1\%$, signal intensity, n=4, 6 sections per animal, **Fig. S3A, Supplemental Materials**).

To elucidate the seemingly counter-intuitive observation that the activation of a small subset of OT neurons resulted in a stronger behavioral response than the activation of axons originating from virtually all OT neurons (OT^{Constitutive} group) in the CeL, we next aimed to investigate the underlying circuitry within the CeA by *ex vivo* electrophysiology in acute brain slices from fear-naïve OT^{Constitutive} and fear-experienced OT^{vGATE} groups (**Fig. 2D**). In the naïve group, all OT neurons constitutively expressed hChr2 (OT^{Constitutive}), whereas in the OT^{vGATE} group, a small fraction of OT neurons was labeled with the vGATE virus cocktail (Virus 3 = **P**_{OT}-FLEX-hChr2-mCherry). In both groups, BL stimulation of OT axons in the CeL (**Fig. 3B**) induced an increase of IPSC frequencies in neurons of CeM, which receive direct synaptic input from GABA-ergic neurons of the CeL (**Fig. 3C1**). Interestingly, the BL-induced increase of CeM IPSC frequencies in the OT^{vGATE} group was almost entirely blocked by the glutamate AMPA receptor antagonist NBQX, while it only had a minor effect on the control group (**Fig. 3C2**). On the other hand, the application of the OT receptor antagonist dOVT diminished the BL-induced increase of CeM IPSCs in the OT^{vGATE} group by only 50%, while it prevented the increase of CeM IPSCs in the control OT^{Constitutive} group entirely (**Fig. 3C2**).

In order to rule out that the observed differences were not a result of the fear experience, we injected an additional group of animals with rAAV-**P**_{OT}-hChr2-mCherry (OT^{Constitutive}) and subjected them to the same paradigm as the OT^{vGATE} group (**Fig. 2A and Fig. S3B1**). The analysis of the fear-conditioned OT^{Constitutive} group revealed that the BL-induced unfreezing effect, onset and duration of mobility time (**Fig. S3B2-B4**), as well as the effects of NBQX and dOVT on IPSC frequencies in CeM neurons (**Fig. S3B5-B7**) were comparable to those observed in the OT^{vGATE} group (**Fig. 2C and 2D, Fig. 3C1, C2**). These findings suggest that the initial exposure to FC induced plastic changes in OT neurons, resulting in prevalent release of glutamate from their axonal terminals within the CeL. Due to the similarity of behavioral and amygdala responses to OT axon stimulation in fear-experienced OT^{Constitutive} and OT^{vGATE} groups, the observed effects might stem from the same population of OT neurons naturally activated in both groups of rats during fear expression (**Fig. 2B**).

To test whether the *ex vivo* findings have a functional relevance *in vivo*, we blocked the OT receptor specifically by an antagonist (OTA) (L-368,899, 1mg/kg i.p.), which crosses the blood brain barrier (Eliava et al., 2016). First, we injected rats bilaterally in the SON and PVN with **P**_{OT}-hChr2-mCherry (OT^{Constitutive}) for optogenetic tagging and implanted optic fibers into the CeA (**Fig. 3D1**). Next, we subjected the animals to the FC paradigm. On day 3, the treatment of animals with OTA, 40 min prior BL stimulation (session 1: OTA + BL1), completely prevented the BL-induced unfreezing effect (**Fig. 3D2**). However, 2 weeks later, when we exposed the very same animals to the second session of

FC (session 2: OTA + BL2), the animals displayed a rapid BL-induced unfreezing response despite prior application of the OTA (**Fig. 3D3**). Interestingly, the onset of BL-induced unfreezing occurred much faster ($6 \pm 0.8s$ vs. $25.9 \pm 10.8s$, $p < 0.0001$, **Fig. 3D4**) than in the fear-naive OT^{Constitutive} group (**Fig. 2D**). To exclude that the observed unfreezing effects were a result of OTA-induced changes in plasticity originating from the first round of FC, we processed an additional group of animals, constitutively expressing hChR2 in OT neurons, which received saline (instead of OTA) in the first session and OTA in the second session, and obtained similar results (**Fig. S3C1-3**) as in the initial group, which was treated with OTA in both sessions (**Fig. 3D**). Thus, our data indicate that fear experience drives a shift from OT to glutamate release from OT axons in the CeL of fear-experienced groups. Moreover, the enhanced responsiveness of CeL neurons to glutamate and its correlated effect that could be abolished by application of NBQX (**Fig. 3C2**), may indicate a transient, fear experience-induced upregulation of AMPA receptors in these cells.

Inhibition of Fear-Activated OT Neurons Impairs Fear Extinction in a Context-dependent Manner

To provide further evidence that OT neurons activated by fear exposure attenuate fear expression, we hypothesized that the silencing of OT^{vGATE} neurons inhibits fear extinction specifically in the context, where these neurons had originally been activated. Therefore, rats (n=6) were injected with a virus expressing the modified human muscarinic receptor hM4D(Gi) under the control of the OT promoter exposed to contextual FC and extinction paradigms (**Fig. 4A**). All four groups of animals (OT^{vGATE} A-A, OT^{vGATE} A-B, OT^{Constitutive} A-A and OT^{Constitutive} A-B) were subjected to FC in context A (d1 shock, d2 shock, d3 Dox injection, d4 exposure). To induce fear extinction, the two groups (OT^{vGATE} A-A and OT^{Constitutive} A-A) were exposed to the same context A for four consecutive days (40 mins each session) without any electrical shock, receiving a daily i.p. injection of clozapine-N-oxide (CNO) 40 minutes prior to testing (control groups received saline). In parallel, we processed two additional groups of animals (OT^{vGATE} A-B and OT^{Constitutive} A-B, n=6) in analogy to the first groups, which were submitted to a first round of FC (in Context A) and then transferred to a novel context (Context B, **Fig. 4** blue star) prior to being submitted to the extinction protocol. We introduced the novel context (Context B) to specifically investigate the potential role of OT in context-dependent fear extinction. This context represented a novel environment that was distinct in terms of visual, tactile and olfactory cues (**Fig. S4A**). Importantly, rats conditioned in context A did not display contextual FC in context B (**Fig. S4B**), clearly demonstrating that animals are able to discriminate between the two boxes (the complete data set on freezing behavior is provided in **Table S4**). Silencing of all OT neurons by constitutively expressed hM4D(Gi) and application of its agonist CNO resulted in impaired fear extinction in both context A and B (**Fig. 4B1**, **Table S4**). We next applied the vGATE technique to express hM4D(Gi) specifically in OT neurons (virus 3 = rAAV-P_{OT}-hM4D-Gi). We discovered that the silencing of OT fear neurons that were tagged in context A impaired fear extinction in context A, but not in the novel context B (**Fig. 4B2**). As CNO has been reported to potentially activate certain neurons (Gomez et al., 2017), we performed control experiments (without virus injection, n=6), where CNO alone had no effect on context-specific fear extinction (**Fig. S4C**). To further rule out that the observed differences in fear extinction dynamics were a result of the two different contexts, we subjected naïve, non-injected animals to fear extinction

sessions in both contexts and found comparable freezing times throughout the extinction sessions (n=6, **Fig. S4D**).

To demonstrate that the observed impairments in fear extinction stem from inhibition of local OT release onto the CeA, we injected rats (n=8) with the vGATE cocktail (virus 3 = rAAV- P_{OT} -hM4D-Gi) and implanted bilateral guide cannulas above the CeA for local infusion of CNO (**Fig. 4C1-C2**). CeA local infusion of 1 μ M CNO (Stachniak et al., 2014) 15 min prior to each session impaired fear extinction in context A, but not context B (**Fig. 4C3**), thereby confirming our previous results. To verify proper targeting of the CeA, we infused retrobeadsTM immediately after the last extinction session and killed the animals 10 mins later to avoid spreading of the tracer (**Fig. 4C2**). The control group (n=8) injected with vGATE-GFP viruses displayed normal fear extinction in both contexts, indicating that local infusion of CNO *per se* does not impair fear extinction (**Fig. 4C3**).

Activity of OT Neurons in the SON is Context-Dependent

To shed light on the cellular mechanisms underlying the context-dependent effect of neuronal inhibition (**Fig. 4B2**), we labeled the OT^{vGATE} neurons in green with the rAAV- P_{OT} -FLEX-GFP (virus 3) and co-stained sections for endogenous c-fos in red to identify the fraction of OT cells that was active during re-exposure to the same context (**Fig. 5A**). In line with previous results (**Fig. 2B2**), the first round of context A FC induced tagging of 10.8% of OT neurons in PVN and 11.9% in SON, visualized by intrinsic GFP (**Fig. 5A**). The second round of context A FC induced c-fos signal in 9.5% of OT neurons in the PVN and 16.2% in the SON (**Fig. 5A**). The overlap of virally expressed GFP and c-fos was 18% in the PVN and 89% in the SON, suggesting a context-dependent activity of OT neurons specifically in the SON as well as a functional difference between the two nuclei. The difference in re-activation of OT cells in context A→A vs. context A→B was significant for the SON (Context A-A: $89 \pm 5.5\%$ vs. Context A-B: $22 \pm 3.6\%$, n=6, $p < 0.0001$), but not the PVN (Context A-A: $18 \pm 2.7\%$ vs. Context A-B: $31 \pm 3.7\%$, n=6, $p = 0.2805$, **Table S5**). It is important to note that the group of animals that was first exposed to context A displayed ~ 5-times more activated neurons in both PVN and SON after a further exposition to context B (PVN: Context A vGATE-GFP - 9.6%, context B c-fos - 47.1%; SON: Context A vGATE-GFP - 11.7% context B c-fos - 56.4%, **Fig. 5B, Table S5**). This 5-fold difference in neuronal OT activity in context B correlated with the dramatically increased OT concentration in the blood ($27.64 \pm 5.4\%$ pmol/ml vs. $86.7 \pm 12.9\%$ pmol/ml, **Fig. S5A**). However, despite the massive recruitment of new cells, the overlap of GFP-positive and c-fos expressing OT neurons in the SON in context B amounted to only 22%. Taken together, these results indicate that re-exposure to a familiar FC context reactivates the same fraction of OT neurons, while exposure to a novel context recruits an entirely different population of cells. Therefore, it is plausible that OT neurons participate in a long-lasting fear memory engram, which coordinates the OT-ergic response to different contextual fear episodes.

Finally, to investigate whether OT engram cells display distinct basic electrophysiological properties than non-engram OT cells, we performed an additional experiment, where we injected animals (n=12) with vGATE (Virus 3 = P_{OT} -FLEX-GFP) and P_{OT} -mCherryChr2 into the SON only. Animals were fear conditioned and received injection of Dox on day 3 and processed for *ex vivo* patch clamp recordings. In these animals, non-engram OT cells expressed red fluorescence, while vGATE-labeled engram cells appeared in yellow (green + red) (**Fig. 5C1**). We analyzed access resistance,

membrane capacitance, membrane potential, spontaneous EPSC amplitudes and frequencies, and discharge profile upon current steps. Interestingly, we found significant differences in membrane capacitance (OT non-engram: 20.81 ± 1.25 pF, $n=16$; OT engram: 26.13 ± 1.52 pF, $n=24$; $p<0.05$, **Fig.5C2**) and frequency of spontaneous EPSCs (OT non-engram: 1.87 ± 0.51 and OT engram: 3.18 ± 0.54 Hz, $p<0.05$, **Fig.5C2**). All other parameters did not significantly differ between OT^{Constitutive} and OT^{vGATE} cells (**Fig. S5B**). Thus, these findings suggest that vGATE-labeled OT engram cells have a larger membrane surface and receive more excitatory inputs, a feature of engram cells that has already been described in the hippocampus (Kitamura et al., 2017; Ryan et al., 2015).

To investigate whether increased glutamatergic input onto OT^{vGATE} could potentially underlie the observed differences in membrane capacitance and increased spontaneous EPSCs, we stained brain slices of vGATE-injected rats with the vesicular glutamate transporter vGluT2 and compared the number of vGluT2-positive puncta in the immediate surroundings (surface of soma and 5 μ m radius around each OT-positive neuron). We found that the number of vGluT2 puncta encompassing OT engram (OT^{vGATE}) cells (18.6 ± 1.2 , 130 cells, $n=3$) was significantly higher ($p<0.001$) compared to non-engram OT neurons (8.3 ± 0.9 , 1044 cells, $n=3$) (**Fig. 5C3**). These results suggest that OT engram cells receive more prominent glutamatergic input than OT non-engram cells, potentially explaining the unique electrophysiological properties of engram OT neurons (**Fig. 5C2**).

