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## A role for *Tac2*, NkB and Nk3 receptor in normal and dysregulated fear memory consolidation

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### Summary

The centromedial amygdala (CeM), a subdivision of the central amygdala (CeA), is believed to be the main output station of the amygdala for fear expression. We provide evidence that the *Tac2* gene, expressed by neurons specifically within the CeM, is required for modulating fear memories. *Tac2* is colocalized with GAD65 and CaMKII $\alpha$  but not with *PKCd* and *Enk* neurons in the CeM. Moreover, the *Tac2* product, NkB, and its specific receptor, Nk3R, are also involved in the consolidation of fear memories. Increased *Tac2* expression, through a stress-induced PTSD-like model, or following lentiviral CeA overexpression, are sufficient to enhance fear consolidation. This effect is blocked by the Nk3R antagonist, osanetant. Concordantly, silencing of *Tac2*-expressing neurons in CeA with DREADDs impairs fear consolidation. Together these studies provide a new understanding of the role of the *Tac2* gene and CeM in fear processing and may provide novel approaches to intervention for fear-related disorders.

### Introduction

Among learning and memory processes, fear memories are crucial in anxiety disorders such as Panic Disorder, Phobia, and Posttraumatic stress disorder (PTSD). PTSD occurs in some individuals after experiencing or witnessing extreme traumatic events. The symptoms of PTSD include re-experiencing memories of these traumatic events through intrusive thoughts, flashbacks, and nightmares (American Psychiatric Association, 2013). PTSD is also generally accompanied by hyperarousal symptoms. Moreover, persistent highly aversive memories related to the trauma, potentially over-consolidated memories, and the

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Supplemental Information

Supplemental information includes Supplemental Experimental Procedures, six figures and two tables.

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inability of these memories to be extinguished are all frequent characteristics of this disorder. Specifically relevant is the memory consolidation phase following emotional learning since it is required to stabilize the initial fear memory trace.

In order to decrease the prevalence of PTSD, it is necessary to identify biological and environmental risk and resilience markers (Norrholm and Ressler, 2009; Vermetten and Lanius, 2012), to find early interventions after trauma exposure (Kearns et al., 2012) and to treat the disorder when it is present and debilitating (Andero and Ressler, 2012; Hetrick et al., 2010). Notably, understanding molecular pathways mediating the initial fear consolidation event is particularly important to target the prevention of PTSD. The only FDA-approved pharmaceutical treatments for PTSD are selective serotonin reuptake inhibitors (SSRI) antidepressants, which have met with limited results in clinical trials (Hetrick et al., 2010). Even when antidepressants are combined with exposure-based psychotherapy, increased effectiveness has not always been demonstrated (Hetrick et al., 2010). Thus, more effective, targeted approaches to prevention and treatment are needed to normalize the functioning of areas key to fear processes such as the amygdala, the hippocampus or the medial prefrontal cortex (mPFC).

The tachykinins refer to two peptides encoded in rodents by the *Tachykinin 1 (Tac1)* and *Tac2 (TAC3)* in humans genes which are involved in neurotransmission and neuromodulation in the central nervous system (Beaujouan et al., 2004). *Tac1* encodes a precursor protein that produces two peptides, substance P (SP) and neurokinin A (NkA), whereas *Tac2/TAC3* encodes neurokinin B (NkB). SP and NkA have been previously implicated in fear processes and PTSD (Dunlop et al., 2012). Unfortunately, clinical trials with pharmaceutical agents targeting the *Tac1* pathway have not previously shown beneficial effects in PTSD treatment (Dunlop et al., 2012). A possible explanation for this lack of effect is that the SP and NkA receptors (Neurokinin 1 receptor, Nk1R, and Neurokinin 2 receptor, Nk2R) are widely expressed in the brain. So, when administering drugs that specifically target Nk1 and Nk2 they interact with multiple brain regions affecting multiple functions (Beaujouan et al., 2004). In contrast, the expression of *Tac2*, NkB, and its specific receptor, Neurokinin 3 receptor (Nk3R), are relatively restricted in rodents to brain regions that regulate emotion, such as the amygdala (Beaujouan et al., 2004; Duarte et al., 2006). Nk3R is a G-protein coupled tachykinin receptor that binds NkB with highest affinity (Gether, 2000; Khawaja and Rogers, 1996). Nk3R couples to the pertussis toxin-insensitive-G proteins Gq/G<sub>11</sub>, the activation of which results in the production of inositol triphosphate and diacylglycerol, and the activation of protein kinase C (Khawaja and Rogers, 1996). Additionally, it has been shown that *TAC3* and Nk3R are expressed in the equivalent areas in rhesus monkeys and humans (Mileusnic et al., 1999; Nagano et al., 2006).

Here, beginning with an unbiased discovery approach, we show that the *Tac2* gene is dynamically regulated during the consolidation of conditioned fear within the central amygdala (CeA). Additionally, Nk3R activation is required for normal consolidation of fear memory formation in mice. Furthermore, increased expression of the *Tac2* gene, NkB peptide and activation of Nk3R may be involved in stress sensitization and over-consolidation of fear. In contrast, genetic silencing of *Tac2*-expressing neurons impairs fear

consolidation. Blockade of this pathway may provide for a novel therapeutic approach for disorders with altered fear learning such as PTSD.

## Results

### ***Tac2* is involved in fear learning**

Using amygdala tissue punches from mice that had been sacrificed 30 minutes or 2 hours after auditory fear conditioning (FC) (CS, acoustic tone; US, electric footshocks, Figure S1A), we performed an mRNA microarray. Using average linkage hierarchical clustering, the microarray heat map shows differential gene regulation at 30 minutes and at 2 hours after fear learning, which is a critical period for consolidation of fear memories (Ressler et al., 2002), Figure 1A. FDR was calculated with SAM 4.01 using a standard 5% cutoff criteria. The cutoff criteria was set with an FDR at the 1.3 fold level for the 2hrs after fear conditioning (FC) group, since with the more conservative 1.5 fold cutoff used in the 30 min after FC group, no genes were initially identified. The criteria followed in Supplemental Table 1 and 2 for a Yes in the column “Specifically highly expressed in the amygdala”: 1) Very high expression in the amygdala (red color, Allen Brain Atlas) 2) No expression of the gene in the hippocampus nor PFC (other key areas related to emotional learning). Moreover, from the top candidates of this microarray, the only gene which is specifically highly expressed in the amygdala and belongs to a ‘druggable’ pathway with available agonists and antagonists which cross the blood-brain barrier and can be used systemically is *Tac2* (See Table S1-2, Figure S1B and S2). Therefore we focused on understanding and manipulating the *Tac2* pathway.

Independent replication studies with additional fear conditioned mice show that *Tac2* is rapidly up-regulated at 30 minutes after FC, returning to basal levels at 2 hours (ANOVA  $F_{3,28} = 5.014$ ,  $P = 0.01$ , Post-hoc \* $P = 0.05$  vs Home Cage (HC) and 2hrs, Figure 1B). Moreover, in an additional replication, *Tac2* mRNA up-regulation only occurred when the conditioned and unconditioned stimuli are paired, but not when they are unpaired, suggesting that within this paradigm, *Tac2* increased expression is specific to associative cued fear learning and independent of non-specific stress and/or contextual learning (ANOVA  $F_{2,36} = 3.93$ ,  $P = 0.05$ , Post-hoc \* $P = 0.05$  vs HC and unpaired, Figure 1C). See Figure S3 for detailed interactions of the *Tac2* gene and Nk3R.

### ***Tac2*, NkB and Nk3R in the amygdala**

Figure 1D and S1B show a radioactive *in situ* hybridization demonstrating that the areas where *Tac2* gene is expressed are quite specific and limited within in the mouse brain: bed nucleus of the stria terminalis, hypothalamus, habenula, central amygdala (CeA), zona incerta and medial mammillary nucleus. *Tac2* is highly expressed in the CeA within the amygdala with no expression in the basolateral amygdala (BLA) nor lateral amygdala (LA) (Figure 1D). The highest expression of *Tac2* within the CeA occurs in the medial subdivision of the central amygdaloid nucleus (CeM), whereas lower expression is observed in the centro-lateral (CeL) and centro-central (CeC) amygdala.

