Opposite-sex effects of the Tac2 pathway blockade in fear memory consolidation: implications for fear-related disorders

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Abstract

Memory formation is key for brain functioning. Uncovering the memory mechanisms is helping us to better understand neural processes in health and disease. Moreover, more specific treatments for fear-related disorders such as posttraumatic stress disorder and phobias may help to decrease their negative impact on mental health. In this line, the Tachykinin 2 (Tac2) pathway in the central amygdala (CeA) has been shown to be sufficient and necessary for the modulation of fear memory consolidation. CeA-Tac2 antagonism and its pharmacogenetic temporal inhibition impairs fear memory in male mice. Surprisingly, we demonstrate here a totally opposite effect of Tac2 blockade on enhancing fear memory consolidation in females. Furthermore, we show that CeA-testosterone in males, CeA-estradiol in females and Akt/GSK3β/β-Catenin signaling in both mediate the opposite-sex differential Tac2 pathway regulation of fear memory. This study may have implications beyond memory functioning in fear-related disorders, including fields related to behavior where opposite-sex effects have not been studied in-depth or at all.

Introduction

Memory is the process by which knowledge of the world is encoded, stored and later retrieved by neural circuits (1). By this assumption, memory is known to involve two different stages: short-term memory (which lasts min to hours) and long-term memory (which can last days, weeks or even years). A major difference between them is that long-term memory requires a consolidation process in which gene regulation and protein synthesis are necessary. Fear memory is related to aversive events, and allows an organism to identify threatening cues previously associated with a negative experience (2). These cues evoke fear responses that allows the organism to preserve its integrity in the face of danger. In the laboratory, we use Pavlovian fear conditioning to assess fear memory, which consists of pairing a neutral cue as a tone with an aversive event as a footshock, resulting in fear responses that may last for days in when the conditioned cue is presented, There are several data that indicate notable differences in fear memory processing between the female and male brain, in both rodents and humans, at molecular, systemic and behavioral levels of analysis (3, 4). Unfortunately, the study of sex differences is still not fully considered in the scientific community researching the brain - including memory - although women are more likely to present a fear-related disorder (4). In the last years, researchers have published 5.5 studies in males per 1 in females (5) pointing out the evident and growing need to change our approach to neuroscience by including female subjects at all levels of research (6). Consequently, the molecular mechanisms underlying sex differences between male and females on memory processing are still largely unclear.

We previously reported that the Tachykinin 2 (Tac2) pathway is sufficient and necessary for the modulation of fear memories (7). The *Tachykinin 2* (*Tac2*) gene encodes the neuropeptide Neurokinin B (NkB) that binds to the Neurokinin 3 receptor (Nk3R). Nk3R couples to the pertussis toxin-insensitive G protein Gq, whose activation results in the production of inositol triphosphate and diacylglycerol, and the subsequent activation of protein kinase C resulting in memory modulation (7, 8). Tachykinins are abundant peptides in the central nervous system and are involved in neurotransmission and

neuromodulation (9). Because of its restricted brain expression in emotional areas and the available tools, Tac2 manipulation allows a high degree of precision for modulating specific fear memory circuits (7). Moreover, Nk3R antagonists, such as osanetant, are safe and well-tolerated drugs in humans meaning that these findings could rapidly be translated into humans (9). Interestingly, Nk3R agonists or antagonists are not approved for the treatment of human disorders yet. It is known that there are differences across species in the distribution and pharmacology of the Nk3R in humans and rats (10), but both species have a similar expression of Nk3R in important areas for memory regulation (10–12). Furthermore, the binding affinity for NkB, in rats and humans is equivalent (12) suggesting similarities in the pharmacological properties of the Nk3R in both species.

Men and women have previously demonstrated differential life prevalence of fear related disorders (13). Specifically, females present higher life prevalence of fear-related disorders as posttraumatic stress disorder (PTSD) or specific phobias. Notwithstanding, the studies of the Tac2 pathway have been mostly performed only with male subjects. Here, we have discovered opposite-sex effects mediated by the Tac2 pathway in behavioral and molecular mechanisms of long-term memory in mice.

Results

Nk3R antagonism decreases memory consolidation in adult males and increases it in adult female mice.

Systemic administration of the Nk3R antagonist osanetant (5 mg/kg) has previously shown to decrease fear memory consolidation in adult male mice when administered 30 min after fear acquisition (FA) by using a cued-fear conditioning (FC) paradigm (Fig. 1A) (7). After replication of these results in our laboratory (Fig. 1B), we tested whether the administration of osanetant after FA was effective for modulating fear memory consolidation in adult female mice. Surprisingly, 24 hours after the administration of osanetant, female mice presented higher rates of freezing than the control group, thus showing increased memory consolidation in a totally opposite effect than in males (Fig. 1C).

We explored whether an intrinsic variable among females could be modulating the effect of the drug. Based on previous works by Milad (14, 15) showing that the estrous cycle modulated the consolidation of fear extinction memories, we divided the animals of the previous experiment according to their estrous cycle stage on the day of FA and drug administration. We used the vaginal smear cytology method for the estrous cycle determination (16). We found that osanetant is effective for increasing fear memory consolidation when FA and drug administration happen during the proestrus – presenting high concentrations of estradiol and progesterone in comparison to the rest of the cycle –, but lack any effect during the rest of the stages (estrus, metestrus or diestrus) (Fig. 1D-E). In males, we used the Confrontation Tube Test (CTT) to investigate whether social hierarchy could be influencing the effect of the drug on fear memory consolidation as suggested (6). The CTT is a tool that may be used to infer the dominance status of an animal and thus, differential testosterone levels in males housed in groups (Fig. S1A) (6, 17, 18). We found that both dominant and submissive mice presented the same amount of fear

expression 24 hours after the administration of osanetant (Fig. S1B-C), showing no effect of social hierarchy in the effects of osanetant.

After demonstrating that the 5mg/kg dose in proestral females and in males, independent of social hierarchy, was modulating fear memory consolidation, we explored whether other doses had similar effects. For this, we tested both 1mg/kg and 10mg/kg finding that none of them altered fear memory consolidation (Fig. S1D-F). Thus, the drug describes an inverted U shape dose-response curve which is similar to other drugs modulating memory (19).

Due to the suggested role of sex hormones in modulating the effect of osanetant on fear memory consolidation, we wanted to test whether this modulation was dependent on the presence of adult-levels of sex hormones. For that end, we tested 5mg/kg of osanetant on prepubertal postnatal day (P) P28 male and female mice, which have not reached sexual maturity yet (20). Results showed that male and female mice at day P29 had similar freezing rates irrespective of treatment, thereby suggesting that osanetant does not modulate fear memory consolidation in the absence of adult-levels of sex hormones. An alternative explanation may be that the Tac2 pathway circuits are not mature yet although the neuropeptide is expressed in the brain. Further, both females and males, express similar levels of Nk3R in the CeA (Fig 1G).