Parvocellular OT Neurons Activate Magnocellular OT Neurons in a Novel Context

Since the OT system is composed by two types of neurons, magnocellular (magnOT) and parvocellular (parvOT) neurons (Rhodes et al., 1981; Swanson and Kuypers, 1980; Swanson and Sawchenko, 1983), we next aimed to investigate which hypothalamic OT cell types are reactivated during the two fear episodes. To distinguish between magnOT and parvOT neurons, we used the retrograde marker Fluorogold (FG), which labels magnOT, but not parvOT cells (Naumann et al., 2000). Injecting the cocktail of vGATE viruses (virus 3 = rAAV-**P**_{OT}-FLEX-GFP), we found that virtually all FG-negative parvOT neurons of the PVN (parvOT neurons are not present in the SON) were labeled with GFP in context A and contained c-fos in context B, while only a small fraction (14-20%) of FG-positive magnOT neurons of the PVN and SON labeled with GFP in context A were c-fos positive in context B (**Fig. 6A**; **Table S6**).

Following previous findings that PVN parvOT neurons provide synaptic inputs onto OT neurons in the SON to activate magnOT neurons (Eliava et al., 2016), we aimed to silence parvOT neurons to elucidate their functional relevance in the novel context B during repeated fear exposure. To achieve this, we expressed hM4D(Gi) in a Cre-dependent manner in the PVN via a CAV2-Cre virus injected into the SON (**Fig. 6B**) to silence parvOT inputs onto magnOT neurons. We found that silencing of parvOT neurons in context B prevented the massive induction of c-fos expression in the SON (**Fig. 6C-G**, **Table S6**), indicating a critical role of parvOT neurons during repeated fear exposure. Similar results were obtained in the PVN (**Fig. S6A-E**), suggesting that parvOT neurons might also directly or indirectly control magnOT activity within the PVN neurons. To exclude that the prevented rise in c-fos expression was a result of the CNO treatment, we subjected animals to the same paradigm, where they received CNO, but were not injected with Cre-dependent hM4D(Gi) rAAV, and found no differences in c-fos expression in OT neurons of the SON and PVN (**Fig. S6F**).

DISCUSSION

Here, we provide first evidence that the vGATE-assisted OT-tagged neurons during fear expression are sufficient for controlling fear behavior and necessary for fear extinction. Furthermore, fear experience drives enormous plasticity, mediating a shift from OT to glutamate signaling in the CeL. We propose that repeated fear exposure activates the OT-ergic fear engram cells for rapid adaptive behavioral responses.

Context-dependent Fear Memory Engram and its Plasticity in the Hypothalamic OT System

The hypothesis that learning-activated cell ensembles form memory engrams has received substantial attention (Kitamura et al., 2017; Liu et al., 2012; Reijmers et al., 2007). While the memory engrams have been discovered in higher brain regions such as the cortex, hippocampus or basolateral amygdala (Kitamura et al., 2017; Liu et al., 2012), no study – to our knowledge – has addressed the potential role of the lower brain regions such as the hypothalamus in the modulation of fear memory. In a number of studies, the OT system has been highlighted as a key structure modulating various aspects of fear, such as acquisition, expression and extinction.

To investigate whether OT neurons encode for context-specificity, we applied the vGATE method to identify and manipulate potential OT engram cells and labeled a small fraction (~11-14%) of hypothalamic OT neurons by contextual FC. Remarkably, majority of OT^{vGATE} neurons project to the CeA and optogenetic activation of the OT neuronal axonal fibers/terminals in the CeA elicited rapid onset of unfreezing. We further found that upon fear exposure, OT neurons show enhanced glutamatergic over OT-ergic transmission in the CeM (**Fig. 3D1**) with increased membrane capacitance (**Fig. 5C2**) and elevated vGluT2-levels in OT axons terminating in the CeA (**Fig. 5C3**), consistent with previous studies (Cicchi et al., 2010; Li et al., 2013; Penzo et al., 2015). Thus, our results provide the first evidence for fear-induced long-term plasticity (15 days) of the OT-CeL circuit. It seems indeed possible that a single episode of fear conditioning permanently alters OT/glutamate balance, perhaps for the strengthening of synaptic connections for rapid response and memory maintenance. These findings supports the general notion that coordinated release of slow-acting neuromodulator peptides and fast-acting amino acid transmitters could be a likely mechanism to modulate cognitive, emotional and metabolic processes (van den Pol, 2012).

Based on our findings, we hypothesized that OT^{vGATE} engram neurons might be also involved in fear extinction. Given the key role of CeA in fear extinction, we next investigated if the context-specific OT neuronal circuits would control context-specific fear extinction. While inhibition of virtually all OT neurons resulted in a context-independent impairment of fear extinction, the inhibition of OT^{vGATE} neurons exclusively affected fear extinction in the context in which the cells had originally been labeled. These OT^{vGATE} neurons might be required for the suppression of memory expression, safety cues and priming for extinction. This could explain the unaffected fear extinction of animals in context B after silencing of OT^{vGATE} neurons that were tagged in context A, where inhibition of these fibers might no longer be relevant, since newly recruited OT neurons in a different context could compensate for the reduced release of OT in the CeA, by release of this neuropeptide in other brain regions (**Fig. 4B2 and 4C3**). Therefore, we concluded that the silencing of OT^{vGATE} engram cells impaired fear extinction, demonstrating necessity, a likely result of blocking OT/glutamate-mediated neural modulation in the

CeA. We propose that the OT^{VGATE} cells represent a “neuromodulatory” engram, which plays a vital role in controlling a simple “contextual memory” representations for context-specific behavioral expression and the extinction phenotype.

Context-independent Control by Parvocellular OT Neurons of the Hypothalamic OT System

OT neurons are divided into two types: magnOT and parvOT neurons. Despite their relatively low number (~ 70 neurons per rat), parvOT neurons modulate important physiological processes, such as cardiovascular functions, hunger and pain (Althammer and Grinevich, 2017). The present study highlights the functional relevance of parvOT neurons in contextual FC because virtually all parvOT neurons that were activated in context A were also reactivated in context B, and the silencing of parvOT neurons almost entirely blocked the recruitment of novel magnOT neurons in an unfamiliar context. Therefore, we hypothesize that parvOT neurons operate as a class of 'master-cells', orchestrating magnOT neuron activity and subsequent OT release of into the blood. Although the precise role of OT neurons during fear conditioning remains to be determined, it is plausible that the coordinated activity of parvOT neurons may facilitate global priming effects and/or induce plasticity of magnOT neurons in various physiological demands (Theodosios et al., 1986; Tobin et al., 2012). In line, the global activation of the OT system, driven by parvOT neurons, can be crucial for metabolic, autonomic, and behavioral adaption to exacerbated fear-induced stress. In our current study, the 5-fold increase in c-fos expression quite accurately matches the 5-fold elevation of OT levels in the blood (**Fig. S6A**). This indicates that newly activated OT neurons in context B are magnOT cells, which release the neuropeptide from the neurohypophysis into the blood. Thus, the newly recruited OT neurons in both hypothalamic nuclei provide the massive release of OT into the peripheral circulation to regulate various physiological demands to cope with an exacerbated stress response (Yang et al., 2013).

Conclusion

We have identified and validated a “neuromodulatory” engram, composed specifically of OT cells, that fulfills the key criteria of the synaptic plasticity memory hypothesis (Martin et al., 2000): sufficiency, necessity and plasticity. We demonstrated that OT neurons of the SON participate in a fear memory engram (**Fig. 7**), while the parvOT neurons coordinate OT release in a context-independent manner. Our findings will facilitate the investigation of pathophysiological mechanisms underlying emotion-associated mental disorders, especially PTSD's symptoms, their potential treatment by exogenous OT (Althammer et al., 2018; Frijling et al., 2014) and virus-delivered genetic-based targeted therapeutics.

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AUTHOR CONTRIBUTION

Design and development of the vGATE system (MTH), characterization of vGATE (MTH, J-MW, GKD, IB), Project conception (MTH, AC, VG), Implementation of other methodologies (MTH, J-MW, RH, MEL, RS, PP, HB), *ex vivo* patch-clamp electrophysiology (SG, JW, PD, YT, XL, AC), Viral injections (FA, MdSG, SG), Fear behavior (FA, MdSG, SG, DK, AJ, AR, JS, CP), Immunohistochemistry (FA, MdSG, AL, ME, HSKB), HPLC MS/MS (VC, YG), Data analysis (FA, MSdG, SG, AR, J-MW, AC), Confocal microscopy (FA, MEL, MdSG, AL), Manuscript preparation (MTH, AC, VG), Extended manuscript preparation (FA, HSK-B, RH, SCH, RS, HB, AC, MTH and VG), Supervision (MTH, AC, and VG), Project administration (MTH, AC, VG), Funding acquisition (MTH, AC, VG).

CONFLICTS OF INTEREST

The authors declare no competing interests.

FIGURES

Figure 1. Operating Principle of the vGATE System and Activity-dependent Tagging of OT Neurons

(A) Scheme of the vGATE system: Virus 1 (rAAV-(tetO)₇-P_{Fos}-rtTA), Virus 2 (rAAV-P_{tet}bi-Cre/YC3.60) and Virus 3 (rAAV-P_{OT}-FLEX-hChR2-mCherry). **(B)** Rats were injected with vGATE viral cocktail Virus 3 (rAAV-P_{OT}-FLEX-hChR2-mCherry) into the hypothalamic SON and treated with Dox in combination with osmotic stimulation (salt loading). **(C)** Dox-dependent rtTA expression is only detected in the Stimulus +Dox group. To verify correct viral targeting of the SON, rAAV-(tetO)₇-P_{Fos}-rtTA virus was mixed 1:1 with an rAAV expressing Venus under the control of oxytocin promoter (rAAV-P_{OT}-Venus; Knobloch et al., 2012) **(D)** In euhydrated rats (Basal; upper panel), Dox injection induces rtTA-dependent YC3.60 (via Virus 2, green) and Cre-dependent ChR2-mCherry (via Virus 3, red) signals in a few scattered neurons. In contrast, both signals were detected in the majority of SON OT neurons (blue) after Dox injection to osmotically challenged, salt-loaded rats (Stimulated, low panel). **(E)** The expression of c-fos signal (visualized via immunohistochemistry, green) overlays vGATE-assisted ChR2-mCherry signal (red) in virtually all SON OT neurons of Dox-treated osmotically-challenged rats.

Figure 2. Fear Learning and Optogenetic Control of Fear Behavior

(A) Scheme of the fear condition setup, Dox administration and blue light stimulation of the CeA. **(B1)** The vast majority of OT neurons ($99.4 \pm 0.8 \%$, green) of the PVN and SON were labeled via a constitutive OT promoter driving hChR2-mCherry (red). **(B2)** Fear expression induced tagging (ChR2-mCherry, red) of a small population of OT neurons (green). **(B1, B2)** Vertical panels depict ChR2-mCherry containing axons (red) with OT-immunopositive puncta (green, appear in yellow) in the CeL of both groups. **(C1, C2)** The freezing time before and after blue light illumination of the CeA (top graph: constitutive hChR2 expression, OT^{Constitutive} group; bottom graph: OT^{vGATE} group). The black bars (control) show the freezing time immediately prior to the BL stimulation, whereas the blue bars (BL) indicate the freezing time after the BL stimulation. $***p < 0.001$, T-test. **(D)** The onset and duration of the BL-induced unfreezing effect. Data presented as mean \pm SEM.

Figure 3. Functional Plasticity of the OT System Upon Fear Learning

(A1, A2) GFP-labeled axons of OT neurons in CeL of naive OT^{Constitutive} (**A1**) and OT^{vGATE} (**A2**) rats. Overview and enlarged fragments, showing co-localization of GFP (green) with OT (blue) and vGluT2 (red). **(A3)** Quantification of total axonal lengths and vGluT2-immunoreactive puncta; green: OT axons containing no vGluT2, yellow: co-localization of OT and vGluT2; st - *stria terminalis*. $*p < 0.05$; $***p < 0.001$, T-test. **(B)** Scheme of the *ex vivo* recordings setup. **(C1)** Effect of optical stimulation of OT-ergic axons present in the CeL (BL) on IPSC frequencies recorded in CeM neurons in both fear-naive and fear-experienced vGATE animals. Pie charts depict the proportion of CeM neurons responding to BL (naive OT^{Constitutive}: $n=27/56$; OT^{vGATE}: $n=23/78$, n.s.). Bar plot shows the change in CeM current frequencies (Hz) in response to BL. Data are expressed as means across slices plus SEM. Individual values are indicated as white circles. $**p < 0.01$, $***p < 0.001$, two-way ANOVA followed by Sidak post-hoc test. **(C2)** Pharmacological dissection of BL effect. Left, quantification of the effect of NBQX (green; naive OT^{Constitutive} $n=7$; OT^{vGATE} $n=5$) or dOVT (red; OT^{Constitutive} $n=7$; OT^{vGATE} $n=6$) application on the initial BL effect on IPSC frequencies recorded in the same CeM neurons. Data are expressed as means across slices plus SEM. Individual values are indicated as white circles. $***p < 0.001$, Mann-Whitney test. Right, Z-scores illustrating the time course and modulation of BL effects by NBQX or dOVT. **(D1)** Scheme of the BL stimulation of the CeL in OT^{Constitutive} animals pretreated with OTA. **(D2, D3)** The bar charts display the freezing time in the corresponding group before and after blue light stimulation of the CeA after OTA administration. Top graph: the first conditioning session (OTA-BL1), bottom graph: the second conditioning session (OTA-BL2). The black bars (control) show the freezing time immediately prior to the BL stimulation, whereas the blue bars (BL) indicate the freezing time after the BL stimulation. $***p < 0.001$, T-test. **(D4)** Graph displays the onset and duration of the BL-induced unfreezing effect.