Recently, specific cell populations within the central nucleus have received attention for distinct roles in fear learning. For example, *PKCd* has been suggested to be part of a microcircuit in which the CeL amygdala neurons inhibit neuronal output to the CeM during the conditioned stimulus, which drives fear expression, called CeL<sub>off</sub> units (Haubensak et al., 2010). Moreover, around 40% of Protein Kinase C Delta (*PKCd*) expressing neurons also express Enkephalin (*Enk*) in the CeL (Haubensak et al., 2010). Of note, *Enk* mRNA levels are increased after FC in the CeL (Petrovich et al., 2000). Since other neuronal populations have been previously related to fear processes in the CeA, we wished to examine if *Tac2* mRNA colocalized with them suggesting functional interactions. Using double fluorescent *in situ* hybridization (FISH), we show that *Tac2* gene expression is mostly not colocalized with *PKCd* nor *Enk* and is expressed primarily within the CeM (Figure 2A-F). Thus, given the lack of colocalization and regional and functional specificity of these cell populations, we have uncovered a subdivision-specific cell population that may be involved in the consolidation of fear memory. Additionally, the colocalization of *Tac2* mRNA levels and the 65-kDa isoform of glutamic acid decarboxylase (GAD-65) peptide in the CeM (Figure 2G) may provide deeper understanding of the functions of gamma-aminobutyric acid (GABA) in fear learning. CaMKII $\alpha$ , a well characterized neuronal population involved in synaptic plasticity, is also colocalized with *Tac2* mRNA in the CeM (Figure 2H). Interestingly, GAD65 and CaMKII are associated with the consolidation of fear memories in the amygdala although little is known about the specific role of these peptides in each substructure (Bergado-Acosta et al., 2008; Lepicard et al., 2006).

We also examined detection of the NkB peptide in amygdala cell culture, demonstrating that the peptide is highly present in both soma of neurons and dendrites (Figure 3A). Moreover, NkB peptide is also highly expressed in the CeA (Figure 3B). Interestingly, NkB is up-regulated in the amygdala 2 hours after FC (Student's t test,  $t = -2.902$ ,  $**P = 0.01$  Fear Conditioning vs Home Cage, Figure 3C). The Nk3R antagonist osanetant has already been used in humans in clinical trials for schizophrenia. Although it appears to have no beneficial effects in the treatment of schizophrenia, these studies show that it is well tolerated and safe in humans (Meltzer et al., 2004). Here, in amygdala cell culture, osanetant inactivates the Nk3R and leads to a compensatory increase in Nk3R expression as suggested by dose-dependent enhanced *Nk3R* mRNA levels (ANOVA,  $F_{3,5} = 10.014$ ,  $P = 0.05$ ; Post-hoc  $*P = 0.05$  vs Veh,  $**P = 0.01$  vs Veh, Figure 3D).

### Osanetant and emotional learning

The above studies suggest that osanetant may be an ideal candidate to target the Nk3R in the amygdala *in vivo*, and we wished to examine its effects behaviorally. Osanetant given systemically, 30 minutes before open field, elevated plus maze and the conditioning chamber elicits no changes in anxiety-like behavior, locomotor activity or electric shock reactivity (Figure S4). Notably, when osanetant is dosed from 30 min before auditory FC up to 1 hour after training it does not affect fear acquisition, but impairs fear memory consolidation as shown by decreased freezing in the fear expression test (Figure 4A and S4G; Student's t test, 30 min  $t = 3.042$ ; 10 minutes after FC,  $t = 2.277$ ; 1 hour after FC,  $t = 2.872$ ;  $*P = 0.05$  vs vehicle).

Recently, the PACAP-PAC1R pathway has been associated with PTSD in humans as well as in animal models (Ressler et al., 2011, (Stevens et al., 2014). These prior data showed that expression of the *ADCYAP1R1* gene (encoding the PAC1 receptor) is increased following FC. Here we found that osanetant given before FC also normalizes the levels of *ADCYAP1R1* mRNA levels in the amygdala (ANOVA  $F_{2,31} = 5.541$ ,  $P = 0.01$ ; Post-hoc  $*P = 0.05$  vs Veh-FC;  $**P = 0.01$  vs Veh-FC, Figure 4B). These data suggest that inhibition of the *Tac2*/NKB/Nk3R pathway may prevent activation of a stress-related gene pathway previously associated with PTSD. Concordantly, bilateral infusion of osanetant in the CeA also impairs fear memory consolidation, suggesting that CeA-NK3R are required for the formation for emotional memories (Student's *t* test;  $t = 2.268$ ,  $*P = 0.05$  vs vehicle, Figure 4C and D).

We have shown in previous studies that mice exposed for 2 hours to a severe one-time stressor, immobilization to a wooden board (IMO), present long-term PTSD-like symptoms: impaired fear extinction and spatial memory, and enhanced anxiety-like behaviors (Andero et al., 2013; Andero et al., 2011). Additionally, IMO in rats elicits alterations of the hypothalamic-pituitary-adrenal (HPA) axis which may be similar to the process initiating PTSD in humans (Armario et al., 2008). Notably, *Tac2* mRNA levels were more robustly up-regulated in IMO treated mice than in naïve mice after FC, consistent with enhanced *Tac2*-dependent fear processing (ANOVA  $F_{3,53} = 6.242$ ,  $P = 0.001$ , Post-hoc  $*P = 0.05$  vs HC,  $**P = 0.01$  vs IMO, Figure 4F). Additionally, osanetant given systemically after FC impaired memory consolidation in IMO treated mice, as shown by decreased freezing in the fear expression test (ANOVA repeated measures  $F_{1,13} = 6.072$ ,  $*P = 0.05$ , Figure 4G). This suggests that Nk3R antagonism reduces enhanced fear memory consolidation in a PTSD-like model.

### ***Tac2* overexpression and blockade by osanetant**

We next developed a viral vector to overexpress the *Tac2* gene in an inducible fashion within the brain, the lentivirus-*Tac2* (LV-*Tac2*). We first tested its functional expression by infecting HEK 293 cells with the LV-*Tac2* compared to control LV-GFP lentiviruses, demonstrating that the NkB peptide was robustly expressed (Figure 5A and B). We then examined the behavioral effects of *Tac2* overexpression in mice. LV-*Tac2* or LV-GFP were bilaterally infused in the CeA (Figure 5C and D) and 14 days later *Tac2* was found to be overexpressed by 42%, as determined by mRNA levels with *in situ* hybridization, compared to mice that had received LV-GFP (Student's *t* test;  $t = -3841$ ,  $**P = 0.001$  vs LV-GFP, Figure 5E). Mice infected with the LV-*Tac2* or LV-GFP received systemic osanetant or vehicle immediately after FC, and then fear expression was tested 24 hours later. Specific CeA-*Tac2* overexpression elicited a significant enhancement of fear memory consolidation (ANOVA  $F_{3,20} = 8.512$   $P = 0.05$ ; Post-hoc  $**P = 0.05$  vs LV-GFP-Veh, Figure 5I). Interestingly, we found that *Tac2* overexpression in the CeA did not induce changes in anxiety-like behavior nor fear acquisition (Figures 5G and H). Replicating our previous findings, osanetant impaired fear memory consolidation when given to mice with the control LV-GFP (Post-hoc,  $*P = 0.05$  vs LV-GFP-Veh, Figure 5I). Additionally, the enhanced fear memory consolidation caused by CeA-*Tac2* overexpression was reversed by osanetant

(Post-hoc, \*\* $P < 0.01$  vs LV-*Tac2*-Osa and LV-GFP-Osa Figure 5I). See Figure S5 for a graphical representation of the *Tac2*-LV overexpression.