Osanetant decreases males' testosterone and increases females' estradiol during the consolidation window of fear memories.

Because adult sex hormones appear to be necessary for osanetant to modulate memory consolidation in males and females, we next tested the hormonal dynamics after FA and injection of osanetant or vehicle. In males and females, testosterone was reduced 30 min after the injection of the drug (1 hour after FA) (Fig. 2A-C). When assessed 330 min after receiving osanetant or vehicle (6 hours after FA), males that received vehicle presented a peak (P=0.051) of testosterone above the baseline while the osanetant treated group presented lower levels than the vehicle group (Fig. 2A), thus showing that the injection reduced the testosterone levels during the consolidation window of fear memories. Similar results were obtained with amygdalae micropunches; osanetant decreased amygdalar concentration of testosterone compared to vehicle 330 min after the administration of osanetant (6 hours after FA) (Fig. S2A). Furthermore, linear regression analyses showed that seric testosterone was highly predictive of amygdalae testosterone (Fig. S2A,C) as well as corticosterone (Fig S2B,D). In contrast, neither proestral or metestral females presented an effect of the drug in testosterone when compared to the vehicle groups (Fig. 2B-C). Proestral females presented increased seric estradiol concentration compared to the vehicle and baseline 330 min after receiving the drug (Fig. 2P), showing increased estradiol during the consolidation window of memory, while metestral females presented no effect of the drug (Fig. 2Q).

We also tested whether the drug produced an effect over stress-related hormones corticosterone, dihydrocorticosterone or deoxycorticosterone, as well as in progesterone. None of these hormones were modulated by the administration of osanetant in any of the two chosen time-points (30 or 330 min after the drug injection) (Fig. 2D-O). This lack of modulation was also found in amygdala micropunches for

corticosterone. (Fig. S2B). Indeed, we also found that seric corticosterone was mildly predictive of amygdalae corticosterone (R^2 = 0.344) (SFig. 2D). Males and females presented a similar response of corticosterone to the stress induced by FA in both groups, that decreased over time between the two time points (Fig. 2D-F). Interestingly, progesterone was increased in males and decreased in proestral females independently of the treatment 30 min after receiving the injection, but no changes were observed in females during metestrus (Fig. 2G-I).

Nk3R expression is similar in males and proestral and metestral females, with increased colocalization of Nk3R with GAD65 during proestrus.

Nk3R is highly expressed under basal conditions in limbic and prefrontal cortical areas. Especially in the amygdala, it is highly localized in the central amygdala (CeA) (21). Because we have previously shown that the modulation of Nk3R in the CeA is crucial for the regulation of memory consolidation (7), we next determined whether differences exist in the expression of this receptor in different neuronal types of the CeA among males and females across the estrous cycle. We found a similar number of Nk3R+ neurons immunolocalized in the CeA in males and females in both proestrus and metestrus (Fig. 3A). We explored the colocalization of Nk3R+ neurons with Glutamic Acid Decarboxylase 65 (GAD65), Calmoduline Kinase II a (CaMKIIa) and vesicular Glutamate Transporter 2 (vGLUT2) as markers of inhibitory neurons (GAD65), synaptic plasticity (CAMKIIa) and excitatory neurons (vGLUT2) (Fig 3B-E). Further, colocalization of GAD65 with Nk3R was increased in females during proestrus when compared to metestral females and males (Fig. 3B-C). CaMKIIa immunocolocalization with Nk3R was also increased in females in comparison to males (Fig. 3B and D). Immunocolocalization of vGLUT2 with Nk3R showed no differences among groups (Fig. 3B and E).

Given the evident role of estradiol in the effects of the drug on memory consolidation, an immunolocalization study was performed to assess colocalization of Estrogen Receptors (ERs – ERb and ERa –) with Nk3R in the CeA. We found no significant differences in colocalization of ERs with Nk3R between males and proestral and metestral females neither in ERb (Fig. S3A and C) nor ERa (Fig. S3A-D).

Chemogenetic silencing of CeA-Tac2 neurons after FA decreases memory consolidation in males and increases it in proestral females.

It has been previously shown that local Nk3R antagonism in the CeA decreases fear memory consolidation in male mice (7). Thus, we tested whether the increase of fear memory consolidation shown in proestral females could be replicated by blocking Nk3R with an antagonist specifically in the CeA. Bilateral local injection of osanetant in the CeA of proestral females after FA increased memory consolidation as shown by increased freezing rates during the expression test when compared to vehicle controls (Fig. 4A-B; Fig. S4). This finding demonstrated that a population of Nk3R+ neurons located in the CeA is required for the modulation of memory consolidation in an opposite-sex manner.

Due to the high degree of colocalization between Nk3R and NkB (22) we used Tac2-Cre mice to assess whether chemogenetic silencing of these neurons in the CeA replicated the effects of pharmacological

Nk3R blockade in fear memory consolidation in both sexes. Male and female mice were inoculated in the CeA with the Cre-dependent AAV-DIO-hMD4i-mCherry. All mice underwent the standard procedure above-mentioned (Fig. 4C). Tac2 neurons were silenced with CNO administration (1mg/kg, ip) 30 min after FA (7). The fear expression test revealed reduced freezing in male mice that expressed the reporter mCherry in the CeA when compared to wild-type injected controls (Fig. 4D). In contrast, memory consolidation was significantly increased in female mice that received FA and CNO in proestrus (Fig. 4E), but not in metestrus (Fig. 4F), all compared to wild-type controls also injected with the virus but not expressing the reporter.

The expression of the reporter mCherry was also used to assess the number of Tac2+ neurons in the CeA. Supporting our previous Nk3R+ immunolocalization results, no difference was found in the number of Tac2+ neurons between males and females in the CeA. These data indicated that the opposite-sex effect on memory consolidation was not due to a different number of CeA-Tac2+ neurons (Fig. 4G-H).

Osanetant regulates oppositely the Akt/GSK3β/β-Catenin pathway in the amygdala during memory consolidation.