Figure 4. Pharmacogenetic Inhibition of OT Neurons Impairs Fear Extinction

(A) Scheme of the FC setup in Context A and B (Context A: green wall; context B: red wall). Virally-injected animals were subjected to contextual FC. After two shock sessions (d1, d2), animals received an i.p. injection of Dox on d3, followed by another session on d4, without shocks. Ten days later, animals were subjected to an additional shock day to reinforce the fear memory and afterwards underwent a 4-day fear extinction protocol. During the extinction session, animals received a daily injection of CNO 40

minutes prior to the experiment. After exposure to context B, animals were subjected to 2 days of shock, followed by another 4-day extinction session, where they again received CNO daily prior to the experiment. **(B1, B2)** Bar charts show the average freezing time of animals over the course of a 4-day fear extinction paradigm, as well as the total increase in mobility; e1-e4 indicate extinction days. **(B1)** While control animals underwent normal fear extinction, CNO-mediated silencing of all OT neurons equally impaired the fear extinction in both Contexts. $**p<0.01$, $***p<0.0001$, t-test. **(B2)** CNO-mediated silencing of vGATE-tagged OT neurons in Context A impaired fear extinction in Context A, but not in Context B. $**p<0.01$, $***p<0.0001$, one-way ANOVA. **(C1)** Schematic depiction of the local CNO infusion onto the CeA. **(C2)** Confocal image shows the site-specific infusion of retrobeads™ following CNO infusion to verify proper targeting of the CeA. **(C3)** Local infusion of CNO into the CeA impaired fear extinction in Context A, but not B. $*p<0.05$, $**p<0.01$.

Figure 5. Context-Dependent Tagging of OT Neurons

Percentage of OT neurons, tagged in context A, and reactivated after the exposure to a familiar **(A)** or a novel **(B)** context. Representative confocal images of the PVN and SON with intrinsic GFP signal (green, vGATE-based tagged neurons in context A), co-stained for OT (blue) and c-fos (red, indicates neurons activated in context B). Pie charts show the relative numbers and percentages of GFP-positive and c-fos expressing OT neurons in each hypothalamic nucleus. In the PVN, the initial exposure to Context A resulted in GFP-based tagging of 10.8% of all counted OT neurons (see Table **S5**), while re-exposure to the same context led to c-fos expression in 9.5% of OT neurons, with 18% of reactivated cells (i.e. OT cells expressing GFP and c-fos). In the SON, the exposure to context A activated 11.9% of OT neurons and re-exposure to the same context induced c-fos in 16.2% of OT cells, displaying 89% overlap in GFP and c-fos signals. For context B, the initial exposure activated 9.6% of OT cells in the PVN visualized by intrinsic GFP expression and the second exposure recruited a massive 47.1% of OT cells, with a reactivated GFP-expressing fraction of 31%. In the SON, first exposure to novel context B activated 11.7% of OT neurons, whereas re-exposure to the same context resulted in the drastically increased activation of 56.4% of cells, yet reflecting only 22% overlap of GFP and c-fos. **(C1-C3)** Electrophysiological properties of OT^{vGATE} vs OT^{Constitutive} neurons. **(C1)** Experimental scheme showing OT^{vGATE} in yellow (co-localization of green and red signals) and OT^{Constitutive} in red in the SON. **(C2)** Bar plots and individual data (dots) of sEPSC frequency (left) and membrane capacitance (right) recorded in OT^{vGATE} (yellow; n=24) and OT^{Constitutive} (red; n=16). $*p<0.05$, t-test. **(C3)** VGluT2-immunoreactive puncta (green) in close vicinity of OT neurons (blue) labelled by vGATE viruses (FLEX-mCherry, red) and non-labelled OT cells of the SON. The number of vGluT2-positive puncta in vicinity of OT vGATE neurons is significantly higher compared to non-labelled OT neurons ($p<0.001$).

Figure 6. Pharmacogenetic Silencing of Parvocellular OT Neurons.

(A) Percentage of parvOT and magnOT, tagged in Context A by GFP, and reactivated after exposure to Context B. Representative confocal images of FG-positive (magnOT neurons, in red) and FG negative (parvOT neurons) containing intrinsic GFP and c-fos (in white). Respectively, green arrows indicate OT neurons activated in Context A (GFP), red arrows – magnocellular OT neurons, and white arrows – OT cells containing c-fos. Pie charts between confocal images display the distribution of c-fos labeling in

parvOT and magnOT neurons. **(B)** Experimental setup for exclusive silencing of ParvOT neurons: Animals received injection of CAV2-Cre into the SON and Cre-dependent rAAV, expressing hM4Di-mCherry under the control of OT promoter into the PVN to specifically label parvocellular OT neurons in the PVN. Confocal image shows parvocellular OT cells (blue) expressing hM4Di-mCherry (red). **(C-F)** Images of the SON of naive animals **(C)**, rats conditioned in Context A **(D)**, and rats conditioned in Context A followed by Context B and treated with vehicle **(E)** or CNO **(F)**. **(G)** CNO treatment drastically reduced the number of c-fos expressing OT neurons in the SON of rats exposed to Context B. Black: control, green: exposure to context A, red: exposure to context B plus saline injection, blue: exposure to context B plus CNO injection. *** $P < 0.0001$, one-way ANOVA.

Figure 7. Context-dependent Changes in OT Neuron Activity Following Contextual Fear Experience

The scheme represents a working hypothesis on plasticity and contextual specificity of the central OT system. At the level of OT terminals in the amygdala (top panel), the fear learning process induces drastic changes in the transmitter/neuromodulator balance: while in naïve rats OT axons predominantly release OT, in fear-experienced rats the same axons release glutamate instead of OT. At the level of the hypothalamus (middle panel), the majority of magnOT neurons in the SON exhibit context specificity and are not reactivated in a novel context. In contrast, parvOT neurons of the PVN, synapsing on SON magnOT neurons, get reactivated in a novel context and drive the massive recruitment of new magnOT neurons. The massive activation of new SON OT neurons correlated with OT release to the blood, serving homeostatic adaptation to excessive stress caused by exposure to a novel fearful environment (bottom).

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken anti-GFP primary antibody	Abcam	ab13970
Mouse NeuN primary antibody	Chemicon	A60
Rabbit vGluT2 primary antibody	Synaptic systems	135 103
Rabbit anti-dsRed primary antibody	Clontech	632496
Rabbit anti-Fluorogold primary antibody	Milipore	AB153
Guinea-pig anti-Fluorogold primary antibody	Protos Biotech Corp	NM-101
Rabbit c-fos polyclonal primary antibody	Santa-Cruz	sc-7202
Mouse monoclonal anti-OT primary antibody	Provided by Dr. Harold Gainer	PS 38
Rabbit polyclonal anti-CRH primary antibody	Peninsula Labs	T-4035.0500
Rat monoclonal anti-Somatostatin primary antibody	Chemicon	MAB345
Rabbit polyclonal anti-vGluT2 primary antibody	SYSY	135403
Bacterial and Virus strains		
(tetO) ₇ -P _{c-fos} -rtTA AAV 1/2	This paper	N/A
YC3.60-P _{tet} bi-Cre AAV 1/2	This paper	N/A
P _{OT} -FLEX-hChr2-mCherry AAV 1/2	Eliava et al., 2016	N/A
CAV2-Cre	Bru et al., 2010	N/A
P _{OT} -Venus AAV 1/2	Knobloch et al., 2012	N/A
P _{OT} -FLEX-GFP AAV 1/2	Eliava et al., 2016	N/A
P _{OT} -FLEX-hM4D(Gi)-mCherry AAV 1/2	Eliava et al., 2016	N/A
Chemicals, Peptides, and Recombinant Proteins		
Fluorogold™	Santa-Cruz	sc-358883
Oxytocin Receptor Antagonist	Santa-Cruz	L-368,899

Doxycycline	Cayman Chemical Company	14422
Clozapine-N-Oxide	Tocris Bioscience	4936
NBQX	Abcam	Ab120018
dOVT	Bachem	H2908
Retrobeads™	Lumafloor	N/A
Experimental Models: Organisms/Strains		
Rattus Norvegicus (Wistar)	Janvier	N/A
Recombinant DNA		
(tetO) ₇ -P _{c-fos} -rtTA	This paper	N/A
YC3.60-P _{tet} bi-Cre	This paper	N/A
P _{OT} -FLEX-hChr2-mCherry	Eliava et al., 2016	N/A
P _{OT} -Venus	Knobloch et al., 2012	N/A
P _{OT} -FLEX-GFP	Eliava et al., 2016	N/A
P _{OT} -FLEX-hM4D(Gi)-mCherry	Eliava et al., 2016	N/A
Software and Algorithms		
Graphpad prism 7.0	www.graphpad.com	N/A
Fiji	www.imagej.net/Fiji	N/A
Adobe Photoshop CS5	www.adobe.com	N/A
Adobe Illustrator 16.05	www.adobe.com	N/A
Panlab Fear conditioning software	www.panlab.com	N/A
Other		
Optic fiber implants	www.thorlabs.com	CFMLC52L02
Laser cables for optogenetics	www.thorlabs.com	M106L01
Guide cannula 5.8mm	www.bilaney.com/plastics-one	C313G/spc
Cannula dummy cap	www.bilaney.com/plastics-one	C313DC/1/spc
Internal cannula	www.bilaney.com/plastics-one	C313I/spc
473nm Blue Laser Generator	www.dreamlasers.com	SDL-473-XXXT
Programmable Pulse Stimulator (A.M.P.I.)	www.ampi.co.il	Master-9

CONTACT FOR REAGENT AND RESOURCE SHARING

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STAR METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Anatomical, electrophysiological, optogenetic and behavioral studies were performed with female Wistar rats purchased from Janvier, France (8-10 weeks old on arrival at our facility). All rats were housed under standard conditions with *ad libitum* access to food and water. All experiments have been approved by the German Animal Ethics Committee of the Baden Württemberg (licenses numbers 35-9185.81/G-24/12, 35-9185.81/G-26/15 and 35-9185.81G-102/17).

METHOD DETAILS

The vGATE system

Generation and cloning: We engineered a synthetic c-fos promoter linked to its first exon (Exon 1; (Schilling et al., 1991) with ATGs in the Exon 1 converted to TTGs by site directed mutagenesis and heptamerized tetracycline (tet) operators, (tetO)₇, added upstream of it. These operators drive the

expression of a humanized reverse tet transactivator (rtTA) (Dogbevia et al., 2015) named $(tetO)_7$ - P_{fos} -rtTA. The entire cassette was subsequently cloned in a plasmid to produce recombinant adeno-associated viruses (rAAVs) packaged with serotype 1 and 2 (Dogbevia et al., 2015) to generate Virus 1 (rAAV- $(tetO)_7$ - P_{fos} -rtTA). Next, an AAV equipped with bidirectional tet promoter (P_{tetbi}) expressing the Cre-recombinase was linked to a genetically-encoded calcium indicator (YC3.60) (Virus 2, rAAV- P_{tetbi} -Cre/YC3.60, (Lutcke et al., 2010)). As the last component of our viral technique (Virus 3), we generated rAAVs under the OT promoter to drive expression of hChr2-mCherry, GFP or hM4D(Gi)-mCherry (Eliava et al., 2016; Grund et al., 2017) in a Cre-dependent manner. The entire system comprising rAAV- $(tetO)_7$ - P_{fos} -rtTA (Virus 1), as the key element of the system, combined with rAAV- P_{tetbi} -Cre/YC3.60 (Virus 2) and the Cre-dependent 'FLEX' viruses (Virus 3, see Key Resource Table) for 'virus-delivered, Genetic Activity-dependent Tagging of cell Ensembles' or vGATE. In the vGATE method, the c-fos promoter drives rtTA expression only when neurons are activated. The rtTA generated by transient c-fos promoter activity binds to the upstream $(tetO)_7$ only in the presence of Dox. This way, the rtTA drives its own expression, thus establishing an autoregulatory loop, even when the induced c-fos promoter activity declines to baseline levels as neuronal activity subsides. Therefore, only in the presence of Dox, the rtTA can activate the expression of the bidirectional tet promoter (P_{tetbi}) to express any gene of choice, for example, the Cre recombinase, for permanent tagging of activated cells via a Cre-dependent FLEX cassette.