### Silencing of *Tac2*-expressing cells and emotional learning

To further understand the role of the *Tac2* gene, we temporarily silenced the activity of neurons expressing this gene in the CeA during fear learning using designer receptors exclusively activated by designer drugs (DREADD) technology. The B6.129-*Tac2*<sup>tm1.1(cre)Qima/J</sup> (*Tac2*-cre) (Mar et al., 2012) mice were infected with a DREADD Gi-coupled receptor via the pAAV-hSyn-double floxed hM4D-mCherry virus (hM4Di-mCherry AAV) (Figure 6A). This elicited specific expression of the mCherry reporter in *Tac2* cells within the CeA, but not any other area of the brain, suggesting the insertion of the DREADD receptor on the plasma membrane (Figure 6B and 6C, (Krashes et al., 2011). 14 days later, clozapine-N-oxide (CNO), which binds to the inserted receptor but otherwise is pharmacologically inert, was given systemically 30 minutes before FC in both groups, *Tac2*-Cre-hM4Di-mCherry and *Tac2*-Cre+/hM4Di-mCherry. CNO had no effect on fear acquisition as shown by equivalent amount of freezing in both groups (Figure 6D). However, when animals were tested for fear expression, 24hrs later in the absence of CNO, the *Tac2*-Cre+/hM4Di-mCherry mice presented less freezing, suggesting impaired fear memory consolidation (Student's *t* test,  $t = 3.257$ , \* $P < 0.05$  *Tac2*-Cre-hM4Di-mCherry vs *Tac2*-Cre+/hM4Di-mCherry, Figure 6D). This demonstrates that the animals expressing *Tac2*-Cre+/hM4Di-mCherry and inducible Gi to temporally silence the activity of *Tac2*-expressing neurons exhibit significantly less fear consolidation when tested for fear learning. Mice were then retrained with a different CS and a different context in the same FC apparatus, as in previous experiments but without dosing CNO. *Tac2*-Cre-hM4Di-mCherry and *Tac2*-Cre+/hM4Di-mCherry mice showed similar amount of freezing in the FC and fear expression test (Figure 6E). This suggests that when *Tac2*-expressing neurons are not silenced there is normal fear memory consolidation in both *Tac2*-Cre-hM4Di-mCherry and *Tac2*-Cre+/hM4Di-mCherry groups. Moreover, when given CNO, these two groups presented equivalent levels of anxiety-like behavior and pain sensitivity (Figure 6F and Figure S6).

### Discussion

Previous reports have shown that Nk3R is associated with memory processes in hippocampus-dependent tasks in rodents (de Souza Silva et al., 2013; Siuciak et al., 2007; Zlomuzica et al., 2008). Here, we provide novel evidence that *Tac2*-NkB-Nk3R signaling within the CeA is required for the modulation of fear memory consolidation. Other studies have shown that the CeA is required for the acquisition, consolidation and expression of fear memories (Wilensky et al., 2006). Here we show novel mechanisms that may also be involved in those processes within the CeA. To the best of our knowledge, this is the first evidence that a neuronal population specifically highly expressed in the CeM, *Tac2* and its product NkB peptide, are required for the modulation of fear memory consolidation affecting neither unconditioned fear nor anxiety-like behavior. All that is known about NkB release is from *in vitro* experiments, where it is suggested that NkB release is potassium-evoked and calcium-dependent, fulfilling the criterion of a neurotransmitter or a

neuromodulator (Lindfors et al., 1985). More studies about this topic would be desirable to further understand the mechanisms of the *Tac2*/*NkB*/*Nk3R* pathway.

Our findings also suggest that CeA-*Tac2* lentiviral overexpression enhances fear memory consolidation but *Nk3R* antagonism prevents it. This shows that *Nk3R* antagonism within the CeA is able to normalize dysregulated functioning induced by the *Tac2* gene. We believe that it is possible that osanetant given systemically or intracranially within the CeA may be acting in the *Nk3R* in all areas of CeA and not only in the CeM (Smith and Flynn, 2000; Yip and Chahl, 1997). This impairment of fear memory consolidation by *Nk3R* antagonism is consistent with previous reports where *Nk3R* activation with senktide in the hippocampus and cortex leads to enhanced post-synaptic depolarization and long-term-potential (LTP) in slice physiology studies, and this effect was blocked by *Nk3R* antagonism (Gallopín et al., 2006; Rekling, 2004). However, specific electrophysiological experiments in the amygdala should be performed in the future to study if activation or blockade of the *Nk3R* in this structure is involved in LTP or other types of activity-dependent plasticity.

In agreement with these prior findings, specific and temporal pharmacogenetic silencing of *Tac2*-expressing neurons in the CeA with DREADDs leads to impaired fear memory consolidation. Of note, no effects in fear acquisition, pain sensitivity nor anxiety-like behavior were detected when over-expressing *Tac2*, inhibiting *Tac2*-expressing neurons, or with the *Nk3R* antagonist, which is consistent with previous findings suggesting that the *Tac2*-*NkB*-*Nk3R* pathway is not directly involved in these processes, although it may modulate them (Ebner et al., 2009; Mar et al., 2012; Siuciak et al., 2007). Interestingly, in our fear paradigm, the *Tac2* gene within the amygdala is involved in auditory (CS+US paired) but not stress nor contextual memories (US only). However, this does not preclude the hypothesis that different fear paradigms might reveal a role of *Tac2* in the amygdala in contextual fear conditioning.

Thus, we believe that enhanced *Tac2* gene expression in our fear models enhanced *NkB* production in the amygdala, binding to *Nk3R* and promoting fear memory consolidation. This up-regulation of *Tac2* mRNA levels primarily within the CeM suggests several possible non-mutually exclusive scenarios. The first is that the *Tac2* gene synthesizes *NkB* in the CeM amygdala, acting on local *Nk3R* within the CeM specifically. The second is that *Tac2* mRNA and/or *NkB* are transported from the CeM to other nuclei within the amygdala such as CeL, CeC or BLA where they bind to the *Nk3R*. Our data also suggests that amygdala cell culture with osanetant increases *Nk3R* mRNA levels. The most likely interpretation is that osanetant antagonizes amygdala *Nk3R* and due to its decreased availability, *Nk3R* mRNA is increased to synthesize more *Nk3R* in a compensatory manner. The current data provide intriguing support for a specific role of *Tac2* gene, via *NkB* activation of *Nk3R* in fear consolidation within the CeA.

CeLon neurons essentially serve as disinhibitory cells for CeLoff neurons. Both CeLon and CeLoff neurons release GABA when activated. CeLon neurons send projections to CeLoff neurons, and, therefore, the increased firing of CeLon neurons during CS presentation results in inhibition of CeLoff neurons (normally inhibiting the CeM), and, thus, in disinhibition of CeM, promoting conditioned fear responses (Ciocchi et al., 2010).

Specifically, CeLoff neurons largely overlap with PKCd+ neurons (Haubensak et al., 2010). Moreover, we also show that *Tac2* gene is not colocalized with *Enk* in the CeM. *Enk* is colocalized with *PKCd* in the CeL (Haubensak et al., 2010) and specific CeA-*Enk* deletion decreases fear expression during FC without affecting fear memory consolidation (Poulin et al., 2013). Thus, the *Tac2*-CeM neuronal population appears to be independent of, and complementary to, other previously described neuronal populations involved in FC. The GAD65 peptide, abundantly found at nerve terminals and synapses, plays a key role in GABA neurotransmission (Pinal and Tobin, 1998). Additionally, CaMKII is a well-known marker for synaptic plasticity. Thus, the colocalization of *Tac2* mRNA levels and GAD65 and CaMKII $\alpha$  peptides in the CeM suggest that *Tac2* gene may have a role in neurotransmission within the GAD65 and CaMKII $\alpha$  expressing neurons, in agreement with our data that suggest that this CeM population may be critically involved in fear memory consolidation.