Nk3R is a G Protein-Coupled Receptor (GPCR) upstream of different signaling pathways regulating the synthesis of inositol triphosphate (8). To determine the signaling pathways affected by osanetant, we extracted both amygdalae 30 min after receiving osanetant in males and females during proestrus and metestrus (Fig. 5A). A qPCR mRNA array of 84 GPCR-related genes was performed, revealing downregulation of *Agt*, *Agtrap*, *Bcl2*, *Calcrl*, *Ccnd1*, *Ccne1*, *Cdkn1a*, *Cdkn1b*, *Cflar*, *Elk4*, *Galr2*, *Gnas*, *Bcl2ll* in males that received osanetant compared to vehicle controls; while *Akt1*, *Gcgr*, *Fgf2*, *Lhcgr*, *Mmp9*, *Ptgdr*, *Rho*, *S1pr3*, *Vcam1* gene expression was upregulated in females that received osanetant only during proestrus (Fig. S5A). In females during metestrus, only *Adrb2* and *Lpar1* were downregulated and *Kcnh8* upregulated after the treatment (Table S1). Ingenuity Pathway Analysis (IPA) of these genes revealed the Akt/GSKb/bCatenin signaling pathway as the main molecular route affected by the drug (Fig 5B, Fig. S5B).

Since PI3K/Akt signaling targets different pathways, we next analyzed the activation and protein levels of its three main downstream pathways – CREB, mTOR and GSK3b – following a similar approach. 10 min after the injection of the drug, amygdalae were extracted and levels of total and phosphorylated levels of CREB, mTOR, GSK3b and Akt were analyzed by Western blotting. Our results showed no effect of the drug in the levels of total and phosphorylated CREB (Ser 133) or mTOR (Ser 2448) in males and proestrus females. Notwithstanding, total GSK3b protein levels were significantly decreased in males (Fig. 5C, Fig. S5C-D) while total Akt, but not its phosphorylation (Ser 473 and Thr 308), and the ratio GSK3b(Ser 9)/GSK3b were increased in proestral females, with a statistical tendency towards stabilization of the downstream protein b-Catenin (Fig. 5D, Fig. S5E-F). These results are in agreement with the observed increase of Akt1 transcript levels in the gene expression analyses and indicate that osanetant-mediated Nk3R blockage results in upregulation of Akt leading to inactivation of GSK3b and upregulation of b-Catenin in proestrus females.

Blockade of osanetant effect in memory consolidation by androgen receptor (AR) agonism or Akt activation in males and ERs antagonist or Akt inactivation in females.

Altogether, osanetant showed to reduce testosterone in males and to increase estradiol in proestral females during memory consolidation. Further, we also found that osanetant decreased the Akt/GSK3β/β-Catenin pathway in males while it increased it in proestral females. Therefore, we tested whether pharmacological manipulation of AR in males or ERs in females, or Akt in both, could block the effects of osanetant in memory consolidation (Fig. 6; Fig. S6).

As previously hypothesized, males that received systemic osanetant together with intra-CeA AR agonist or an Akt activator presented increased memory consolidation in comparison to those that only received osanetant, as shown by higher freezing rates in the expression test 24 hours later. In contrast, proestral females that received intra-CeA ERs antagonist or an Akt inhibitor together with systemic osanetant presented lower memory consolidation when compared to those proestral females that only received osanetant, as expressed by lower freezing rates in the expression test 24 hours after receiving the treatment. Taking together all the results, the effects of osanetant in memory consolidation in both males and females require the modulation of the Akt/GSK3b/b-Catenin interacting with sex hormones.

Discussion

Taken together, our data showed that the temporal inactivation of the Tac2-CeA pathway results in impaired fear memory consolidation in males but increases fear memory in females. This memory effect is dependent on sex hormones and its specific receptors in the CeA; testosterone in males and estradiol in females. Additionally, Tac2 pathway inactivation is sufficient for amygdala Akt/GSK3b signaling downregulation in males and upregulation in proestral females, being these molecular changes necessary for the memory modulation effect in both sexes. See Figure 7 for a graphical summary of the results.

There are numerous previous studies that have found sex differences in long-term memory formation and its mechanisms (4). For example, cyclin-dependent kinase 5 (CDK5) mRNA and histone H3lysine 9/14 acetylation in the CA1 region of the hippocampus are upregulated in male mice but not in females (with the estrous cycle unmonitored) using a contextual fear conditioning paradigm (23). Other studies have focused on long-term potentiation (LTP) which is an electrophysiological correlate of synaptic efficacy required for long-term memory consolidation (24). Male and female rats show equivalent LTP, although the proven mechanisms in females involve cAMP-activated protein kinase (PKA) whereas they are not necessary in males (25). Also, it has been shown a sex hormone-dependent modulation of memory consolidation. Intraperitoneal injection of a dopamine receptor 1 agonist in female rats during low estradiol phases enhances fear memory recall whereas females trained during high estradiol estrous phases present impaired FE recall (26). However, our data provide the first evidence that there are opposite-sex effects in behavioral and molecular mechanisms in memory formation.

Dominance/submission, estrous cycle, hormones and fear memory.

Typically, the manipulations in rodent fear memory studies only involve one sex and generally do not account for traits or hormonal status which are relevant for brain functioning (6). We have tested for the first time the effect of a drug in fear memory depending on both dominance/submission in males and estrous cycle in females. Results suggest that dominance status as assessed by CTT in males is not relevant for the impairment of memory consolidation induced by osanetant. However, an alternative explanation is that the stress associated with the evaluation of dominance/submission in rodents interferes with subsequent memory formation processes. In females, it is only when osanetant is given during the proestrus stage that fear memory consolidation is enhanced. Of note, it has been already reviewed that female rodents in proestrus present enhanced fear extinction retention (4). Furthermore, in regard to healthy women, it has been previously demonstrated that a higher concentration of circulating estradiol enhances extinction recall, without an effect on FA or fear extinction itself (27). However, we show that osanetant given after FA at different stages of the estrous cycle can result in different effects such as enhancement of memory or no effect. Of note, osanetant had no effect on fear generalization to the context.

Sex hormonal but not stress hormonal response to FA is altered by systemic osanetant.

Our hormonal profile after FA showed a novel testosterone modulation with an initial decrease after fear acquisition and an increase hours later in those animals treated with vehicle. Of note, there is no agreement in the literature regarding testosterone regulation after exposure to mild stressors such as the footshocks that mice received in our experiments. Some reports show increased seric concentration of testosterone after noise stress (85 dB) (Armario, Campmany, & López-Calderón, 1987) whereas others show a decrease after 4 days of chronic stress by paradoxical sleep deprivation, footshocks or cold (29). It is possible that the nature of the stressor, its intensity and duration may be crucial factors in testosterone modulation after stress exposure. It is noteworthy in our data that osanetant prevented the peak of testosterone 360 min after FA. This is not surprising since it has been previously shown that Nk3R antagonism in humans reduces circulating sex hormones (30). In females, FA did not change the estradiol levels in the vehicle group, concordantly with previous studies that show no changes on estradiol after acute stress even 6 hours after the offset of the stressor (31). Also, osanetant produced an increase in seric estradiol in proestral females 6 hours after FA (330 min after the administration) but not in metestral females. Of note, progesterone was increased in males and decreased in proestral females 1 hour after FA, and remained unaltered in metestral females, with no differences between vehicle and osanetant treated groups in any of the cases mentioned. Thus, we can discard a role of progesterone in the Tac2-CeA pathway for fear memory consolidation.