Validating in cultured neurons: Dissociated rat hippocampal cultured neurons were treated with two viruses (rAAV- $(tetO)_7$ - P_{fos} -rtTA + rAAV- P_{tetbi} -Cre/tdTOM) for two weeks. Afterwards, cells were treated with bicuculline (bic) for 20 minutes and replaced with fresh medium supplemented with Dox (1 μ g/ml) for 24 hours. Only in the presence of bic and Dox, a strong rtTA signal was detected by immunohistochemistry (**Fig. S1**, bottom panel). By live fluorescence imaging of fixed cells, rtTA-dependent tdTOM expression was also clearly detected in +bic/+Dox treated cells. In a few neurons, however, tdTOM expression was also detected under condition of -bic/+Dox. This is likely a result of spontaneous activity in some neurons. Western blot analyses validated tdTOM expression (**Fig. S1**).

Novelty of the method: We describe here a genetic method (vGATE) that uses c-fos promoter elements to drive expression of a reverse tetracycline transactivator (rtTA). The key novelty of our method is that the 'recording period' can be rapidly opened within a few hours by a single intraperitoneal Dox injection before the tagging of activated neurons. In previous methodological studies, we carefully characterized, both *ex vivo* and *in vivo*, the regulation of Dox-controlled gene expression by rtTA expressed under a human synapsin promoter (Dogbevia et al., 2015; Dogbevia et al., 2016). We determined an optimal Dox concentration for *in vivo* application, and the time course of gene activation and inactivation by a single intraperitoneal Dox injection. In our previous studies, we found that the bidirectional Tet promoter (P_{tetbi}) has highly reduced leakiness (Dogbevia et al., 2016) compared to the uni-directional Tet promoter (P_{tet}) (Dogbevia et al., 2015). Indeed, P_{tet} is very leaky (Dogbevia et al., 2015), due to its close proximity close to an inverted terminal repeat (ITR) of AAV, which appears to have intrinsic enhancer-like activity (Dogbevia et al., 2015). This raises potential concerns that AAVs equipped with a uni-directional tet promoter (P_{tet}) might be leaky enough to non-specifically tag non-engram cells. With these considerations, we characterized the vGATE method both *ex vivo* and *in vivo*. The vGATE method is

very tightly controlled and highly flexible; different combination of gene modules in rAAVs can be simultaneously delivered to the targeted brain region(s). With this approach, we performed selective and cell type-specific tagging of OT neurons activated during contextual fear learning and expressed in the tagged neurons gene(s)-of-interest for pharmacogenetic and optogenetic manipulations, while others might easily target different neuronal populations.

Different methods exist to induce time-dependent conditional protein expression, namely the TetTag (Reijmers et al., 2007) and the TRAP (Guenther et al., 2013) methods. The TetTag method drives tTA expression under c-fos and animals have to be fed Dox to keep the system inactive. However, to open the recording period, TeTag animals have to be switched to diet without Dox for a few days before performing learning-dependent neuronal labeling. However, this non-recording period of a few days is sensitive for non-specific labeling of neurons. In the case of vGATE, the presence of Dox is requested to activate detectable gene expression within a few hours and achieved the maximum expression after 24 hours (Dogbevia et al., 2015, Dogbevia et al., 2016) and gene expression is switched-off in 6 days (Dogbevia et al., 2016). In a previous systematic study (Dogbevia et al., 2016), we found similar time course of 6 days to “re-activate” (with the tTA system) by Dox removal and “activate” (with the rtTA system) gene expression by Dox addition. The discrepancy for 3 days for Dox clearance by others using the tTA system is likely due to the sensitivity of the detection method; we used a highly sensitive reporter, the firefly luciferase, for gene expression assay as a proxy for Dox clearance from the brain (Dogbevia et al., 2016), while other labs use GFP (Liu et al., 2012), which are much less sensitive.

In the TRAP method, the inducer, tamoxifen, is needed to facilitate release cytoplasmic ER-Cre for transport to the nucleus to permanently tag neurons by Cre/loxP dependent gene modules. However, TRAP is in itself leaking, since it is known that a fraction of ER-Cre can already move to the nucleus even without tamoxifen treatment. There is even a bigger concern that tamoxifen as a selective estrogen modulator might interfere with various estrogen-sensitive cell types in the brain. The use of tamoxifen is especially critical for OT neurons, which are sensitive for estrogens (Somponpun and Sladek, 2003) and tamoxifen itself triggers the activity of the OT gene promoter (Koohi et al., 2005). Finally, tamoxifen induces c-fos expression in OT neurons (Althammer and Grinevich, unpublished data), thereby precluding the use of the ER-Cre method for this particular neuron type. With our novel vGATE technique, we achieved highly specific labeling of OT neurons and precise temporal control, which we believe to be superior to other comparable, c-fos based tagging methods. Therefore, the development of the vGATE method should be able to overcome the key inherent concerns of the previous two main strategies to induce time-dependent conditional protein expression.

Infecting rat hypothalamic neurons in vivo with rAAVs

Cloning of the OT promoter, as well as the production and purification of rAAVs, has been previously described (Knobloch et al., 2012). rAAV genomic titers were determined with QuickTiter AAV Quantitation Kit (Cell Biolabs, Inc., San Diego, California, USA) and RT-PCR using the ABI 7700 cycler (Applied Biosystems, California, USA). rAAVs titers were between 10^{10} - 10^{11} genomic copies per 1 μ l. Infection of OT neurons was achieved by complementing the two viruses indicated above by an rAAV, driving genes of interest (Venus, hChR2-mCherry, FLEX-GFP, FLEX-hM4D and hM4D(Gi). A cocktail of the three rAAVs

(i.e. rAAV-(tetO)₇-P_{fos}-rtTA, rAAV-P_{tet}bi-Cre/YC3.60, and rAAV-P_{OT}-Venus) was injected bilaterally into the hypothalamic nuclei, the PVN and SON, using a previously described protocol (Knobloch et al., 2012).

Neuroanatomy

To trace, label and manipulate the hypothalamus-amygdala connections, rAAVs expressing vGATE viruses were injected into the PVN and SON. Alternatively, CAV2-Cre was injected into the SON, while the Cre-dependent "FLEX" P_{OT}-hM4D(Gi) rAAV was injected into the PVN to specifically label and manipulate parvOT neurons. After transcardial perfusion with 4% PFA, brains were sectioned and stained with antibodies against OT, vGluT2, GFP, FG and dsRed. Images for qualitative and quantitative analyses were taken on the confocal microscope Leica SP5.

BEHAVIOR

Salt loading

To validate the vGATE system *in vivo*, we performed salt-loading (SL) as a stimulus to activate c-fos expression in OT neurons (Kato et al., 2010). Here, we used a mixture of two rAAVs (rAAV-(tetO)₇-P_{fos}-rtTA and rAAV-P_{OT}-Venus) and co-injected them unilaterally into the SON of rats. The animals were divided into four groups (n=3/group): Group 1 (Untreated control animals (-Dox/-SL)), Group 2 (-Dox/+SL, 7 days with SL (2% NaCl in drinking water)), Group 3 (+Dox/-SL, normally hydrated animals, which received single Dox i.p. injection) and Group 4 (+Dox/+SL, animals received a single i.p. injection of Dox on the 5th day of SL and were kept under SL for 2 more days before being sacrificed). Expression patterns of the individual groups are depicted in **Fig. 1D**.

Contextual fear conditioning and optogenetics

We used a mixture of three vGATE viruses as depicted (**Fig. 1A**). Two weeks after the viral infection, adult female rats were subjected to either a 3-day (single fear) or 15-day (double fear) contextual fear conditioning protocol, comprising a conditioning session on the first 2 days and a recall session on the 3rd day (**Fig. 2A**). All sessions lasted for 20 minutes with 7 random foot shocks (1.6mA, 1s) dispersed between the 10th and 17th min of protocol during the fear conditioning session.

As shown in **Fig. 2A**, animals were treated with Dox (5 mg/kg b.w., i.p.) 24 hours after the 2nd day of fear acquisition to tag OT neurons activated during fear expression. The experimental group (animals injected with the vGATE system: rAAV-(tetO)₇-P_{fos}-rtTA + rAAV-P_{tet}bi-Cre/YC3.60 + rAAV-P_{OT}-FLEX-hChR2-mCherry) received another re-conditioning session (double fear), two weeks after Dox injection and recovery, for one day, to achieve a higher level of freezing (above 50 s/min) and 24 hours later were tested for the recall of fear behavior with blue light (BL) illumination of the CeA. The CeA was illuminated bilaterally with BL (473 nm, 10 ms pulse, 20 s duration, 30 Hz) via 200 micrometer optical fibers from Thorlabs (BFL37 200) at the 10th min of the recall session. The rats were evaluated for freezing responses and freezing durations were measured through freezing software (Panlab) and manually through an offline video in addition. In **Fig. 2C**, the 2 different graphs display the average freezing time per minute in the corresponding groups (OT^{Constitutive} and OT^{vGATE}) before and after BL illumination. The black bars (control) show the average freezing times in minutes immediately prior to the BL illumination, whereas the grey bars (BL) indicate the average freezing times per minute during the minute in which

the BL-induced onset of the unfreezing effect (i.e. where the first signs of mobility appear). All groups of animals were sacrificed 90 minutes after the start of the optogenetic session.

Estimation of the estrous cycle

To monitor ovarian cycle, we performed vaginal smear collections. Animals in metestrus, proestrus and estrus phases were excluded from experiments and reintroduced once they reached diestrus.

Pharmacogenetics (DREADD) and fear extinction in Contexts A and B

In this experiment, animals were subjected to a series of fear conditioning and fear extinction paradigms that we performed in two different contexts (A and B). For this purpose, two visually distinct contextual fear conditioning chambers were used, which were comparable in size, shape of the grid and power of the electrical shocks (1.6 mA). The chambers were located in two different institutes (chamber A, Max Planck Institute of Medical Research (MPI), Heidelberg, Panlab; chamber B, Interdisciplinary Neurobehavioral Core (INBC), Heidelberg, Med Associates) and the animals were exposed to them for the first time in both cases. Animals were injected with P_{OT} -hM4D(Gi) ($OT^{Constitutive}$) or rAAV-(tetO)₇- P_{fos} -rtTA, rAAV- P_{tet} -Cre/YC3.60 + rAAV- P_{OT} -FLEX-hM4D(Gi) (OT^{vGATE}) and subjected to the experimental procedure after recovery and handling. For the contextual fear conditioning, animals were placed in a fear conditioning chamber (Panlab, Harvard Apparatus) with a metal grid for application of electrical foot shocks (1.6 mA). We used a 3-day fear conditioning protocol (2 days shock, 1 day testing, each 20 min) to fear condition the animals. On the first two days, the animals, after a 10 min habituation period, received 7 electrical foot shocks within 7 min (randomly distributed, on average 1 shock per min), followed again by 3 min without shocks. For activation of the viral system, animals received Dox (5 mg/kg b.w., i.p.) on day 3 of the fear conditioning (24 hours after the 2nd shock day of the first round of fear conditioning). On the fourth day (24 hrs after injection), animals were placed in the same box for 20 min without any shocks. The experiment was recorded with a video camera and sensors in the grid measured the total movement of the animals during the procedure. After a 2-week break (Dox clearance and viral expression) animals were exposed to the box in a second session. Here, we used a 5-day fear extinction protocol with one re-conditioning day (in order to maintain a high level of freezing (above 50 s/min) and identical in all groups) and 4 testing days. Due to the 2-week interval in between the two rounds, animals received electrical shocks on the first day (recall session), followed by 4 days on which the animals were placed in the box for 40 min each without any shocks (extinction sessions). Animals expressing the hM4D(Gi) receptor in all OT neurons ($OT^{Constitutive}$) or tagged OT neurons (OT^{vGATE}) received a daily injection of Clozapine (3 mg/kg bw i.p., Tocris Bioscience, Bristol, UK, dissolved in 1xPBS) 30 min prior to placing them into the Context A chamber. The control groups ($OT^{Constitutive}$ and OT^{vGATE}) received the same volume of 0.9% NaCl solution. After the first round of fear extinction in Context A, animals were transferred to Context B. Here, an additional round of fear extinction was performed, which comprised two shock sessions followed by 4 consecutive daily extinction sessions, 40 minutes prior to which the animals received a daily injection of CNO (**Fig. 4A**), whereas the control group received the same volume of 0.9% NaCl solution 40 min prior to the start of the session. All animals were sacrificed and perfused 90 mins after the fear conditioning session in Context B. In the two control groups, the time of freezing

in both contexts was almost identical (**Fig. S4B**). Therefore, for the sake of simplicity, only the freezing time of OT^{Constitutive} and OT^{vGATE} control groups in Context A is depicted in **Fig. 4B1** and **B2**.