Finally, one of the most interesting aspects of our data is the potential use of the Nk3R antagonist osanetant as a pharmacological agent to block fear memory consolidation shortly after exposure to a trauma. Additionally we found that osanetant prevented the up-regulation of the *Adcyap1r1* gene, which encodes the PAC1 receptor. The PACAP-PAC1R pathway is involved in PTSD, fear conditioning, amygdala excitatory neurotransmission and stress (Almli et al., 2013; Cho et al., 2012; Hashimoto et al., 2011; Ressler et al., 2011; Uddin et al., 2013). All this could be relevant in PTSD prevention since it has previously been found that osanetant is safe in humans, although additional preclinical studies, such as those described herein are needed first to establish the mechanisms involved. This gives our findings an exciting potential approach to translation to human patients. Although other molecular pathways have previously been associated with PTSD we believe that there will be a number of different mechanisms identified that eventually will synergistically be used to target emotional memory modulation.

In summary, these studies provide a new understanding of the role of the *Tac2* gene and the CeM in fear processing and provide novel approaches to intervention for fear-related disorders.

## Experimental procedures

Procedures are described in detail in Supplemental Experimental Procedures.

### Mice

Amygdala cell culture experiments were performed with male wild-type (WT) C57BL/6J p21 mice. All other experiments were performed on adult WT C57BL/6J or B6.129-*Tac2*<sup>tm1.1(cre)Qima/J</sup> (*Tac2*-Cre) (Mar et al., 2012) from Jackson Labs (Stock # 018938), male mice that were group-housed in a temperature-controlled vivarium, with *ad libitum* access to food and water. Animals were maintained on a 12-hour/12-hour light/dark cycle, with all behavioral procedures being performed during the light cycle. All procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.



### **Immobilization to wooden board (IMO)**

Mice were exposed once for 2 hours to IMO, which was performed as previously described (Andero et al., 2013; Andero et al., 2011).

### **mRNA extraction and microarray**

Total mRNA was isolated and purified from the tissue with the RNeasy Mini Kit (catalog 74106, Qiagen). Illumina MouseWG-6 v2 Expression BeadChip microarray (Illumina Inc.) was assayed for 45,281 transcripts as previously described (Andero et al., 2013). FDR was calculated with SAM 4.01 using a 5% cutoff for the FDR rate. The heat maps were created with Genesis 1.4.0 (Sturn et al., 2002). The pathway analysis was generated through the use of IPA (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)). The microarray data are publicly available in the Gene Expression Omnibus database under accession number [GSE57465](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57465).

### **Behavioral experiments**

Elevated plus maze, open field, cued-fear conditioning and fear expression tests were performed as previously described (Andero et al., 2013; Andero et al., 2011). The CS were 30 seconds 0.6 KHz tone and the US were 1 mA 500 ms electric footshocks. Retraining of mice was performed with a 12 kHz tone.

### **Complementary DNA synthesis and qPCR**

Total mRNA was reverse-transcribed with the RT<sup>2</sup> First Strand Kit (catalog 330401, Qiagen). The primers used for the qPCR were TaqMan *Tac2* Mm01160362\_m1, *ADCYAP1R1* Mm01326453\_m1 and *NK3R* Mm00445346\_m1 from Applied Biosystems. The qPCR was performed and analyzed as previously described (Andero et al., 2013).

### **Radioactive in situ hybridization**

Tissue was fixed in 4% paraformaldehyde, pretreated, hybridized with 36SUTP labeled cRNA riboprobes prepared from linearized constructs for antisense sequence of *Tac2* (T7 RNA polymerase) as previously described (Rattiner et al., 2004).

### **Fluorescent in situ hybridization (FISH)**

cRNA riboprobes prepared from linearized constructs for antisense sequences of *Tac2*, *PKCd* and *Enkephalin* (T7 RNA polymerase) as previously described (Jasnow et al., 2013). The *Tac2* riboprobe was labeled with fluorescein and the *PKCd* and *Enkephalin* with digoxigenin. Signals were amplified with the TSA Plus Fluorescein Fluorescence System or TSA Plus Cy5 Fluorescence System (PerkinElmer) following each series of primary antibodies. Sections were then stained with DAPI (1:1000), washed, and coverslipped with Mowiol mounting medium (Jasnow et al., 2013).

### **Amygdala cell culture**

Amygdala primary cell culture was performed as previously described (Mou et al., 2011).

## Immunohistochemistry

Pep2/ProNkB, IS-39 ab (1:500) was the antibody used to detect NkB. The procedure was followed as previously described (Kallo et al., 2012). The procedure for detecting Gad65 (AB5082, Chemicon, 1:500) and CaMkII $\alpha$  (Cell Signalling Solutions, 1:250) was similar as previously described (Jasnow et al., 2013) after performing the *Tac2* FISH. After the ISH and IHC sections were stained with DAPI (1:1000) washed, and coverslipped with Mowiol mounting medium.

## Immunocytochemistry

Immunocytochemistry was performed as previously described (Mou et al., 2011). The antibody used was Pep2/ProNkB IHC (IS-39 ab, 1:500) (Kallo et al., 2012) and DAPI or NeuN (1:1000).

## ELISA

Purchased from Mybiosource, Mouse Neurokinin B ELISA Kit (NKB), Catalogue #MBS744693. Inter-assay CV%: 7.5-8.6, Intra-assay CV%: 8.2-9.5, Spike Recovery: 95-103%. Procedure was followed as indicated by the manufacturer.

## Production of Recombinant Viral Vectors

Mixture for transfection was 250 $\mu$ g of FUGW or FUGW-*Tac2* + 187.5 $\mu$ g of pCMVdelta 8.9 + 75 $\mu$ g of pV-SVG + 12ml of ddH<sub>2</sub>O + 12.5ml of 0.5M CaCl<sub>2</sub> + 25 ml of 2x HeBS to total volume 50ml, this solution was vortexed a few seconds and incubated for 20 min at room temperature. Procedure was followed as previously described (Huang et al., 2013). The pAAV-hSyn-double floxed hM4D-mCherry (hM4Di-mCherry AAV) was purchased from UNC Gene Therapy Center, NC, USA.

## Surgery and injection of virus

Mice were anesthetized and placed in a stereotaxic frame. CeA coordinates were as follows: anteroposterior, -1.34mm; dorsoventral, -4.4mm; mediolateral, -2.4mm relative to bregma. For the LV-*Tac2* experiments the animals received bilateral intra-CeA amygdala injections of lentiviral vectors expressing *Tac2*-FUGW or FUGW (GFP) in 1% BSA in phosphate buffered saline (PBS). 0.5  $\mu$ l of virus/side. 1  $\mu$ l of virus/side of the pAAV-hSyn-double floxed hM4D-mCherry (hM4Di-mCherry AAV) was injected in the CeA of *Tac2*-Cre- and *Tac2*-Cre+ mice. For all experiments the rate of injection was 0.1  $\mu$ l/min and the needle was left in place for 10 min following injection and the skin was closed using a 6-0 Vicryl suture.

## Drugs administration

The Nk3R antagonist osanetant (Axon Medchem, Axon 1533) was dissolved in physiological saline and 0.1% Tween 20 which was also the vehicle. Intraperitoneally (i.p.) dose was 5 mg/kg for systemic administration and 0.5  $\mu$ l with 625 ng dose per side for the intra-CeA studies. Cannulation of the mice was performed as previously described (Andero et al., 2013). Clozapine-N-oxide (CNO, Sigma Aldrich C0832) was given i.p. at 1mg/kg (Krashes et al., 2011).

## Statistics

Statistics were performed with IBM SPSS Statistics 19.0. Detection of outliers was performed and, when necessary, removed from analyses. ANOVA followed by post-hoc analyses were appropriate, repeated-measures ANOVA or Student's t test (two-tailed) for independent samples was tested. The results are presented as means  $\pm$  or + SEM, and statistical significance was set at  $P < 0.05$ .