Our studies with cannulated animals demonstrate that a single intra-CeA dose of an AR agonist, blocks the effect of osanetant to reduce fear memory consolidation in male mice. Furthermore, the infusion into the CeA of a dual ERs antagonist together with systemic osanetant inhibits the increase in memory consolidation in proestral female mice. Altogether, these experiments conclude the necessary role of the inactivation in males and activation in females of AR or ERs, respectively, for osanetant to modulate memory consolidation in males and females.

In regard to stress hormones, it has been previously shown that Tac2 levels are increased after chronic stress exposure (32). However, we rule out a role of the stress response in our FA experiments by showing no differences in corticosterone and related hormones after osanetant injection during the consolidation window of fear memories. Because adrenocorticotropin releasing hormone (ACTH) and corticotropin-releasing factor (CRF) levels have not been taken into account, we cannot discard their effects modulating the response to osanetant.

GAD65 and CaMKIIa expression in CeA-Nk3+ neurons.

Here, we demonstrate that there are no differences in the number of Nk3+ neurons between males and females neither in proestrus nor metestrus. Thus, we discard the possibility that the number of CeA-Nk3R+ neurons are important for the difference between males and females in fear memory consolidation. Notwithstanding, there are important physiological differences in these neurons in terms of colocalization with enzymes involved in synaptic plasticity such as GAD65 and CaMKIIa.

GAD65 is one of the main enzymes that synthesizes g-Aminobutyric Acid (GABA) in the brain, together with GAD67. There have been previously reported sex differences in GAD65 synthesis, with increased synthesis in female rats dorsomedial hypothalamus and medial amygdala, while GAD67 appeared to be higher in males (33) . GABAergic transmission is abundant in the CeA, and key in GABA-dependent synaptic plasticity (34–36). Previous studies have highlighted that estradiol increases GAD65 mRNA while decreasing GAD67 mRNA as measured by in situ hybridization in rat brain areas like the magnocellular preoptic area or the dorsomedial hypothalamus (37), but not in the amygdala. Further, it has been proven that estradiol is necessary for GAD enzymes to produce GABA in females (38). Interestingly, we are not aware of reports studying sex differences in GABAergic transmission in the CeA nor its role in fear memory. Herein, we found that GAD65 synthesis is enhanced in Nk3R+ neurons in females exclusively during the proestrus stage of the estrous cycle but not during metestrus, that present similar colocalization than males. These data suggest increased tendency to GABAergic dependent synaptic plasticity in proestral females.

CaMKIIa is postsynaptic effector critical for memory because of its involvement in long-term potentiation through phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors (39, 40). In the CeA, CaMKIIa is expressed in cell bodies and neuropil (41). To the best of our knowledge, no studies have addressed differences in CaMKIIa expression between males and females. Here, proestral and metestral females have shown increased colocalization of CaMKIIa with Nk3R in the CeA in comparison to males. This result may indicate a tendency to increase synaptic plasticity related to fear memories in proestral females than in males.

The CeA Akt/GSK3β/β-Catenin pathway is oppositely regulated between sexes in memory consolidation.

The Akt/GSK3β/β-Catenin pathway is widely known for its role in amygdalar- and hippocampal-dependent learning and memory (42, 43) and cognitive flexibility (44). Of note, hippocampal memory has shown to require estradiol for PI3K activation (45). PI3K/Akt is upstream of important memory targets

like mTOR, CREB and GSK3b (46). The PI3K/Akt/GSK3b pathway has been evidenced for its role in cued-fear memory consolidation through stabilization of b-Catenin (47). Further, there is increasing evidence of inhibitory interactions between estradiol and GSK3b towards the b-Catenin stabilization through ERa promoting memory in the hippocampus (48).

In our study, we show an opposite-sex regulation of the Akt/GSK3β/β-Catenin pathway in males and treated with osanetant when high seric estradiol is present during the consolidation window of fear memory. Furthermore, we discarded CREB and mTOR as the main effectors of osanetant-induced effect in memory consolidation, showing a decrease in total GSK3b in males while, in females, total Akt and the phosphorylated GSK3b(Ser9) are increased in response to the drug after FA. Of note, and because b-Catenin is a downstream target of GSK3b, a tendency towards increased levels of phosphorylated (Ser 33/37/Thr 41) b-Catenin appeared. This results agrees with previous findings indicating a role of b-Catenin in cued-fear memory consolidation (47), suggesting that activation of Akt/GSK3β/β-Catenin mediates long-term stabilization of the recently acquired memory.

In addition, we show in males that the pharmacological activation of Akt in the CeA, together with systemic osanetant, blocks the effects of the Nk3R antagonist to decrease fear memory consolidation. In females during proestrus, the local administration in the CeA of an anti-Akt blocks the effects of osanetant of increasing memory consolidation. Altogether, these results evidence the opposing role between sexes of the Akt/GSK3β/β-Catenin in the regulation of amygdala-dependent memory consolidation.

Conclusion

These data thereby provide the first evidence that there are opposite-sex effects in behavioral and molecular mechanisms in memory formation. Thus, our discovery proposes a novel and diverse perspective on why and how memory processes differ between females and males. This information is highly relevant for designing new treatments in fear-related disorders. Furthermore, most drugs in neuropharmacological studies have not been tested in all the different stages of the estrous/menstrual cycle. Thus, this study suggests that some drugs may present different effects depending on the specific stage of estrous/menstrual cycle. Thus, it is possible that some drugs studied to treat fear-related disorders may not present the expected outcome regarding women, which present higher life prevalence of fear-related disorders. Therefore, the estrous/menstrual cycle should be routinely monitored when possible to fully understand the memory mechanisms of drugs that could potentially treat psychiatric disorders that present memory alterations. Moreover, our findings may increase awareness on sex-differences and promote more basic and clinical studies including females.

Materials And Methods

Ethics and biosecurity protocols.

Ethics protocols approved for the experiments in mice ref. CEEAH 3603 and biosecurity protocols 345-16 and 407-17. All procedures were approved by the Committee of Ethics of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya. They were also carried out in accordance with the European Communities Council Directive (2010-63-UE) and Spanish legislation (RD 53/2013).

Mice.

All experiments used male and naturally cycling female adult mice (2-6 months of age) housed in groups of 4 (except when mentioned) in a room with a 12:12h light/dark cycle (lights on from 8am to 8pm). All animals were housed with controlled temperature 22 ± 1 ºC and humidity (~ 40%). Behavioral procedures and pharmacological manipulations begun early in the light phase of the cycle. Tac2-Cre (stock# 018938 Jackson Labs) were bred within our animal facility. Wild-type C57BL/6J were purchased from Charles River (Barcelona, Spain). Male and female mice were housed separately in the same room.