Context A vs. Context A - mapping of OT neuronal activity via GFP followed by immunostaining for c-fos

Animals were injected in all nuclei with the vGATE system (rAAV-(tetO)₇-P_{fos}-rtTA + rAAV-P_{tet}bi-Cre/YC3.60 + rAAV-P_{OT}-FLEX-GFP) and subjected to the 3-day contextual fear conditioning paradigm after recovery and handling. Following the first two shock sessions, animals received Dox (5 mg/kg b.w., i.p.) on the 3rd day of the fear conditioning paradigm. On the 4th day, animals were exposed to the fear conditioning chamber, this time without electrical shocks. Two weeks after the 1st round of fear conditioning in Context A (1), the animals were exposed to Context A(2) for a second time, where they underwent an additional session of fear conditioning. All animals were sacrificed 90 minutes after the beginning of the test session in Context A (2).

Context A vs. Context B - mapping of OT neuronal activity via GFP followed by immunostaining for c-fos

Animals were injected with the vGATE system into all nuclei (rAAV-(tetO)₇-P_{fos}-rtTA + rAAV-P_{tet}bi-Cre/YC3.60 + rAAV-P_{OT}-FLEX-GFP) and subjected to the 3-day contextual fear conditioning paradigm after recovery and handling. Following the first two shock sessions, animals received Dox (5 mg/kg b.w., i.p.) on the 3rd day of the fear conditioning paradigm. On the 4th day, animals were exposed to the fear conditioning chamber, this time without electrical shocks. After the 1st round of fear conditioning in Context A, the animals were transferred to Context B, there they underwent an additional session of fear conditioning in analogy to that in Context A, one week later. All animals were sacrificed 90 minutes after the beginning of the test session in Context B. This point in time was chosen as it is well established that the c-fos protein has its peak expression at around 90 minutes after the initial activation of OT neurons. To label all neuroendocrine cells protruding beyond the blood brain barrier (most importantly, magnOT neurons), animals received a single injection of Fluorogold, Santa Cruz Biotechnology, Dallas, 15 mg/kg bw i.p. dissolved in 1xPBS, 7 days prior to perfusion (Eliava et al., 2016).

DREADD-based inhibition of parvocellular OT neurons of the PVN in Context B

For this experiment, rats were injected with the retrogradely spreading rAAV-CAV2-Cre into the SON and rAAV-P_{OT}-FLEX-hM4D into the PVN to specifically express hM4D in parvOT neurons of the PVN, in analogy to our previous study (Eliava et al., 2016). After one week of recovery, animals were handled 10 minutes per day for 1 week. In the 3rd week after injection, animals were subjected to fear conditioning in Context A in analogy to **Fig. 2A**. Following the successful fear conditioning in Context A, animals were transferred to Context B, where another round of fear conditioning was performed (see **Fig. 4A**). Thirty minutes prior to the experiment of day 3 (exposure day), the three experimental animals received an i.p. injection of clozapine-N-oxide CNO (3mg/kg bw i.p., Tocris Bioscience, Bristol, UK, dissolved in 1xPBS) to inhibit the activity of parvOT neurons. The control animals received the same volume of vehicle (0.9% NaCl). Ninety minutes after the start of the session, the animals were euthanized with isoflurane and perfused to collect the brains. Brain sections containing SON were used for immunohistochemical

staining for OT and c-fos. Quantitative analysis of OT versus c-fos expressing cells was performed and the results are demonstrated (in **Table S4**). Naive animals and animals that were only subjected to Context A served as controls.

Stereotaxic injection of viral vectors and implantation of optic fibers

Injection of viral vectors into the rat brain was performed in analogy to Knobloch et al., 2012. If not indicated otherwise, all hypothalamic nuclei were injected bilaterally using the following coordinates: SON (M-L \pm 1.6mm, A-P -1.4mm, D-V -9.0mm), PVN (M-L \pm 0.3mm, A-P -1.8mm, D-V -8.0mm) and AN (M-L \pm 1.2mm, A-P -2.0mm, D-V -8.5mm). Point of origin for the coordinates was Bregma and the Z level difference of Bregma and Bambda did not exceed 0.1mm (Cetin et al., 2006). Injection volume per injection site was 300 nl (either single virus or cocktail), while all viruses used were in the range of 10^{12} - 10^{13} genomic copies per ml. For the implantation of optic fibers into the CeA, we used the following coordinates: M-L \pm 3.9, A-P -2.5, D-V -8.0 while the optic fibers had a length of 8.5 mm.

EX VIVO ELECTROPHYSIOLOGY

Amygdala

Horizontal slices preparation

Animals were anaesthetized with an intraperitoneally administered mixture ketamine/xylazine (Imalgene 90 mg/kg, Rompun, 10 mg/kg). Transcardial perfusion was then performed using one of the following artificial cerebro-spinal fluids (ACSFs) dissection solutions. For rats between 10 and 11 weeks old, an ice-cold NMDG based ACSF was used containing (in mM): NMDG (93), KCl (2.5), NaH_2PO_4 (1.25), NaHCO_3 (30), MgSO_4 (10), CaCl_2 (0.5), HEPES (20), D-Glucose (25), L-ascorbic acid (5), Thiourea (2), Sodium pyruvate (3), N-acetyl-L-cysteine (10), Kynurenic acid (2). The pH was adjusted to 7.4 using HCl 37%, after bubbling in 95% O_2 and 5% CO_2 gas; bubbling was maintained throughout the duration of use of the various ACSFs. Following decapitation, the brain was swiftly transferred into the same ice-cold ACSFs dissection solution as for transcardial perfusion, and 350 μm thick horizontal slices containing the CeA were obtained using a Leica VT1000s vibratome. After slicing, brain slices were hemidissected and placed in a room-temperature holding chamber with normal ACSF, for a minimum of 1 hour before the conduction of any experiments. Slices of 10-11 weeks old rats were placed in 35°C NMDG ACSF for 10 minutes before transferring them to the holding chamber at room temperature. Normal ACSF, also used during experiments, was composed of (in mM): NaCl (124), KCl (2.5), NaH_2PO_4 (1.25), NaHCO_3 (26), MgSO_4 (2), CaCl_2 (2), D-Glucose (15), adjusted for pH values of 7.4 with HCl 37% and continuously bubbled in 95% O_2 and 5% CO_2 gas. All ACSFs were checked for osmolality and kept for values between 305-312 mOsm/L. For electrophysiology experiments, slices were transferred from the holding chamber to an immersion recording chamber and superfused at a rate of 2 mL/min with normal ACSF unless indicated otherwise.

CeM Neurons recordings

Pipettes were filled with an intracellular solution containing (in mM): KCl (150), HEPES (10), MgCl_2 (4), CaCl_2 (0.1), BAPTA (0.1), ATP Na salt (2), GTP Na salt (0.3). pH was adjusted to 7.3 with KOH and osmolality checked to be between 290-295 mOsm/L, adjusted with sucrose if needed. All cells were hold

at a membrane potential of -70 mV. Series capacitances and resistances were compensated electronically throughout the experiments using the main amplifier. Average IPSC frequencies were calculated in 20s windows, chosen for light stimulation at maximal effect, as determined by the maximal slope of the cumulative plot of the number of currents using SigmaPlot 11.0. Baselines and recovery IPSC frequencies were measured at the beginning and end of each recording. Z-score values were calculated by subtracting the average baseline IPSC frequency established over 70 seconds at the recording beginning from individual raw values and by dividing the difference by the baseline standard deviation. Optical BL illumination of CeL OT axons expressing hChR2 was performed using light source X-Cite® 110LED from Excelitas Technologies through a GFP filter, controlled with a Clampex-driven TTL pulse for 20s at 30Hz with 10ms pulses. To quantify the pharmacological blockade (NBQX or dOVT) of the BL illumination on the CeM IPSC frequencies, a ratio was first calculated between basal and BL modified IPSC frequencies minus one and that for each recording neuron, in order to obtain the BL effect. Second, the percentage of the remaining BL illumination after pharmacological blockade was obtained by dividing the BL effect after and before drug perfusion. This is reported as $\Delta\text{BL}/\text{BL}_0$ (%) in **Fig. 3B2**.

SON OT neurons recordings

For this experiment, rats received viral injections of 300 nl vGATE with rAAV-**P**_{OT}-FLEX-GFP as virus 3 or rAAV-**P**_{OT}-mCherry bilaterally into the SON. Thus, OT engram cells were labeled in green, while OT non-engram cells in red. Recording pipettes were filled with an intracellular solution containing (in mM): KMeSO₄ (125), CaCl₂ (2), EGTA (1), HEPES (10), ATPNa₂ (2), GTPNa (0.3). pH was adjusted to 7.3 with KOH and osmolality checked to be between 290-295 mOsm/L, adjusted with sucrose if needed. After whole-cell patch-clamp of identified OT neuron, the following parameters were recorded: access resistance, membrane capacitance, resting potential, spontaneous EPSC amplitude and frequency, response to current injection (0 to 150 pA for 500ms, with steps of 25 pA).

Optical stimulations

Optical BL illumination of CeL OT-ergic axons expressing hChR2 was performed using light source X-Cite® 110LED from Excelitas Technologies through a GFP filter, controlled with a Clampex-driven TTL pulse for 20s at 30Hz with 10ms pulses.

Analysis of effect of blue light

Average IPSC frequencies were calculated in 20s windows, chosen for light stimulation at maximal effect, as determined by the maximal slope of the cumulative plot of the number of currents using SigmaPlot 11.0. Baselines and recovery IPSC frequencies were measured at the beginning and end of each recording. Z-score values were calculated by subtracting the average baseline IPSC frequency established over 70 seconds at the recording beginning from individual raw values and by dividing the difference by the baseline standard deviation.

Pharmacological assays

To quantify the pharmacological blockade (NBQX or dOVT) of the BL illumination on the CeM IPSC frequencies, a ratio was first calculated between basal and BL modified IPSC frequencies minus one

and that for each recording neuron, in order to obtain the BL effect. Second, the percentage of the remaining BL illumination after pharmacological blockade was obtained by dividing the BL effect after and before drug perfusion. This is reported as $\Delta\text{BL}/\text{BL}_0$ (%) in **Fig. 3B2**.

HISTOLOGY

Animals were perfused through the heart with 1x PBS, followed by 4% paraformaldehyde to fixate the tissue and extracted brains were post-fixed overnight.

The hypothalamus

Brain sections (50 μm) were collected by vibratome slicing and immunohistochemistry was performed with the following antibodies: chicken anti-GFP (Abcam; 1:10000), anti-OT (PS38, 1:1000; mouse; kindly provided by Harold Gainer); anti-c-fos (1:1000; rabbit; Santa Cruz Biotechnology), anti-Fluorogold (1:1000, guinea pig, Protos Biotech) and anti-DsRed (1:1000; rabbit; Clontech). GFP signal was enhanced by FITC-conjugated IgGs, hChR2-mCherry signals by CY3-conjugated antibodies and, for different experiments, other markers were visualized by FITC-conjugated; CY3-conjugated or CY5-conjugated antibodies (1:500; Jackson Immuno-Research Laboratories). Nuclei of cells were visualized with DAPI (1:1000; Roche). All images were acquired on a confocal Leica TCS SP5 and Zeiss LSM5 microscopes; digitized images were analyzed using Adobe Photoshop. To quantify vGATE-assisted labeling of cells, we counted all OT-, hChR2-immunoreactive neurons in the SON and PVN (8-150 neurons/section dependent on the anterior-posterior Bregma level) in 5 animals (3 sections for SON and PVN) with the stereotactic rostro-caudal Bregma coordinates (PVN: -1.5, -1.8, and -2.0 mm; SON: -1.1, -1.4, and -1.7 mm). Statistical significance was determined by Student's test for colocalization experiments, OT axon morphology and statistical analysis was performed with Prism 5 (Mac OS X). Results are presented as mean \pm SEM.