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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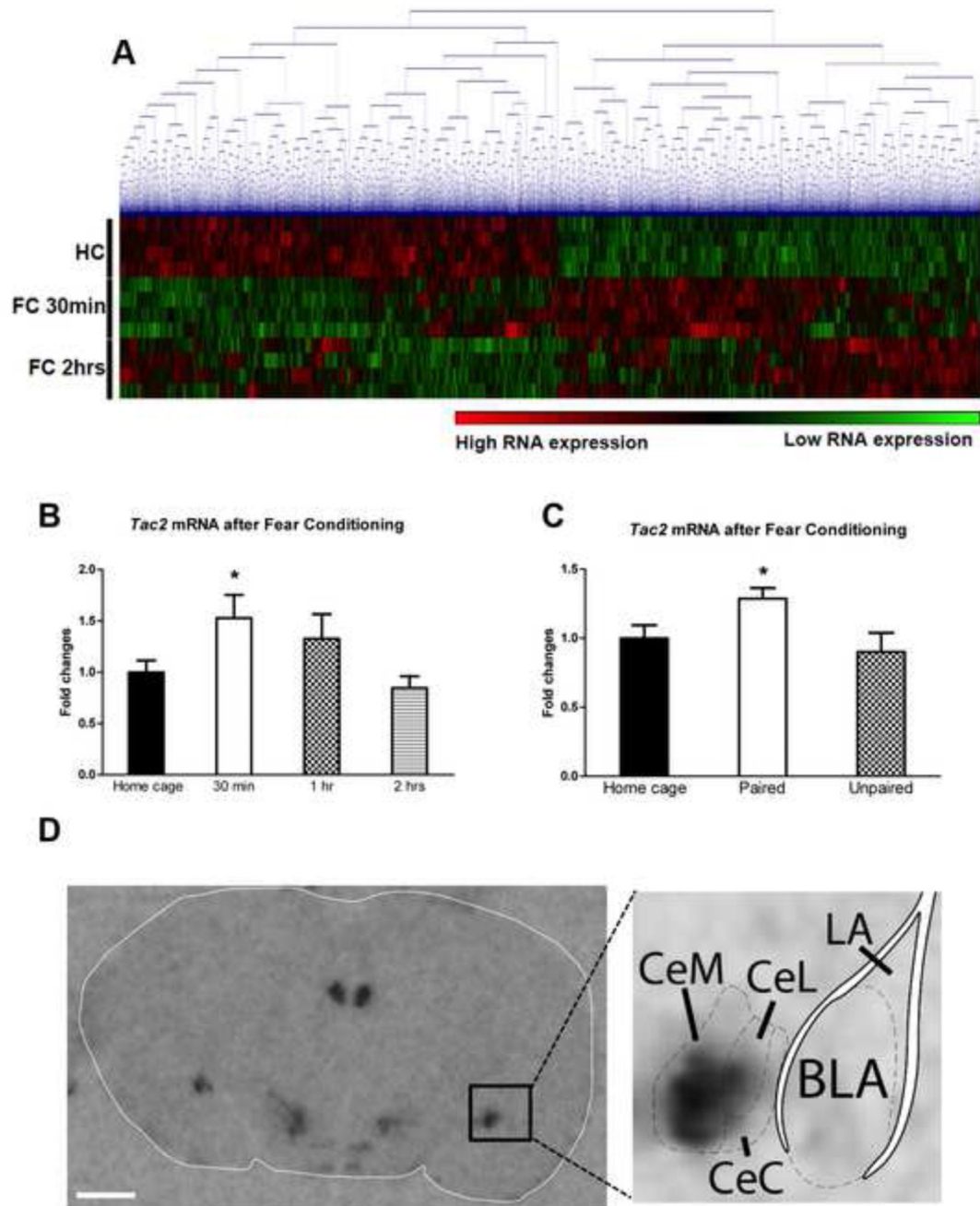
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### Highlights

- *Tac2* is associated with memory consolidation in naïve mice and a PTSD-like model.
- Antagonism of the *Tac2* receptor restores normal and dysregulated fear consolidation.
- *Tac2* colocalizes with GAD65 and CaMKII $\alpha$  but not with *PKCd* nor *Enk* neurons in the CeM.
- *Tac2* neurons are required for the modulation of fear memory consolidation in the CeA.

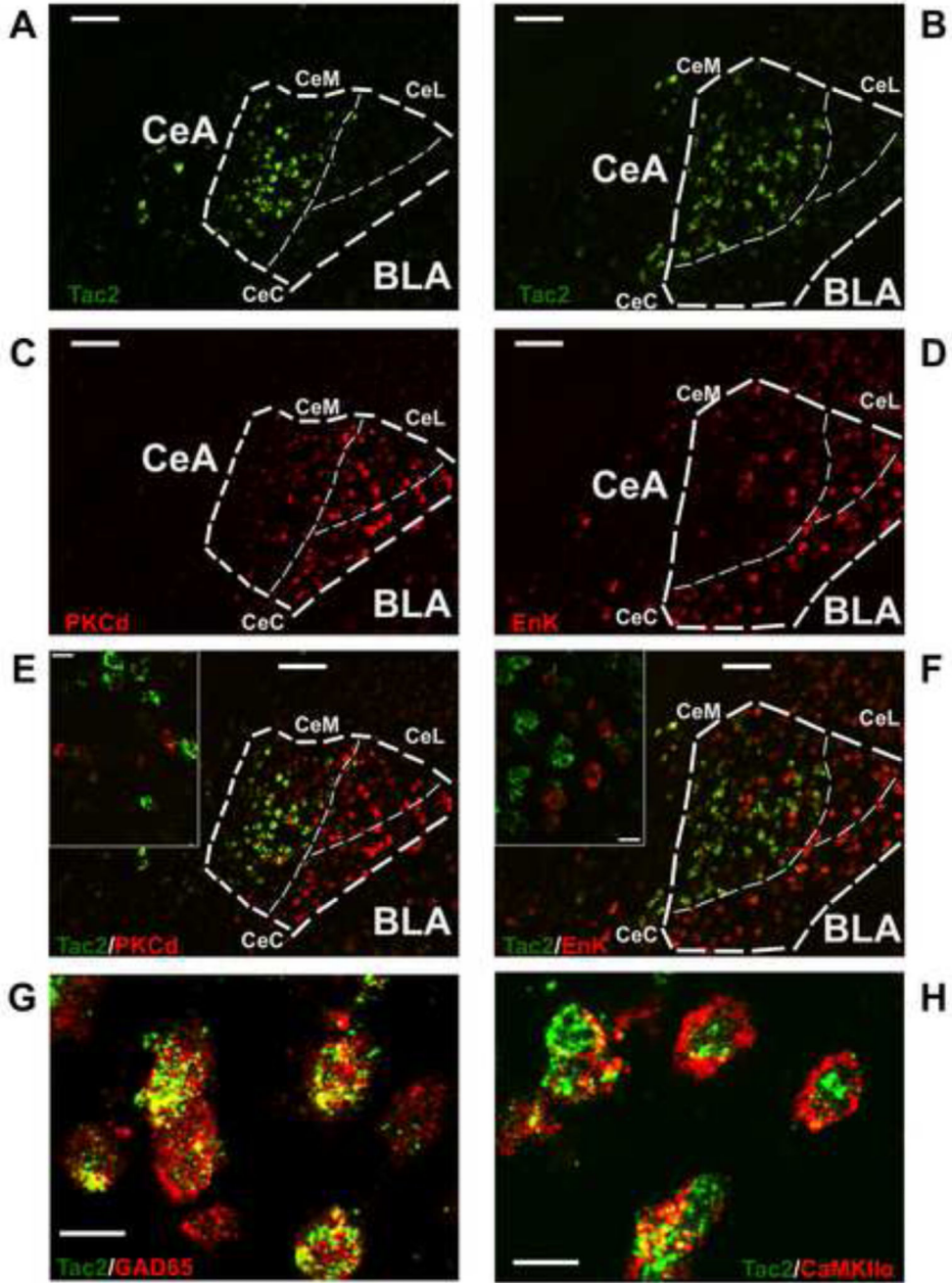


**Figure 1. Differential regulation of *Tac2* gene expression in the amygdala during cued-fear conditioning**

A) With average linkage hierarchical clustering of a RNA microarray there is a differential gene regulation 30 minutes and 2 hours after auditory fear conditioning (FC) when compared to home cage group (no FC). N=4 per group. B) *Tac2* mRNA levels are rapidly up-regulated in the amygdala during fear consolidation 30 minutes after fear conditioning. \*P < 0.05 vs HC and 2 hrs. N=7-8 per group. C) *Tac2* up-regulation occurs when the conditioned stimulus (acoustic tone) and the unconditioned stimulus (electric footshock) are paired but not when they are unpaired. \*P < 0.05 vs HC and unpaired. N=11-15 per group.