Cued-Fear Conditioning.

For Fear Acquisition (FA) and Fear Expression (FE) test, a computerized *Startle* system was employed (Panlab-Harvard, Barcelona, Spain) as previously described (49). Delivery of tones and shocks was simultaneously controlled by *Freezing v1.3.04* software (Panlab-Harvard, Barcelona, Spain). The fear chamber consisted of a black methacrylate box with a transparent front door (25x25x25cm) inside a sound-attenuating chamber (67x53x55cm). The same boxes were used for FA and FE.

Animals were habituated to the chambers for 5 min/day during two consecutive days prior to FA. For cue-dependent fear conditioning, all animals remained 5 min in the fear chamber before the onset of the first tone. During FA, all groups received 5 trials consisting in a tone as the Conditioned Stimulus (CS) (30 s, 6 kHz, 75 dB) that coterminated with a footshock which served as the Unconditioned Stimulus (US) (1 s, 0.6 mA). The intertrial interval (ITI) was 3 min, and 3 additional min followed the last trial. All animals received the treatments 30 min after FA in order to manipulate the consolidation of memory and therefore avoid any effect during acquisition. The FE test was performed 24 hours after FA. Mice remained 5 min in the chamber before trials, and afterwards were exposed to 15 trials of the 30 s CS tone alone (cued-fear) with a 0.5 min of ITI interval. An additional 0.5 min interval followed the last trial of FE. Freezing behavior was used as an index of fear. The *StartFear* system allows recording and analysis of the signal generated by the animal movement through a high sensitivity weight transducer system.

To assure that freezing behavior was exclusive of the previous tone-shock conditioning, different contexts were utilized for FA and FE. FA context consisted of a yellow light source (~10 lux), a grid floor of 25 bars (3 mm Ø and 10 mm between bars) that dispensed the footshocks, a background noise of 60 dB produced by a ventilation fan and a solution of ethanol (EtOH) 70% (v/v) odor was used for cleaning between sessions. FE context consisted of a red light source (~10 lux), a grey floor covering the bars, no background noise and CR36 – bronopol 0.26 % (v/v), benzalkonium chloride 0.08% (v/v) and isopropyl alcohol 41% (v/v) – (José Collado, Barcelona, Spain) for cleaning, with changes in the length and turns of the transportation route from the vivarium to the testing room between FA and FE.

Confrontation Tube Test (CTT).

Male mice were pair-housed for 5 weeks before testing in order to establish stable social hierarchies between them. The confrontation tube consisted of a methacrylate tube (30 cm length, 3.6 cm inner Ø) with two lids at 13 cm from each end. All animals were habituated to the tube for 2 consecutive days before the test. The habituation consisted of 3 crossings from one end to the other of the tube and the tube was always cleaned with EtOH 70% (v/v) between animals. Mice were tested for 6 consecutive days, with 8 trials per day with ~20 min between trials. For each trial, each mouse from each couple entered the tube simultaneously by the extremes. When both animals reached the lids, these were lifted for animals to face each other and that's when the test started. The first animal that put its four limbs outside the tube was considered the looser while the other one won the trial. When both animals remained 2 min inside the tube without confronting each other, the trial was considered null. The animal that presented more wins throughout each session was considered the winner of the session. At the end of the 6 sessions, the animal that presented more won sessions was considered dominant over its cage mate. The day after the 6th session of the tube test, animals underwent the FC protocol as abovementioned. All mice received osanetant 30 min after FA and were tested for memory recall 24 hours later to assess for differences in the effect of the drug between dominant and submissive mice.

50 uL of blood was collected from each animal by tail-nick two days before the first habituation to the tube, 2 hours before the first habituation session, 2 hours before the first test and 2 hours before the first habituation to the fear conditioning chamber, thus, with every two days since the first extraction. Blood was centrifuged (8000 g, 15 min, 4ºC) and serum was stored at -20ºC. Blood was not analyzed since we did not find behavioral differences between groups.

Vaginal smear cytologies.

In order to assess the phase of the estrous cycle that female mice presented during FA, all female mice were monitored for 3 to 4 consecutive cycles (approximately 10-14 days) before learning to test for regularity of the cycle. For that end, a 20 mL pipette was loaded with 10 mL of standard NaCl 0.9% (w/v) solution and later the tip was softly placed on the vaginal aperture. In case of urination when grabbing the animal, urine was cleaned using a regular tissue. The 10 mL of saline were unloaded and collected for 5 consecutive times in order to collect an enough amount cells for the assessment, and later placed on an adhesion slide (Superfrost Plus, Thermo Fisher, Barcelona, Spain). Slides were dried using a hot plate (HI1220, Leica, Madrid, Spain) at 37ºC for 30 min and later stained in Cresyl Violet Acetate (C5042, Sigma-Aldrich, Spain) 0.1% (v/v), washed twice for 1 min in distilled water and read in brightfield microscopy with a 10x or 20x objective in an Eclipse 80i microscope (Zeiss, Spain). Three different cell types may appear in the preparation: cornified epithelial cells, round nucleated epithelial cells or leukocytes. The different stages of the estrous cycle were assessed depending on the proportion of the abovementioned cells. Proestrus is characterized by a high proportion (>80%) of nucleated epithelial cells, that might present very small amounts of cornified epithelial cells or leukocytes. Estrus is typically presented with cornified epithelial cells with a lower grade of staining than leukocytes and nucleated

epithelial cells. Metestrus presents a mixture of cornified epithelial cells and a considerable proportion of leukocytes. Diestrus is characterized by >90% of leukocytes that might present a very small proportion of round nucleated epithelial cells (see Fig S1E). After assessment of regular cycling, females were distributed in groups according to the stage of the estrous cycle they presented before FA, which is also the day they receive the different treatments after FA.

Drugs.

Intraperitoneal osanetant (Sigma-Aldrich, Spain) dose was 5 mg/kg (7), 1 mg/kg or 10 mg/kg, and intra-cerebral dose was 30 nmol per side as in our previous paper (7) using 0.1% Tween 20 in saline as vehicle. The dose of intra-Central Amygdala (CeA) Androgen Receptor (AR) agonist CI-4AS-1 (Tocris, UK) was 100 nM based on previous research showing decreased depolarization-induced suppression of excitation in POMC neurons (50), dissolved in 1% DMSO (v/v). We used the AR agonist instead of testosterone in order to avoid its possible conversion to 17-b-Estradiol by aromatase. The Akt activator SC 79 (Tocris, UK) was used at a dose of 0.1 μM in 1% DMSO (v/v) because it has shown to have a protective effect on dopaminergic neurons against oxidative stress (51). Anti-Akt1/2 (Tocris, UK) was used at 5 μM in 1% DMSO (v/v) due to its ability to block Akt phosphorylation (52). Estrogen Receptors (ERs) antagonist ICI 182,780 (Tocris, UK) was used at a 10 μM in 1% DMSO (v/v) as it has shown previously to be effective decreasing EtOH excitation of DA neurons in the VTA (53). CNO in 0.5% DMSO (v/v) systemically was dosed at 1mg/kg (7).