The amygdala

Staining for the vesicular glutamate transporter vGluT2

Selected sections containing the CeA were then immunolabeled for darkfield and brightfield microscopy. For darkfield fluorescent staining, the cocktail containing different combinations of primary antibodies: anti-vGluT2 (1:2000; rabbit; SySy), anti-OT (1:2000, mouse), anti-DsRed (1:1000; rabbit; Clontech), anti-GFP (1:10,000, chicken, Abcam) were used. The named antigens were detected using appropriate secondary antibodies conjugated with fluorophores of various excitation ranges (Alexa488, Alexa459, Alexa680, Thermofisher). All tissue samples, labeled with fluorescent markers were imaged using Leica SP5 CLSM, and digitized using Adobe Photoshop software. To evaluate the CeL vGluT2 expression level, we processed the tissue for triple GFP, OT and vGluT2 immunolabeling in rats of OT^{Constitutive} and OT^{vGATE} groups. In total, 2000 varicosities per animal group were counted and visually examined to detect the presence of double GFP-vGluT2 signals. The final numbers were computed as a proportion (percentage). For brightfield immunostainings, secondary antibodies conjugated to biotin were used. Final visualization of labeling was carried out with standard ABC HRP Kit (Vector) using DAB as a chromogene. The bright microscopic images were captured using a Nikon Eclipse microscope E200 (Software: Nikon NIS-Elements Version 4.30). We analyzed the number of varicosities and the mean

length of OT fiber segments residing in the CeL to trace possible structural changes. Altogether, 72 coronal planes of 6 rats in the OT^{vGATE} group (and 7 in control) were used for this type of analysis. Length of GFP-positive fibers and number of axonal varicosities were measured and counted using free hand tracing and touch-count options in the latest version of ImageJ software (NIH). All statistics were processed in group-specific comparisons between two groups (t-Test, $p < 0,05$, one-tailed), always in one and the same plane of CeL (Bregma: -2,4 to -2,8).

Staining for Somatostatin, Corticotropin-Releasing-Hormone and c-fos

For triple labeling of c-Fos, (Abcam, 1: 1 000), CRH (Peninsula Labs., 1: 10 000), and SOM (Chemicon, 1:500), combination of DAB technique (c-Fos detection) and fluorescent labeling (for CRH and SOM) was applied. First, the sections were incubated with c-Fos antibody and developed using biotinylated secondary antibody, ABC kit (Vector) and conventional DAB detection protocol. Second, the c-Fos DAB-developed tissue was processed for anti-gene retrieval procedure via “boiling” sections in Tris Buffer Saline pH10 at 95°C for 1h for optimal detection of CRH and SOM in the somas of CeA neurons. Afterwards all primary and secondary labeling steps for CRF and SOM detection were performed as described. The actual figures represent digital overlays of confocal bright-field scans of DAB-detected c-Fos and florescent-labeled CRH and SOM. All images were obtained using Leica SP5 CLSM (Imaging Facility, DKFZ, Heidelberg).

Fluorogold treatment and visualization

To discriminate between magno- and parvocellular OT neurons, animals received a single injection of Fluorogold (Santa Cruz Biotechnology, Dallas, 15 mg/kg bw i.p.) 7 days prior to the perfusion. Brain sections were stained with a primary antibody for Fluorogold (Guinea pig anti-FG, dilution 1:1000, Protos Biotech Corp, New York) and Fluorogold immunosignal was visualized by secondary antibodies conjugated with CY3 (Donkey anti-rabbit, dilution 1:500, Jackson Immuno Research, Newmarket Suffolk, UK) or Alexa 680 (Alexa 680: Goat anti-guinea-pig, 1:1000, ThermoFisher Scientific, Waltham, Massachusetts). The colocalization of Fluorogold, OT (or c-fos) and GFP signals was quantified in the PVN (SON contains only magnOT neurons), (n=10; 4 sections/brain). Quantitative analyses of parvo- and magnocellular OT cells expressing GFP are presented in Table **S6**.

Retrobeads infusion

For the retrograde labeling of vGATE projections terminating in the CeA we used retrobeadsTM from LumaFluor. We used the following coordinates for infusion in accordance with Bregma: (CeA left/right): ML: ± 4.0 mm, AP: 2.5 mm and DV: -8 mm, without angle and an injection volume of 140 nl.

Cannula implantation

Animals were bilaterally implanted with guide cannulas for direct intra-central lateral amygdala infusions. We used the C313G/SpC guide metallic cannulae (Plastics one, VA, USA) cut 5.8 mm below the pedestal. For this purpose, animals were deeply anesthetized with 5% isoflurane and their heads were fixed in a stereotaxic frame. The skull was exposed and two holes were drilled according to coordinates that were adapted from a rat brain atlas (2.3 mm rostral-caudal; 4 mm lateral; 7.5 mm dorso-ventral relative to bregma) by comparing the typical bregma-lambda distance (9 mm) with the one measured in

the experimental animal. Two screws were fixed to the caudal part of the skull in order to have an anchor point for the dental cement. Acrylic dental cement was finally used to fix the cannula and the skin was sutured. CNO infusion (1 μ m) occurred at 20 nl/s with a final infusion volume of 100nl per CeA.

MEASUREMENT OF PLASMA OT CONCENTRATIONS

Plasma preparation for LC-MS/MS analysis

50 pmol of D5-oxytocin internal standard was added to 200 μ l of lithium heparin plasma. Plasma was acidified with an equal volume of 5% H₃PO₄ (v/v) and was centrifuged (14,000 x g, 5min). The resulting supernatants were collected and adjusted to 1% H₃PO₄ with H₂O prior to solid phase extraction (SPE). The SPE procedure was performed with a positive pressure manifold (Thermo Electron). OASIS HLB SPE-cartridges (1cc, 30mg, Waters, Guyancourt France) were first activated with 1ml of acetonitrile (ACN) and then washed with 1ml of H₂O 99% / H₃PO₄ 1% (v/v). The sample was loaded and the SPE-cartridge and the cartridge was washed with 1ml of H₂O 99% / H₃PO₄ 1%. After a 1ml wash with H₂O/formic acid 0.1% (v/v) and with 1 ml of ACN 5% / H₂O 94.1% / formic acid 0.1% (v/v/v), elution was performed with 500 μ l of acetonitrile 60% / H₂O 40% (v/v). Eluates were collected and dried under vacuum prior to MS analysis (see below).

LC-MS/MS instrumentation and analytical conditions

LC-analyses were used to determine the presence of oxytocin in the selected reaction monitoring mode (SRM). Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, CA, USA) coupled with a triple quadrupole Endura (Thermo Scientific). The system was controlled by Xcalibur v. 2.0 software (Thermo Scientific). Extracted plasma samples were solubilized in 100 μ l of H₂O/formic acid 0.1% (v/v) and 20 μ l of the solution were loaded into an Accucore RP-MS column (ref 17626-102130; 100 x 2.1 mm 2.6 μ m, Thermo Electron) heated at 35°C. Oxytocin and D5-oxytocin elutions were performed by applying a linear gradient of buffers A/B. Buffer A corresponded to H₂O 98.9% / formic acid 0.1% (v/v), whereas buffer B was ACN 99.9 %/ formic acid 0.1% (v/v). A linear gradient of 20%-95% of solvent B at 400 μ L/min over 2.5min was applied followed by a washing step (0.5min at 95% of solvent B) and an equilibration step (1min of 20% of buffer B). Qualitative analysis and quantification were performed in SRM using an Endura triple quadrupole mass spectrometer and deuterated internal standards. For ionization (positive mode), 3500V of liquid junction voltage and 350°C capillary temperature was applied. The selectivity for both Q1 and Q3 was set to 0.7Da (FWHM). The collision gas pressure of Q2 was set at 2mTorr of argon. For oxytocin and D5-oxytocin, the selection of the monitored transitions and the optimization of the collision energy were preliminarily and manually determined. The transitions and the corresponding collision energies (CE) used for SRM were the following: m/z 504.2 \rightarrow m/z 285.1 (CE = 15.9 eV), m/z 504.2 \rightarrow m/z 487.2 (CE = 11.9 eV) and m/z 504.2 \rightarrow m/z 495.7 (CE = 10.2 eV) for oxytocin with 2 charges; m/z 506.8 \rightarrow m/z 290.2 (CE= 16.2 eV), m/z 506.8 \rightarrow m/z 492.9 (CE= 12.9 eV) and m/z 506.8 \rightarrow m/z 498.3 (CE= 10.7 eV) for D5-oxytocin (with 2 charges). Identification of the compounds was based on precursor ion, selective fragment ions and retention times obtained for oxytocin and D5-oxytocin internal standard. Quantification of oxytocin was done using the ratio of daughter ion response areas of the D5-oxytocin.

Measuring of signal intensity via Fiji (ImageJ)

To compare the signal intensity of neurons or axonal terminals we used the Image Intensity Processing function of ImageJ. Following the detailed description of the analysis ([https://imagej.net/Image Intensity Processing](https://imagej.net/Image_Intensity_Processing)), we converted the .lif files from the confocal microscope into TIFF images and opened them with ImageJ. For the signal intensity analysis, we used the raw data with no contrast modifications or gamma corrections. The freehand tool was used to delineate the axonal segments and the average signal intensity of the region of interest (ROI) was calculated via the 'Analyze-Measure' function. For the analysis of signal intensity in axons, the entire length per section was used for the quantifications. The numeric values presented in the manuscript represent the output values of ImageJ obtained from the signal intensity measurements. To specifically identify OT-positive varicosities, we first calculated the average signal intensity (via 'Measure-Analyze') and standard deviation of all images included in the data analysis. Next, we used the freehand tool / line to delineate the ROI of the axonal segments and measured the signal intensity. We defined an axon to be 'OT-positive', if the signal intensity within the ROI exceeded the average signal intensity of the included images by at least 4-times the standard deviation. Axonal terminals that could not fulfill this criterion, were considered OT-negative.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantitative analysis (cell counting, c-fos expression and axon length) was performed in a double-blind manner using Fiji or Adobe Photoshop and a superimposed grid feature. Statistical analysis was performed using GraphPad Prism 7. $P < 0.05$ was considered as statistically significant (Tables S1-S6).