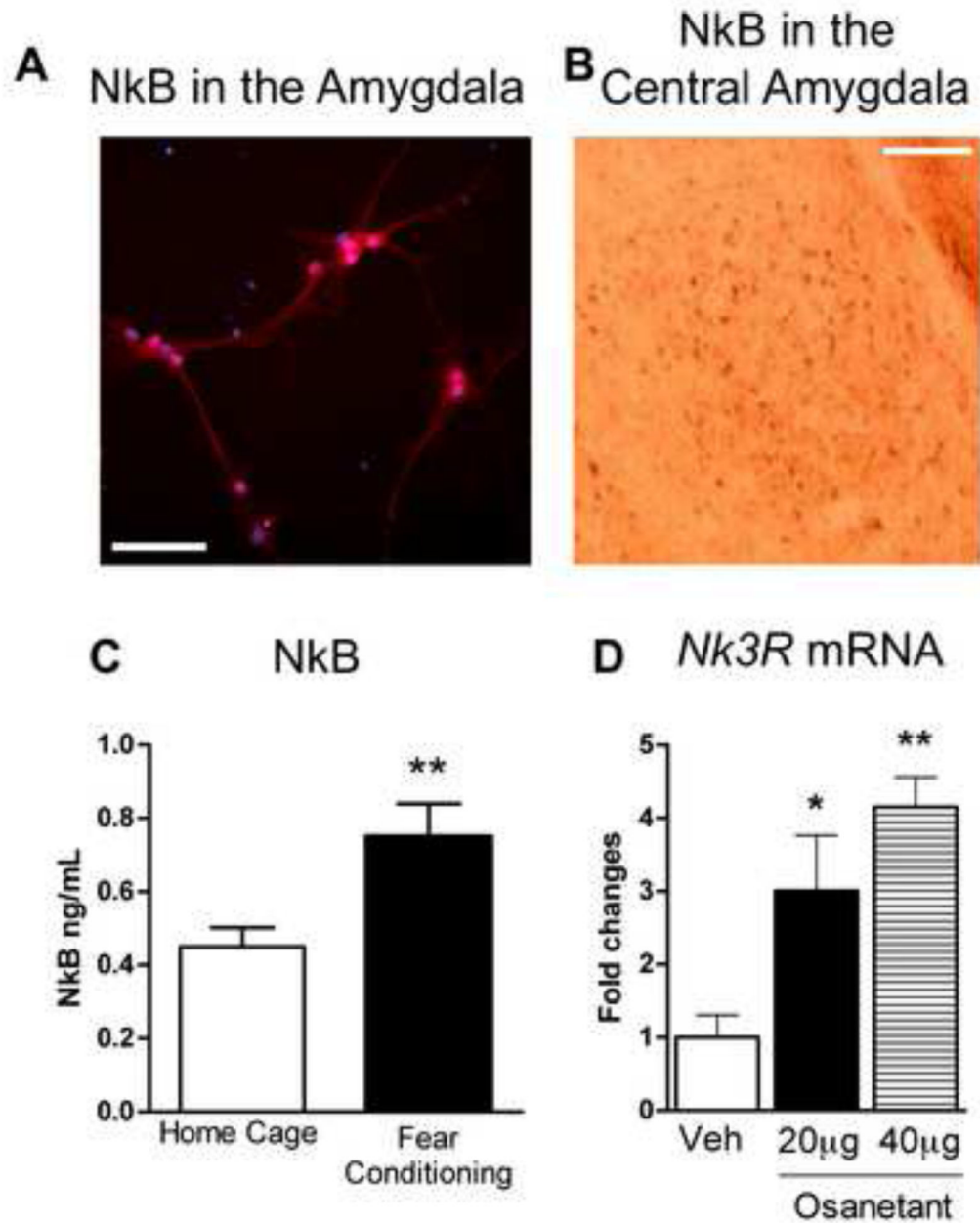
Mean + SEM is shown. D) *Tac2* expression by radioactive *in situ* hybridization in the amygdala is restricted to the central amygdala (CeA) with highest expression in the CeM amygdala. Scale bar = 1 mm. See also Figure S1 and Table S1 and S2.



**Figure 2.** *Tac2* is colocalized with glutamate decarboxylase 65 (*GAD65*) and calmodulin-dependent protein kinase II  $\alpha$  (*CAMKIIα*) but is not colocalized with Protein Kinase C Delta nor Enkephalin-expressing neurons in the CeM

A and B) *Tac2* mRNA expression in the CeA and BLA by non-radioactive fluorescent *in situ* hybridization (FISH). Scale bar = 100  $\mu$ m. C) *PKCδ* mRNA expression by FISH. Scale bar = 100  $\mu$ m. D) *Enk* mRNA expression by FISH. Scale bar = 100  $\mu$ m. E) Right, A and C merged showing different pattern of expression of *Tac2* and *PKCδ* in the CeA. Scale bar = 100  $\mu$ m. Left, confocal image showing no colocalization of *Tac2* and *PKCδ* in the CeM. Scale bar = 15  $\mu$ m. F) Right, B and D merged showing different pattern of expression of

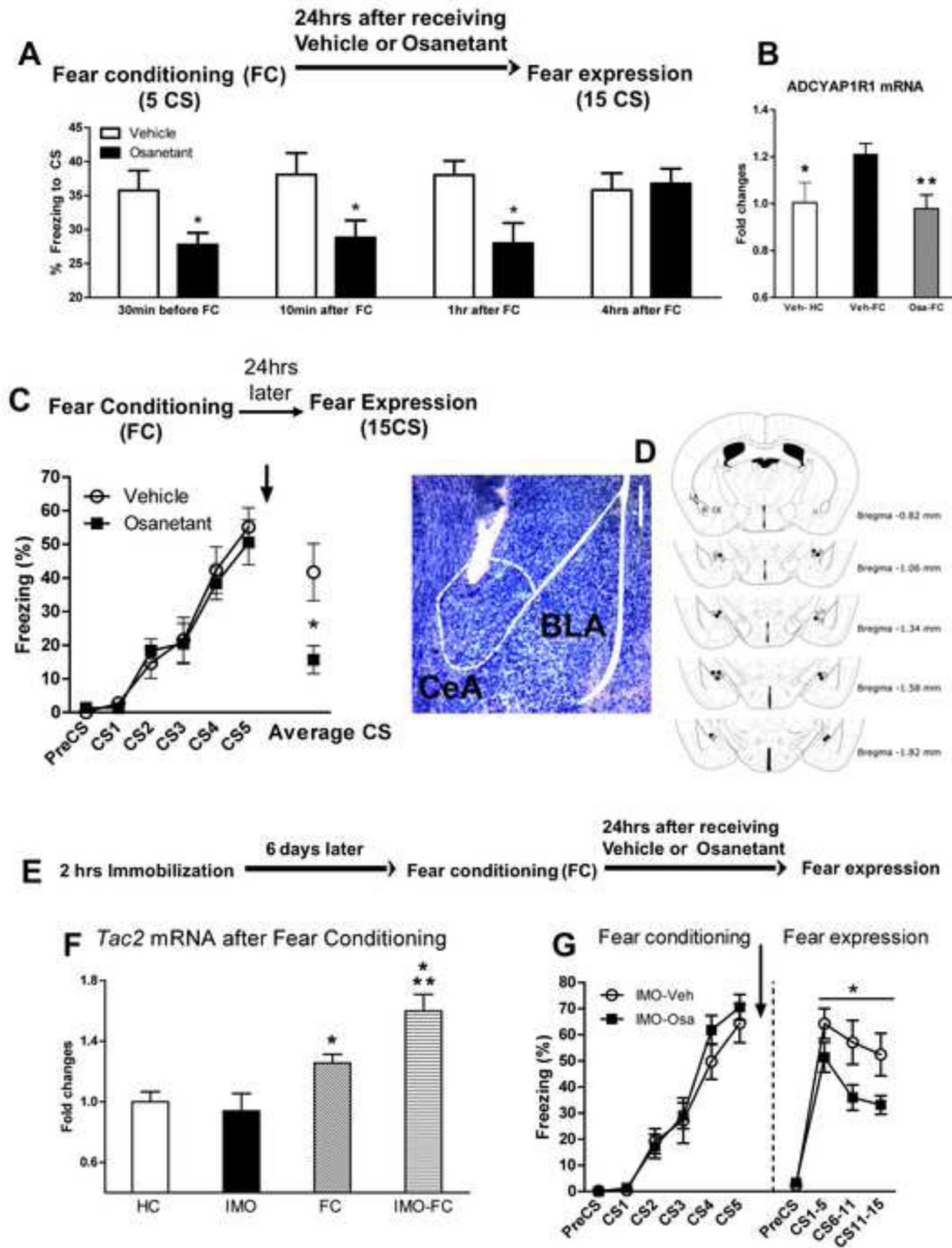
*Tac2* and *Enk*. Scale bar = 100  $\mu\text{m}$ . Left, confocal image showing no colocalization of *Tac2* and *Enk* in the CeM. Scale bar = 15  $\mu\text{m}$ . G) Confocal image showing colocalization of *Tac2* mRNA expression and GAD65 peptide in the CeM. Scale bar = 15  $\mu\text{m}$ . H) Confocal image showing colocalization of *Tac2* mRNA expression and CaMKII $\alpha$  peptide in the CeM. Scale bar = 15  $\mu\text{m}$ . CeM = centro-medial amygdala, CeL = centro-lateral amygdala, CeC = centro-central amygdala, CeA = central amygdala, BLA = basolateral amygdala.