Surgery.

All surgeries were performed using isoflurane 5% (v/v) for induction, and 2-3% (v/v) for maintenance, in oxygen, at a constant rate of 1.5 L/min. After skin shave and skin disinfection with EtOH 95% (v/v) and iodine povidone 10% (v/v), ovariectomies were performed making a bilateral incision on the back of the animal, 1 cm lateral to the midline and right over the backlimbs line. Adipose tissue was extracted, and the ovary was localized and isolated making a knot with sterile absorbable suture thread (1019723, Centauro, Barcelona, Spain) around the oviduct. The ovary was extirpated and the adipose tissue, containing the rest of the oviduct, was returned to the abdominal cavity. Muscle was sewed with sterile absorbable thread and skin was sewed using sterile silk suture (1019717, Centauro, Barcelona, Spain). Mice remained resting for 6 weeks until trunk blood was collected to avoid any effect of previous estradiol.

For stereotaxic surgeries, after induction of sleep, mice were placed in the stereotaxic frame (Kopf Model 962, Harvard-Panlab, Barcelona, Spain). After alignment of the Antero-Posterior (AP) and Latero-Medial (LM) axis with the frame, injections of AAV8-hSyn-DIO-hM4D(Gi)-mCherry to the CeA the following coordinates were employed: AP -1.3, ML ± 2.5, Dorso-Ventral (DV) –4.4 mm from Bregma, as previously described (7). For cannulation, the same AP and ML coordinates were utilized, although cannulae were implanted at DV -3.4 mm due to the need to remain the CeA intact and because of the extra mm projection of the internal cannulae. Cannulae were secured to the skull with anchor screws for mice

(Plastics One, Germany) and dental cement (Fortex Fájula, Cibertec, Spain) For further details on the virus and microinfusions, please see *Adeno associate virus (AAV) infection and microinfusions* section below.

Adeno-associate virus (AAV) infection and microinfusions.

We used AAV obtained from the pAAV-hSyn-DIO-hM4D(Gi)-mCherry (hM4di-mCherry DREADDs). The plasmid was obtained from Addgene and the AAV from the viral vector production unit at Universitat Autònoma de Barcelona. Serotype 8 of the AAV was bilaterally injected (0.5 mL/side) using a microinjection pump (1 mL per 15 min rate) into the CeA (coordinates abovementioned). Cannulae were placed using coordinates mentioned in the surgery section. For microinfusions of drugs in awake animals, internal cannulae projected 1 mm from the tip of the internal guide, reaching DV coordinate -4.4 mm from Bregma for injections, corresponding to the CeA. Bilateral injections were performed simultaneously and manually using two knurled hub, type 3 tip, 1 mL Hamilton syringe (external Ø 0.52 mm and internal Ø 0.26 mm) (Cibertec-Harvard, Madrid, Spain) coupled on one end to a polyethylene (PE) 50 tube (~ 20 cm) (Plastics One, Germany) with the internal cannula coupled to the other end of the tube. The PE-50 tube was filled with distilled water, leaving a 7.5 mm gap of air between the internal cannula and the water to avoid dilution of the drugs with water. Microinfusions were performed at a rate of 0.5 mL over 2 min and internal cannulae remained one extra min in place to prevent the backflow of the drug.

Steroids determination.

Trunk blood was collected after sacrificing the animals. Blood was centrifuged (8000 g, 15 min, 4 ºC). Serum was collected using a 200 µL pipette, transferred into a 2.5 mL Eppendorf tube and stored at -80 ºC for later analysis. Brains from animals euthanized 330 min after treatment (6 hours after FA) were snap frozen using Isopentane cooled with dry ice, and later stored at -80ºC until sectioning. Fresh frozen brains were then coronally sectioned until reaching a coronal plane 0.58 mm behind Bregma. 1 mm Ø micropunches from both Amygdalae were extracted reaching coronal plane 1.94 mm behind Bregma, transferred into a 2.5 mL Eppendorf tube and stored until processing. 400 mL of Formic Acid (F0507, Sigma-Aldrich, Spain) 0.1% (v/v) were added to each tube and sonicated for consecutive five cycles. The sonication probe was then rinsed with 800 mL of Acetonitrile (271004, Sigma-Aldrich, Spain) that were added to the tubes, and these were vortexed for 2 s. Micropunches extracts were stored at -80ºC until analysis. Both plasmatic and amygdala levels of testosterone, progesterone, dehydrocorticosterone and deoxycorticosterone were determined based on previously reported papers (54, 55). Seric and amygdalar testosterone, progesterone, dehydrocorticosterone and deoxycorticosterone were evaluated were evaluated based on previously reported papers (54, 55). Briefly, 20 µL of plasma were mixed with 20 µL of labelled internal standard solution. After proteins precipitation with 100 µL of acetonitrile, samples were centrifuged (3000 g, 5 min) and the supernatant transferred to a clean tube. In the case of micropunches 20 µL of labelled internal standard solution was added to the 1 mL of extract from micropunches. The mixture was vortexed and transferred to a clean tube. Both plasma and micropunches underwent a liquid-liquid extraction by addition of 1 mL of NaCl (saturated solution) and 4 mL of ethyl acetate. Extracts were centrifuged (3000 g, 5 min) and the organic layer was transferred into a clean tube and dried under a

nitrogen stream. Dried extracts were reconstituted with 100 µL of methanol and 10 µL were injected into the LC-MS/MS system consisting on an Acquity UPLC system coupled to a triple quadrupole (TQS Micro) mass spectrometer. Steroids detection was performed by selected reaction monitoring (SRM) including 2 transitions for each analyte. The most specific one was selected for the quantification. Quantification was performed by external calibration approach using the *TargetLynx* module of the *MassLynx* software (Thermo Fisher, Barcelona, Spain). Estradiol was measured with the ELISA kit ES180S-100 (Calbiotech, California, USA). For standard curve preparation, serum from ovariectomized mice was used adding known concentrations of estradiol (E885-250MG, Sigma-Aldrich, Spain) -0, 3, 10, 30, 100 and 300 rg/mL- utilizing denaturalized EtOH (Casa Álvarez, Barcelona, Spain) as a vector for estradiol. Kit instructions were followed as stated, samples were loaded in duplicates and absorbance was read at 450 nm with the microplate reader *Varioskan Lux* (Thermo Fisher, Barcelona Spain) controlled with *Skanit for microplates v6.0* software (Thermo Fisher, Barcelona, Spain). Average of duplicates was used as estradiol determinations for each sample.