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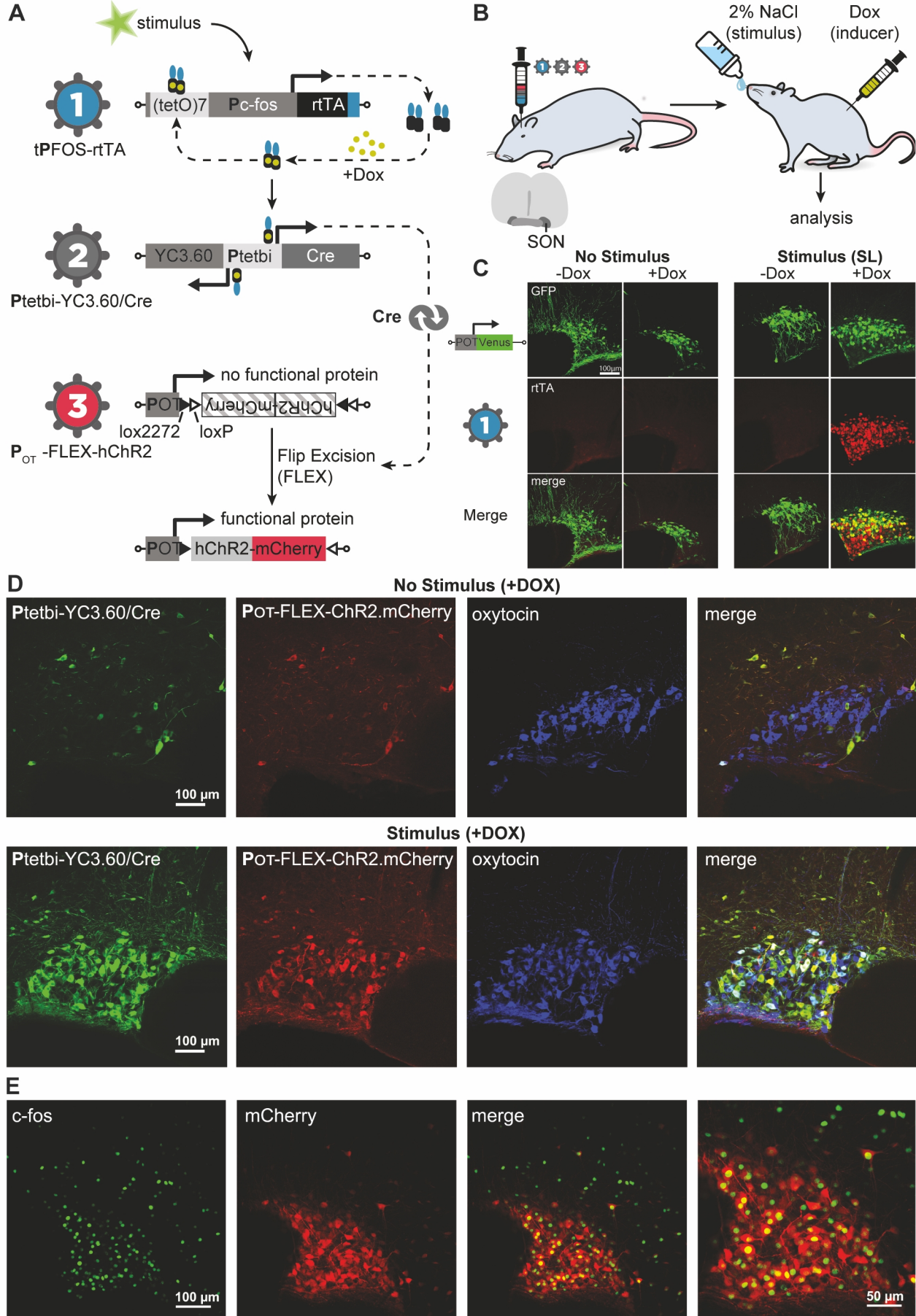
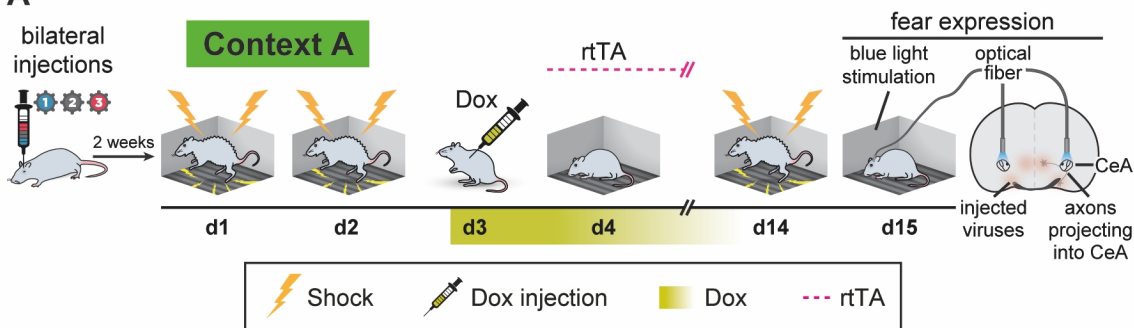
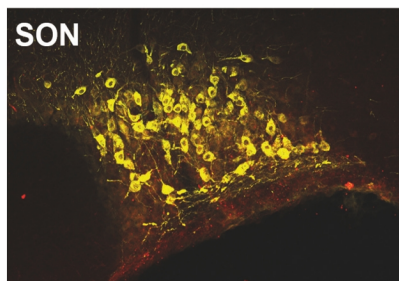
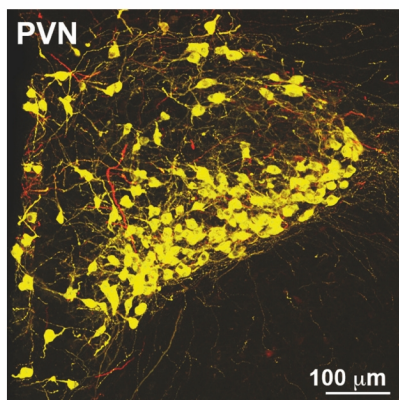


Figure 1



B1 OT^{Constitutive} labeling
POT-hChr2-mCherry



B2 OT^{vGATE} labeling
POT-FLEX-hChr2-mCherry

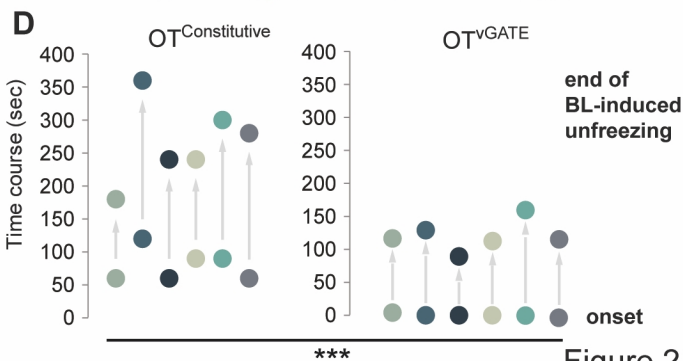
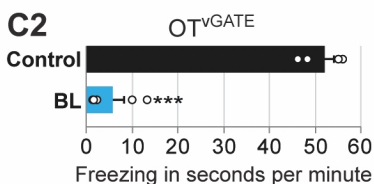
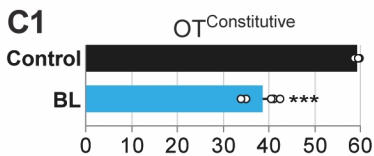
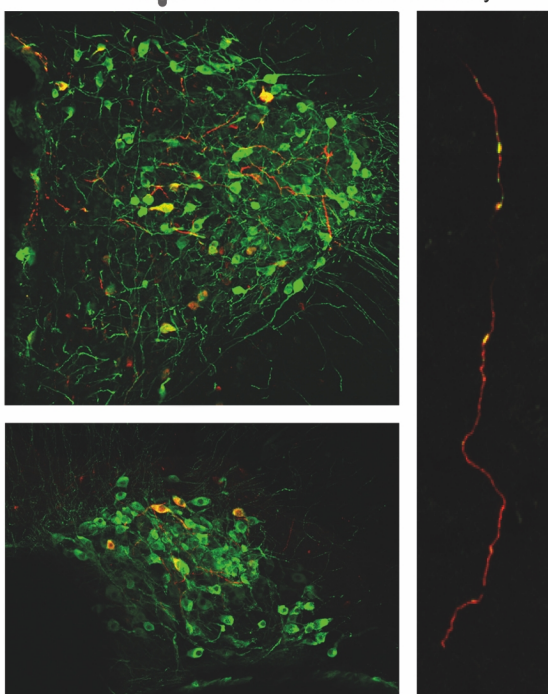