**Figure 3. Fear conditioning, expression of Neurokinin B and Neurokinin 3 receptor in the amygdala**

A) The *Tac2* product, Neurokinin B (NkB) is detected by immunocytochemistry in mouse amygdala cell culture. NkB is highly expressed in the soma and in the dendrites. Red is NkB signal. Blue is neuronal nucleus, NeuN. Scale bar = 25  $\mu$ m. B) Immunohistochemistry studies show high expression of NkB in the central amygdala (CeA). Scale bar = 125  $\mu$ m. C) NkB is up-regulated at 2 hrs in the amygdala after fear conditioning. \*\* $P < 0.01$  vs Home Cage,  $N=6-8$  per group. D) Amygdala cell culture with osanetant, a potent and specific neurokinin 3 (Nk3R) antagonist. Incubation with 20  $\mu$ g and 40  $\mu$ g of osanetant enhances *Nk3R* mRNA levels. This suggests that osanetant activates Nk3R and its downstream

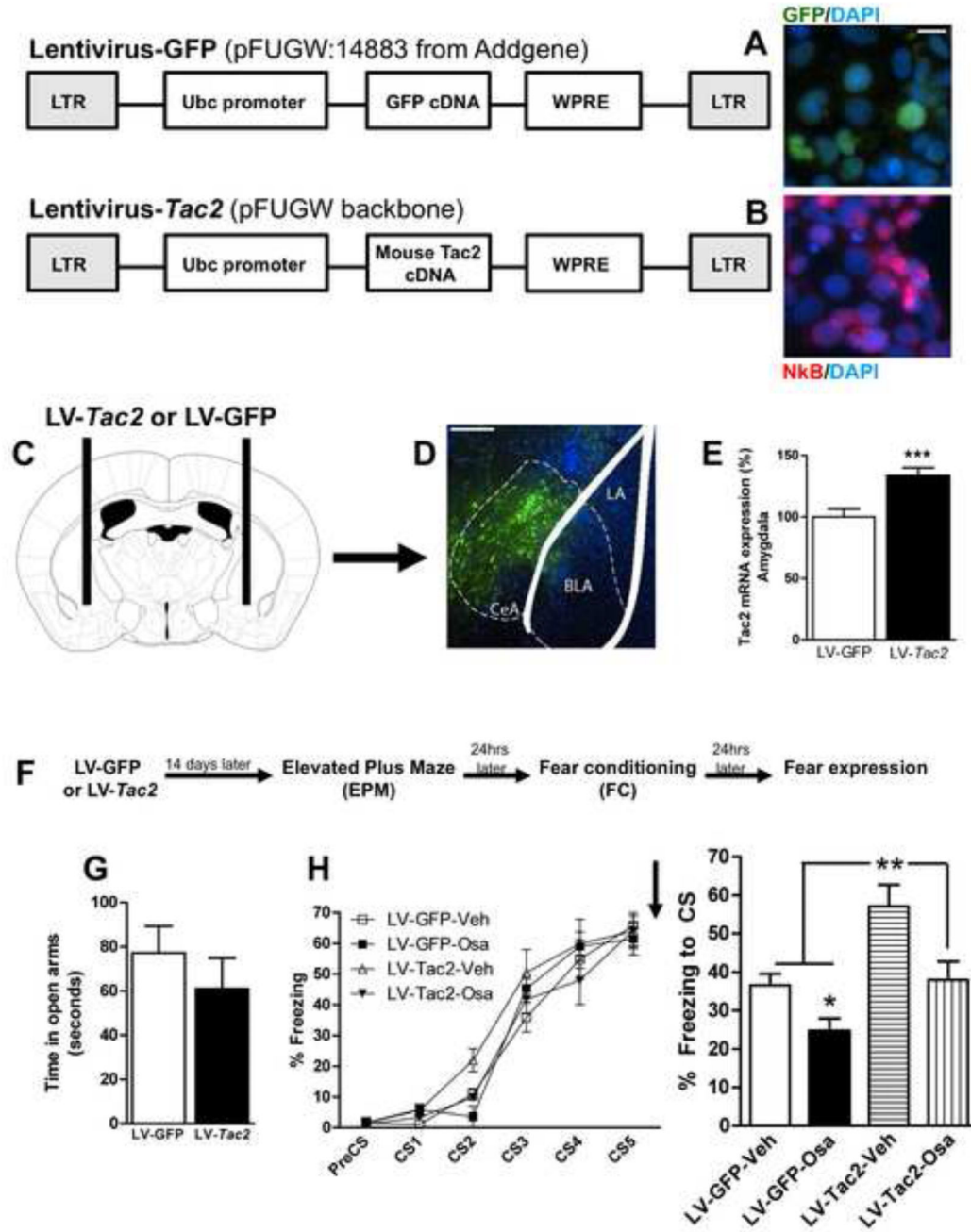
signaling in the amygdala. \*P 0.05 vs Veh, \*\*P 0.01 vs Veh, N=2 per group. Mean + SEM is shown.