Immunohistochemistry.

For immunohistochemistry assays, mice were transcardially perfused with 50 mL of 4% (v/v) paraformaldehyde (PFA) (Casa Álvarez, Barcelona, Spain) for 5 to 6 min, then decapitated and brains were extracted and stored in 4% (v/v) PFA for 24 hours. After this time, brains were rinsed (3 times, 10 min each) with 1x Sorenson's PB consisting of 10.9 g/L Sodium phosphate dibasic (0876, Sigma-Aldrich, Spain), 3.2 g/L Sodium phosphate monobasic (04270, Sigma-Aldrich, Spain) and transferred into 30% Sucrose (84097, Sigma-Aldrich, Spain) in 1x Sorenson's PB in conic Falcon tubes (Thermo Fisher, Barcelona, Spain) until the brain reached the bottom of the tube (~ 48 to 72 hours). Right after, brains were snap frozen in a metal cube containing Isopentane (M32631, Sigma-Aldrich, Spain) cooled with dry ice and stored at -80ºC until sectioning.

For Neurokinin 3 Receptor (Nk3R), Glutamic Acid Decarboxylase 65 (GAD65), Calmoduline Kinase II a (CaMKIIa) and vesicular Glutamate Transporter 2 (vGLUT2) fluorescent immunostaining brain sections (30 mm/section) were rinsed 3 consecutive times with 1x KPBS. All incubations were performed on top of a shaking platform. Right after washing the slices, these were incubated for 60 min in blocking buffer (5% Donkey Serum and 0.4% Triton-X in 1x KPBS) at 4 ºC. After incubation in blocking buffer, sections were incubated overnight with the following primary antibodies: rabbit anti-Nk3R (Donated by Philip Cioffi, INSERM, 1:2500), chicken anti-GAD65 (139958, Abcam, 1:500), goat anti-CaMKIIa (87597, Abcam, 1:500) and mouse monoclonal anti-vGlut2 (ab79157, Abcam, 1:300). Primary antibodies were diluted in 0.4% Triton-X in 1x KPBS. After incubation in primary antibodies solution, brain slices were rinsed 3 times with KPBS 1x and then incubated in a sary antibodies solution for 2 hours at room temperature. The sary antibodies solution was prepared in Triton X (x100, Sigma-Aldrich, Spain) 0.4% (v/v) in 1x KPBS, and included the following sary antibodies conjugated to fluorophores: donkey anti-rabbit AlexaFluor488 (115-546-072, Jackson Immunoresearch, 1:1000), donkey anti-chicken Cyanine3 (703-166-155, Jackson Immunoresearch, 1:1000), donkey anti-goat AlexaFluor594 (705-586-147, Jackson Immunoresearch,

1:1000) and donkey anti-mouse AlexaFluor647 (715-606-150, Jackson Immunoresearch, 1:1000). 4',6-diamidino-2-phenylindole (DAPI) (10236276001, Sigma-Aldrich, 1:10000) was used to stain cell nuclei.

Z-Stacks of the Central Amygdala (0.50 µm/interval) were acquired using a Leica SP5 confocal microscope (Leica, Spain) with a PL APO 40x/1.25-0.75 immersion objective. Colocalization analyses were performed using Fiji for Windows. Z-Projections of Nk3R signal were used to create a mask. This mask was then applied to Z-Projections (0.50 µm/interval) of GAD65, CaMKIIa and vGLUT2 stacks using LungJ plugin. Mander's colocalization coefficient of colocalization between Nk3R and GAD65, CaMKIIa and vGLUT2 was calculated using *Just Another Colocalization Plugin* (JACoP). Average colocalization index between right and left CeA was used a measure of colocalization for each animal.

For Estrogen Receptor b (ERb) and Estrogen Receptor a (ERa) fluorescent immunostaining, the staining protocol was similar to the one mentioned above. Primary antibody solution contained rabbit anti-Nk3R (Donated by Philip Cioffi, 1:2500) and mouse monoclonal anti- ERa (sc-8002, Santa Cruz, 1:50) or mouse monoclonal anti- ERb (288, Abcam, 1:500). Sary antibodies solution included donkey anti-rabbit AlexaFluor488 (115-546-072, Jackson Immunoresearch, 1:1000) and donkey anti-mouse Rhodamine Red-X (715-295-150, Jackson Immunoresearch, 1:1000). DAPI (Sigma-Aldrich #10236276001) was used to stain cell nuclei. Z-Stacks of the Central Amygdala (0.50 µm/interval) are acquired using a Zeiss LSM 700 confocal microscope (Zeiss, Spain) with a PL APO 40x/1.25-0.75 immersion objective. Cell counter plugin for Fiji use employed to measure the number of Nk3R+ cells in the CeM. Mander's colocalization coefficients for ERa and ERb were calculated as abovementioned for Nk3R, GAD65, CaMKIIa and vGLUT2 immunohistochemistry using JACoP plugin in Fiji for Windows.

Cannula placement verification.

For cannula placement verification, amygdala brain sections were stained with a standard Nissl staining protocol. Brains were sectioned (30 µm/section) using a Leica Cryostat (-20 ºC for the chamber, -18 ºC for the sample), direct to mount and stored at -20 ºC until staining. Slides were dried on a hot plate at 37 ºC overnight before staining. First, slides were dehydrated in consecutive decreasing concentrations of denaturalized EtOH (Casa Álvarez, Barcelona, Spain): 3 min in EtOH 100% (v/v), 3 min in EtOH 95% (v/v) and 3 min in 70% (v/v). Right after that, slides were rinsed in distilled water three times for 2 min each to extract any trace of sucrose. Samples were then stained for 8 min in a Cresyl Violet Acetate Solution (C5042) consisting of 1 mg/mL Cresyl Violet Acetate in Walpole Solution, prepared with Glacial Acetic Acid (S2889, Sigma-Aldrich, Spain) 60% (v/v) and Sodium Acetate 0.2M (S2889, Sigma-Aldrich, Spain) 40% (v/v). After staining, slides are rinsed twice in distilled water for 2 min each and then dehydrated using increasing concentrations of EtOH: 3 dips in EtOH 50% (v/v), 3 dips in EtOH 70% (v/v), 3 dips in EtOH 96% (v/v) and 3 min in EtOH 100% (v/v). After this last dehydration, slides are incubated in Xylene (214736, Sigma-Aldrich, Spain) 3 times for 3 min each and then covered using DPX mountant for histology (06522, Sigma-Aldrich, Spain). Slides remain untouched for 24 hours the dark until stored in a regular box for slides at room temperature.