Figure 2

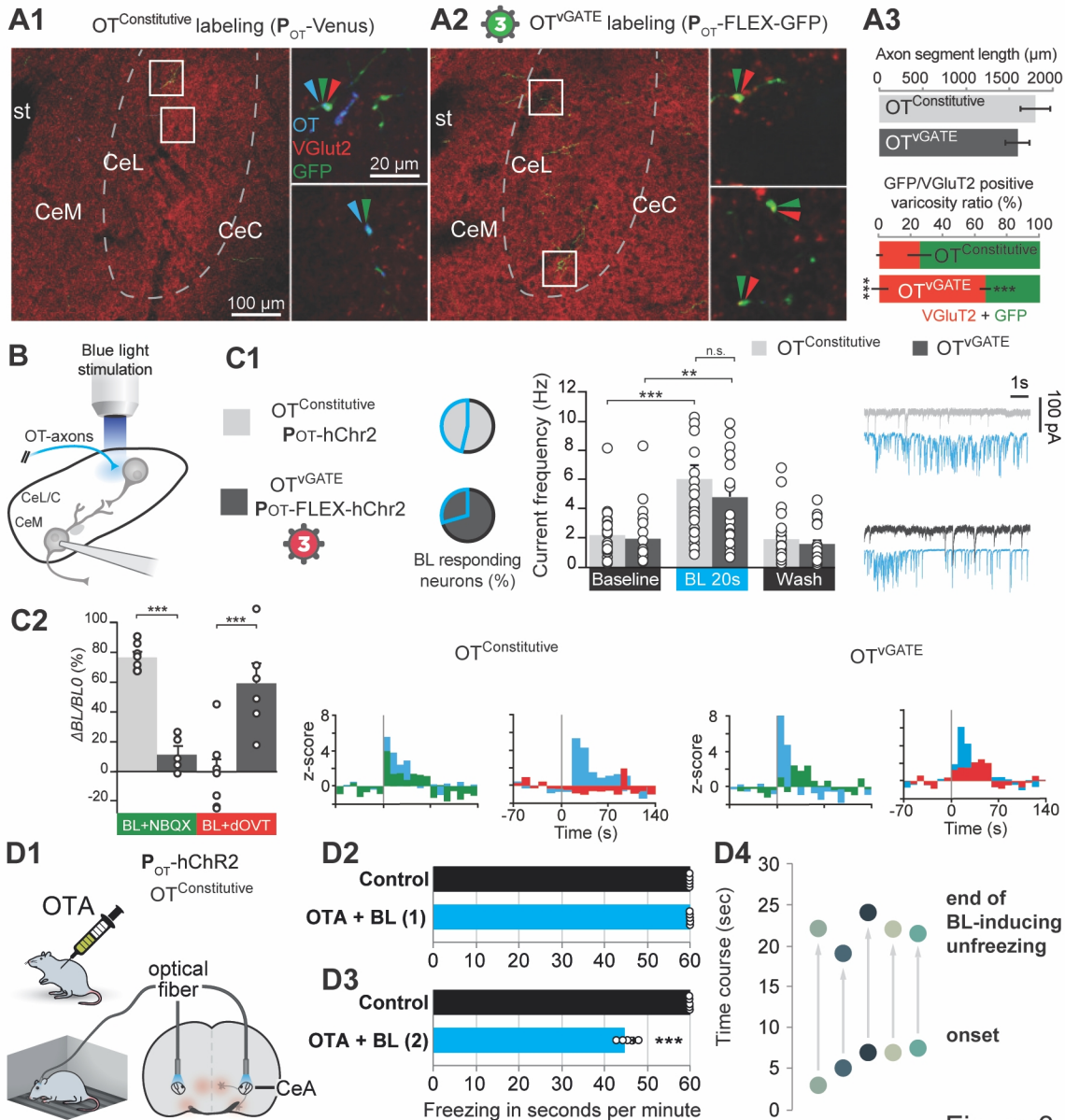


Figure 3

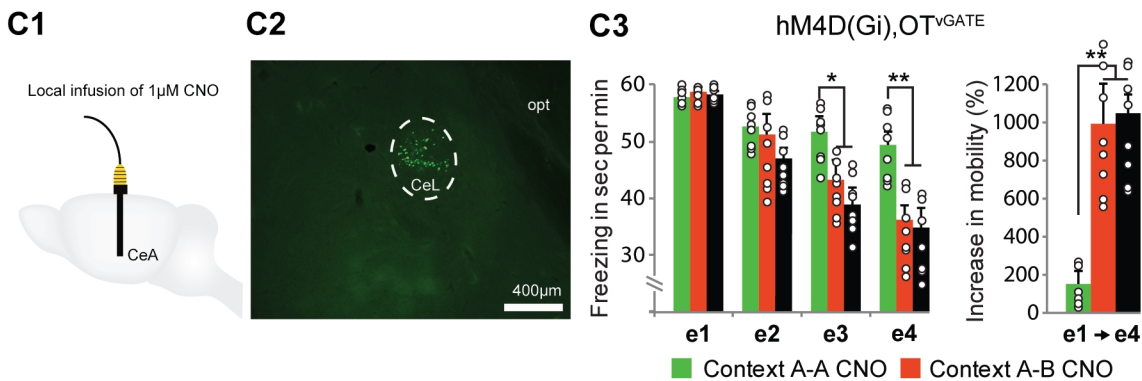
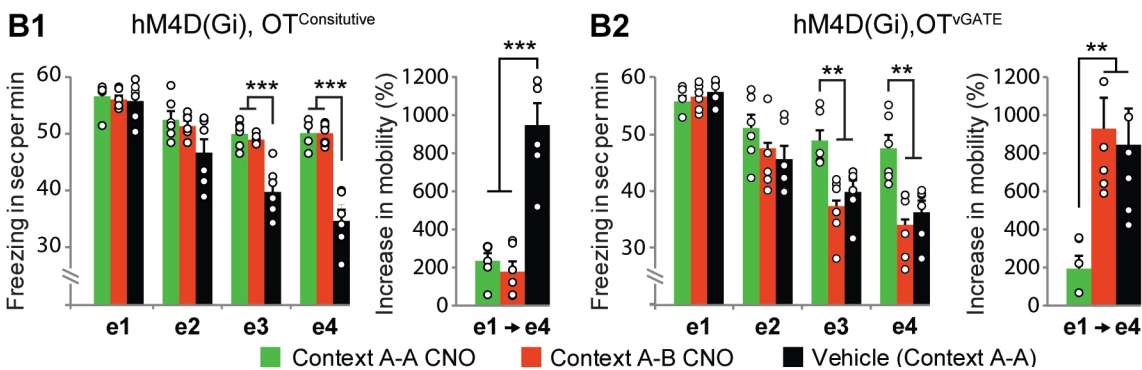
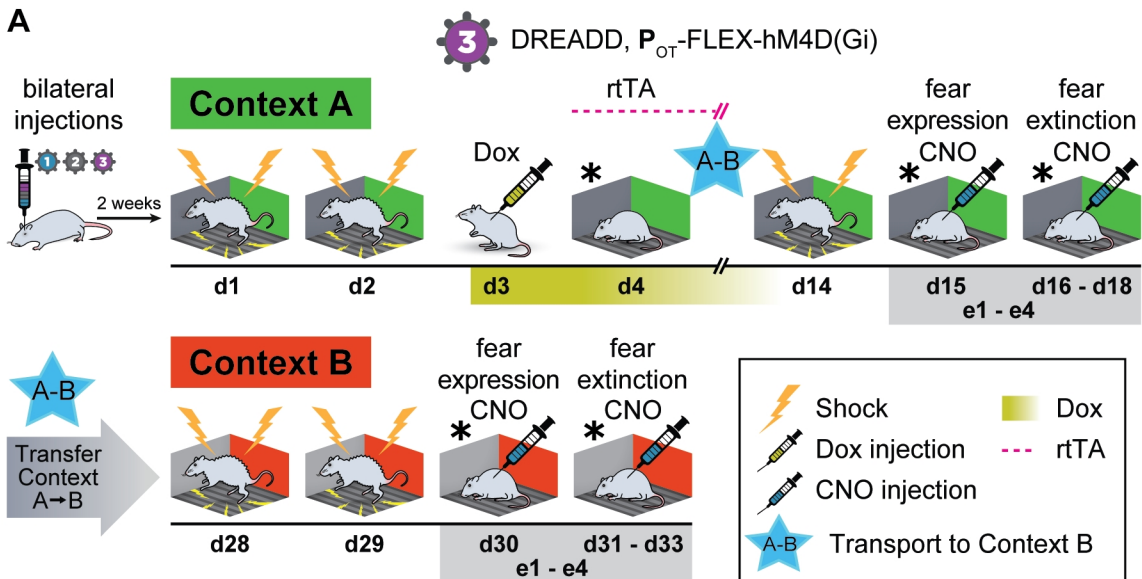
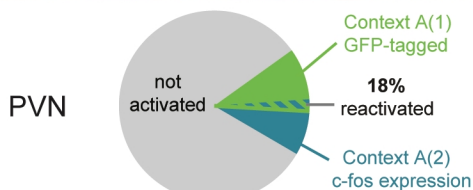
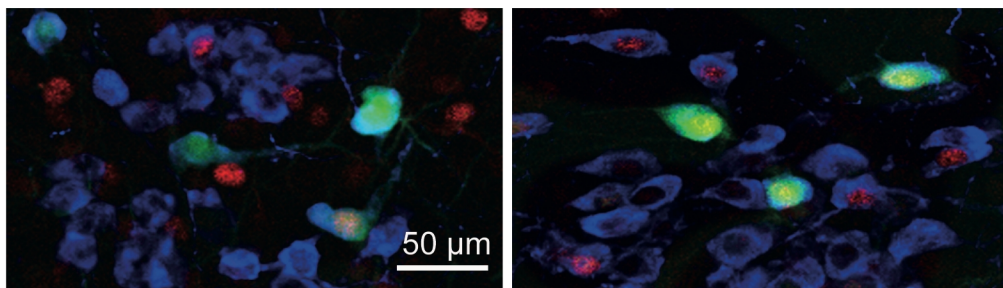


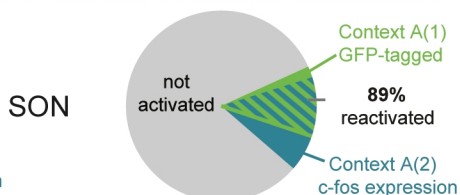
Figure 4

A OT^VGATE (P_{OT}-FLEX-GFP)

GFP (Context A1) c-fos (Context A2) Oxytocin

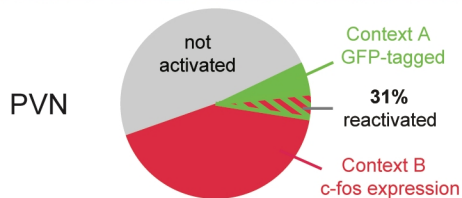
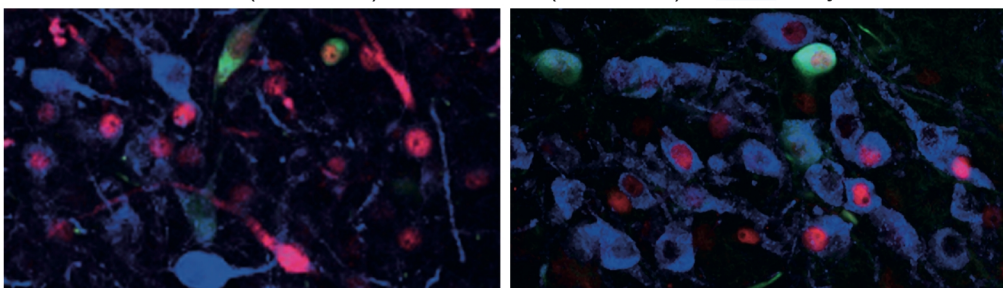


1. Context A(1) → 2. Context A(2)
 (10.8% activated) (9.5% activated)

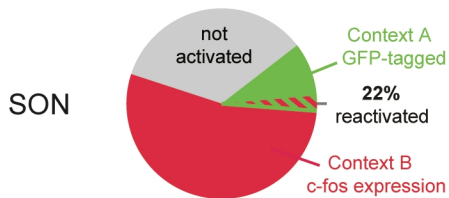


1. Context A(1) → 2. Context A(2)
 (11.9% activated) (16.2% activated)

B GFP (Context A) c-fos (Context B) Oxytocin

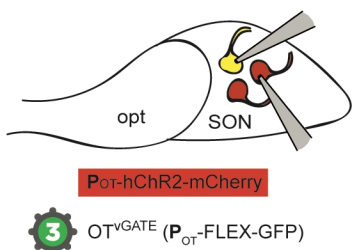


1. Context A → 2. Context B
 (9.6% activated) (47.1% activated)

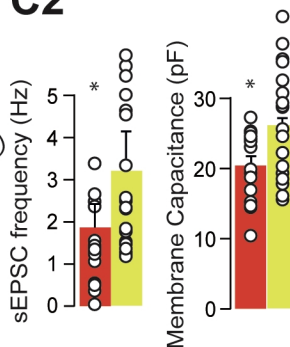


1. Context A → 2. Context B
 (11.7% activated) (56.4% activated)

C1



C2



C3

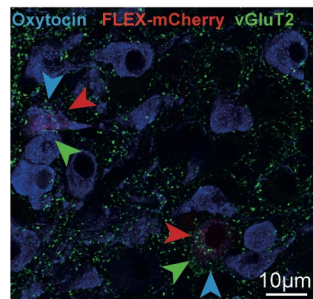
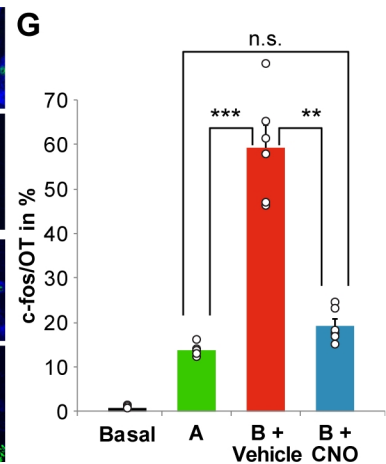
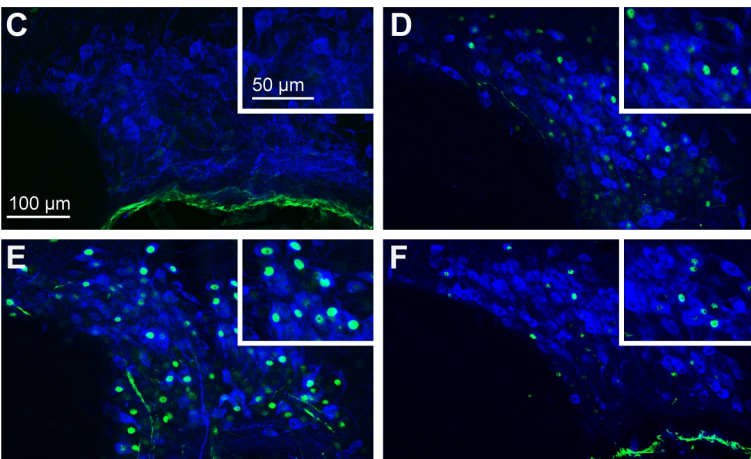
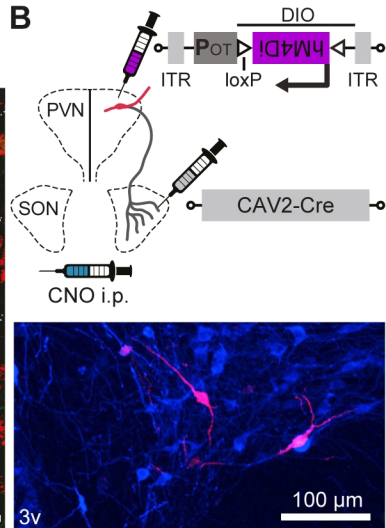
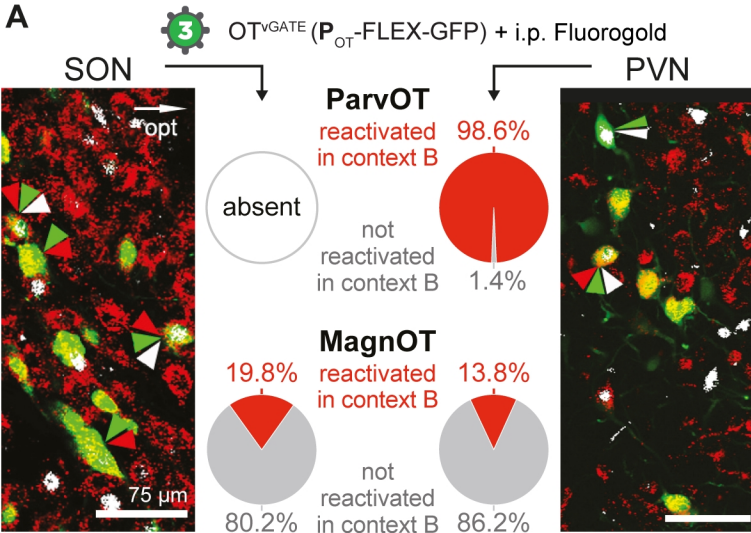


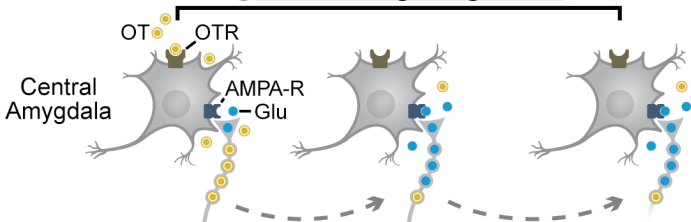
Figure 5



Contextual Fear Experience

Naive → 1st Fear → 2nd Fear

1 OT – Glu Signaling Shift



2 Contextual Distinction

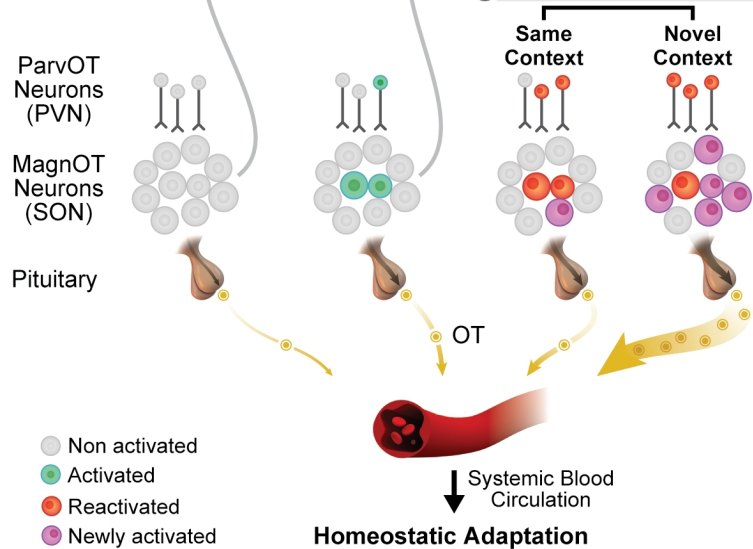


Figure 7