**Figure 4. A Nk3R antagonist impairs cued-fear memory consolidation when infused systemically, in the central amygdala and in a PTSD-like mouse model**

A) Osanetant impairs cued-fear memory when given from 30 minutes to up to 1 hour after fear acquisition. The figure shows the time spent in freezing behavior during the fear expression test when the CS is presented. \* $P < .05$  vs Veh.  $N=4-12$  per group. B) Osanetant given intraperitoneally 30 minutes before fear conditioning impaired the enhancement of mRNA levels of the Pac1 receptor (*Adcyap1r1*), \* $P < 0.05$  vs Veh-FC; \*\* $P < 0.01$  vs Veh-FC.  $N=9-12$  per group. The PACAP-PAC1R pathway is associated with PTSD, fear conditioning and stress C) Osanetant bilaterally injected into the central amygdala

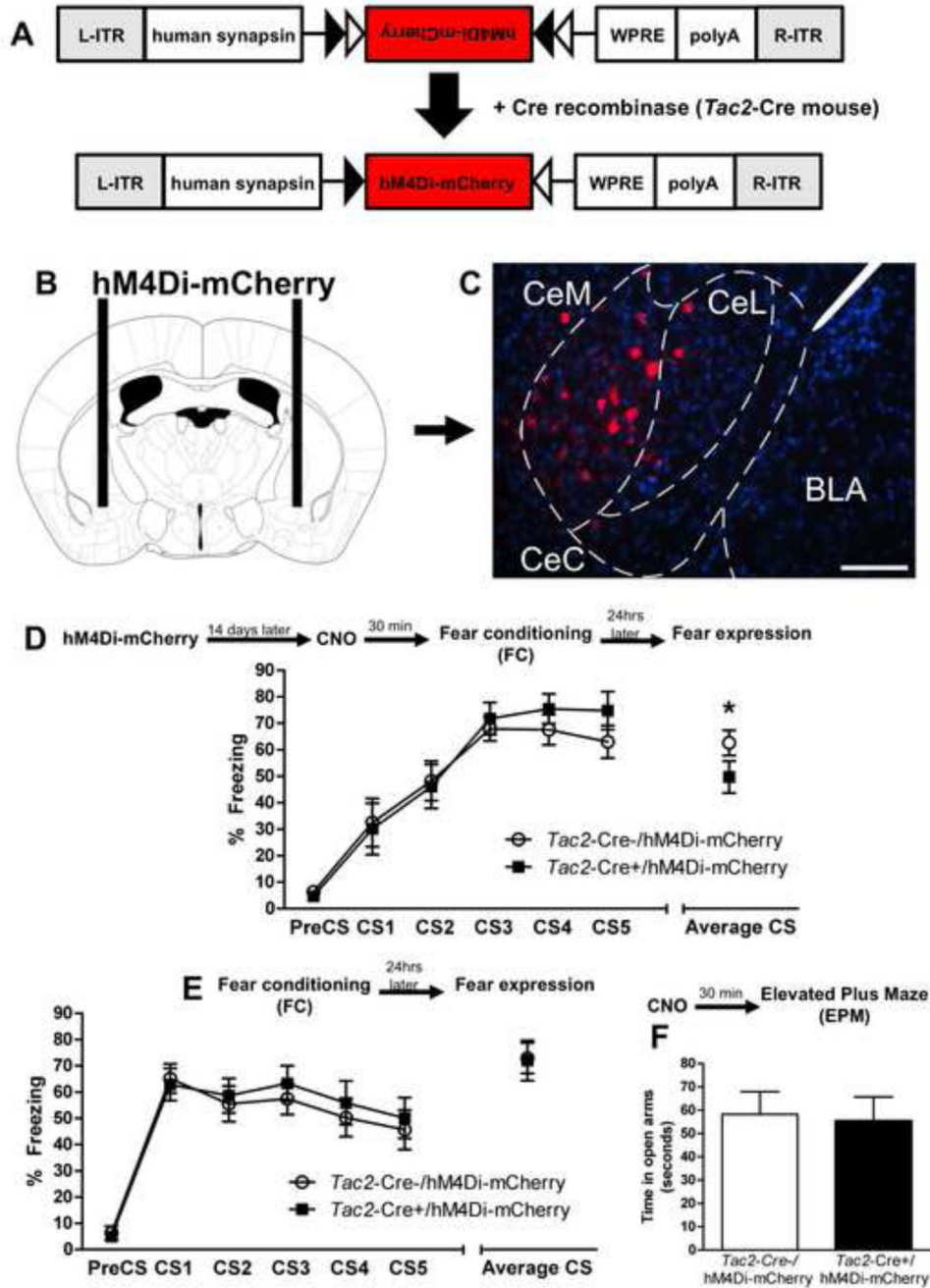
immediately after fear conditioning causes impaired fear memory consolidation as shown by lower freezing in the cued-fear expression test \* $P < 0.05$ ,  $N = 3-9$  mice per group. D) Histological verification of osanetant infusion sites. Left, toluidine blue staining showing an example of the tip of the cannula in the CeA. Scale bar = 250  $\mu\text{m}$ . Right, the dots indicate the lowest point of the injector tip. E) Timeline of the experiment. F) Cued-fear conditioning enhances *Tac2* levels 30 minutes after fear conditioning in naïve mice but more robustly in mice with a previous exposure to immobilization to a wooden board (IMO), a PTSD-like model.  $N = 12-15$  per group. \* $P < 0.05$  vs HC, \*\* $P < 0.01$  vs IMO. G) Osanetant was given immediately after FC and impaired fear memory consolidation in mice which had been previously exposed to a traumatic stress as shown by reduced freezing in the fear expression test, \* $P < 0.05$ .  $N = 8$  per group. Mean + or  $\pm$  SEM is shown. Veh = vehicle; Osa = osanetant.



**Figure 5. *Tac2* overexpression in the central amygdala is sufficient to enhance fear memory consolidation and it is blocked by an Nk3R antagonist**  
 A) The lentivirus GFP-FUGW induces GFP expression but not Neurokinin B (NkB) in Hek293 cells. Scale bar = 10  $\mu$ m. B) The lentivirus *Tac2*-FUGW induces NkB expression in Hek293 cells. DAPI staining (blue) indicates the cellular nuclei. NkB staining (red) is contrasted with GFP fluorescence (green). C) *Tac2*-FUGW or GFP-FUGW were bilaterally infused in the central amygdala and mice were left undisturbed for 14 days. D-E) The lentivirus *Tac2*-FUGW causes a 42% overexpression of *Tac2* in the central amygdala. \*\*\*P 0.001 vs LV-GFP. N= 9-15 per group. Scale bar = 250  $\mu$ m. F) Timeline of the experiment.



G) *Tac2* overexpression in the central amygdala does not alter anxiety-like behavior evaluated by the time spent in the open arms in the elevated plus maze. N= 9-15. H) Left, the lentiviruses GFP-FUGW and *Tac2*-FUGW causes no changes in fear conditioning. Osanetant or vehicle were given systemically immediately after fear acquisition. Right) *Tac2* overexpression enhances fear memory consolidation (LV-*Tac2*-Veh) and osanetant impairs this effect (LV-*Tac2*-Osa). N= 3-8 per group. \*P 0.05 vs LV-GFP-Veh, \*\*P 0.01 vs all other groups. Mean + or ± SEM is shown. See also Figure S5.



**Figure 6. Inducible silencing of *Tac2*-expressing neurons in the CeA with Gi-DREADD decreases conditioned fear**

A) Design of hM4Di-mCherry AAV and *Tac2*-cre mice. B and C) *Tac2*-Cre- or *Tac2*-Cre+ mice were infected with the hM4Di-mCherry AAV in the CeA. The Gi receptor was inserted only on the *Tac2*-Cre cells of *Tac2*-Cre+ mice, CeM = centro-medial amygdala, CeL = centro-lateral amygdala. Scale bar = 125  $\mu$ m. D) CNO was given systemically 30 minutes prior to fear conditioning to *Tac2*-Cre-/hM4Di-mCherry and *Tac2*-Cre+/hM4Di-mCherry. Temporal silencing of the *Tac2*-expressing neurons in the *Tac2*-Cre+/hM4Di-mCherry group did not affect freezing during fear acquisition. However, when mice were tested the

day after for fear expression, without CNO, *Tac2-Cre+/hM4Di-mCherry* mice showed less conditioned fear. \* $P < 0.05$  *Tac2-Cre-/hM4Di-mCherry* vs *Tac2-Cre+/hM4Di-mCherry*, N=10-11 per group. E) *Tac2-Cre-/hM4Di-mCherry* and *Tac2-Cre+/hM4Di-mCherry* mice were retrained to a different acoustic tone (CS) without receiving CNO. Both groups equally acquired fear learning and showed similar levels of fear memory consolidation. F) CNO given 30 minutes before the elevated plus maze showed no effect on *Tac2-Cre-/hM4Di-mCherry* nor *Tac2-Cre+/hM4Di-mCherry* in anxiety-like behavior. See also Figure S6.