Cannulae placement verification was performed using brightfield photographs obtained using a 10x objective in an Eclipse 80i microscope (Leica, Spain). Animals that did not present a bilateral cannula lesion in the central amygdala were discarded from experiments.

mRNA qPCR array.

30 min after receiving osanetant (1 hour after FA), animals were decapitated, and brains were immediately fresh frozen in isopentane cooled with dry ice and stored at -80 °C. Amygdala tissue from both hemispheres was extracted by 1mm micropunch as previously described and each structure from each mouse was individually stored. Total RNA was isolated and purified from the tissue with the RNeasy Mini Kit (74106, Qiagen) following the manufacturer's instructions. Total RNA was isolated with Maxwell RSC simplyRNA Tissue Kit (AS1390, Promega). Quantus Fluorometer (E6150 Promega) was used to ensure the quality of RNA before the qPCR array (PAMM-071ZC-24, Qiagen). Gusb was the housekeeping gene use for normalization of qPCR results. qPCR array was performed following the instructions of the kit as stated, using the thermocycler *7500FAST*, controlled by *7500FAST v2.0.6* software both from Applied Biosystems (Thermo Fisher, Barcelona, Spain).

Ingenuity Pathway Analysis – Bioinformatics.

The gene list obtained on the mRNA qPCR array was analyzed with Bioinformatics. We used Ingenuity IPA version #44691306. The male and female lists of genes selected according to their significant p-value were uploaded separately. All genes were introduced with their respective expression fold change value. The female's gene list contained the genes *Akt1*, *Gcgr*, *Fgf2*, *Lhcgr*, *Mmp9*, *Ptgdr*, *Rho*, *S1pr3*, *Vcam1*. The males' gene list contained the genes *Agt*, *Agtrap*, *Bcl2*, *Calcrl*, *Ccnd1*, *Ccne1*, *Cdkn1a*, *Cdkn1b*, *Cflar*, *Elk4*, *Galr2*, *Gnas*, *Bcl2l1*. We performed a Core Analysis/Expression Analysis using the fold change as a measurement to base the analysis on. These analyses were performed separately for males and females by using most of the predetermined criteria by IPA. The general settings of this analysis included both direct and indirect relationships based on the references set "Ingenuity Knowledge Base (Genes Only)". For the networks analysis we selected 35 molecules per network and 25 networks per analysis. We also choose all Node Type and all Data Sources. For Confidence we selected only "Experimentally observed". For Species we selected all. The results we obtained with this IPA analysis were focused in two categories: Canonical Pathways (Fig 3) and Upstream analysis (Fig S5). For the Canonical Pathways we customized the charts by using a criterion of only selecting the categories that presented more than 4.5-fold changes in both males and females. The graphic in Fig 3 was created opening the PI3K/Akt in males and the G-coupled protein receptor signaling in females. Both graphics were combined using the Path Designer tool.

Biochemical studies.

10 min after receiving osanetant (40 min after FA), male and proestral female mice were decapitated and brains were snap frozen in isopentane right before storage at -80ºC. Both amygdalae were microdissected as abovementioned with a 1 mm micropunch, homogenized by sonication in 80 µL of

cold-lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% Na-deoxycholate, 2.5 mM EDTA, 1 mM Na_3VO_4, 25 mM NaF) containing protease and phosphatase inhibitors (Roche España, Barcelona, Spain). Protein concentration was quantified with the BCA protein assay kit (Thermo Fisher Scientific, Barcelona, Spain), resolved on SDS-polyacrylamide gel electrophoresis and detected by Western blotting with the following antibodies: rabbit anti-phosphorylated Akt (Thr308, 1:1000; Cell Signaling; Ser743, 1:1000; Cell Signaling) and goat anti-total Akt (1:1000; Santa Cruz Biotechnology), rabbit anti-CREB (1:700; Cell Signaling) and anti-phosphorylated CREB (Ser133; 1:1500; Cell Signaling), rabbit mTOR (1:1000; Cell Signaling) and anti-phosphorylated mTOR (Ser2448; 1:1000; Cell Signaling), mouse GSK3β (1:2500; BD Biosciences) and rabbit anti-phosphorylated GSK3β (Ser9; 1:1000; Cell Signaling), rabbit anti-β-Catenin (1:6000; Sigma-Aldrich) and anti-phosphorylated β-Catenin (Ser33/37/Thr41; 1:1000; Cell Signaling) or mouse anti-GAPDH (1:100000; Life Technology). Then, protein bands were detected with sary antibodies coupled to peroxidase enzyme (Bio-Rad, Madrid, Spain) and enhanced chemiluminescent reagent were captured in *ChemiDoc MP* System (Bio-Rad, Madrid, Spain) and quantified in a linear range using the *ImageLab v5.2.1* software (Bio-Rad, Madrid, Spain) as reported (56).

Data analyses.

Statistics analyses were performed using *IBM SPSS Statistics 23.0*. Detection of outliers was using the Grubb's test and removed when it was appropriate. For experiments involving independent samples, when these presented normal distributions and equality of variances, one-way ANOVA (GLM) was employed; otherwise non-parametric analyses were utilized for one factor analyses. Wald's c^2 with pairwise comparisons was used in Generalized Linear Model for multifactorial analyses that did not accomplish normality or homocedasticity. Additional individual comparisons were performed when appropriated.

For related sample analyses, repeated measures ANOVA was performed, using trials for FA or mean of groups of 5 trials for FE test. Equality of variances and sphericity were tested. When sphericity could not be assumed, Greenhouse-Geisser statistics were used for assessing significance and the corrected degrees of freedom were provided. ANOVA was used for evaluation of simple main effects and when interaction between main effects was significant. The results are presented as means ± or + SEM, and statistical significance was set at $P \leq 0.05$.

Normality and homocedasticity tests, as well as statistics with main effects, interactions, pairwise analyses and effect size, when appropriate, are represented in Dataset S1. In and Dataset S2, 95% confidence intervals are represented.

Declarations

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AUTHOR CONTRIBUTIONS.

This study was planned and conceptualized by RA and AF. AF carried all the behavioral, stereotaxic surgeries, vaginal cytologies, ELISA, qPCR, immunohistochemistry, histology, DREADDs and helped with Western blot experiments. EV contributed with help for the vaginal cytology readings. AG and OP contributed with the mass spectrometry data. CMS and CAS contributed with the biochemical experiments. The paper was written by RA and AF and commented on and discussed by all authors.

DECLARATION OF INTERESTS.

RA declares potential conflict of interest with the patent PCT/US2015/037629.

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Figures

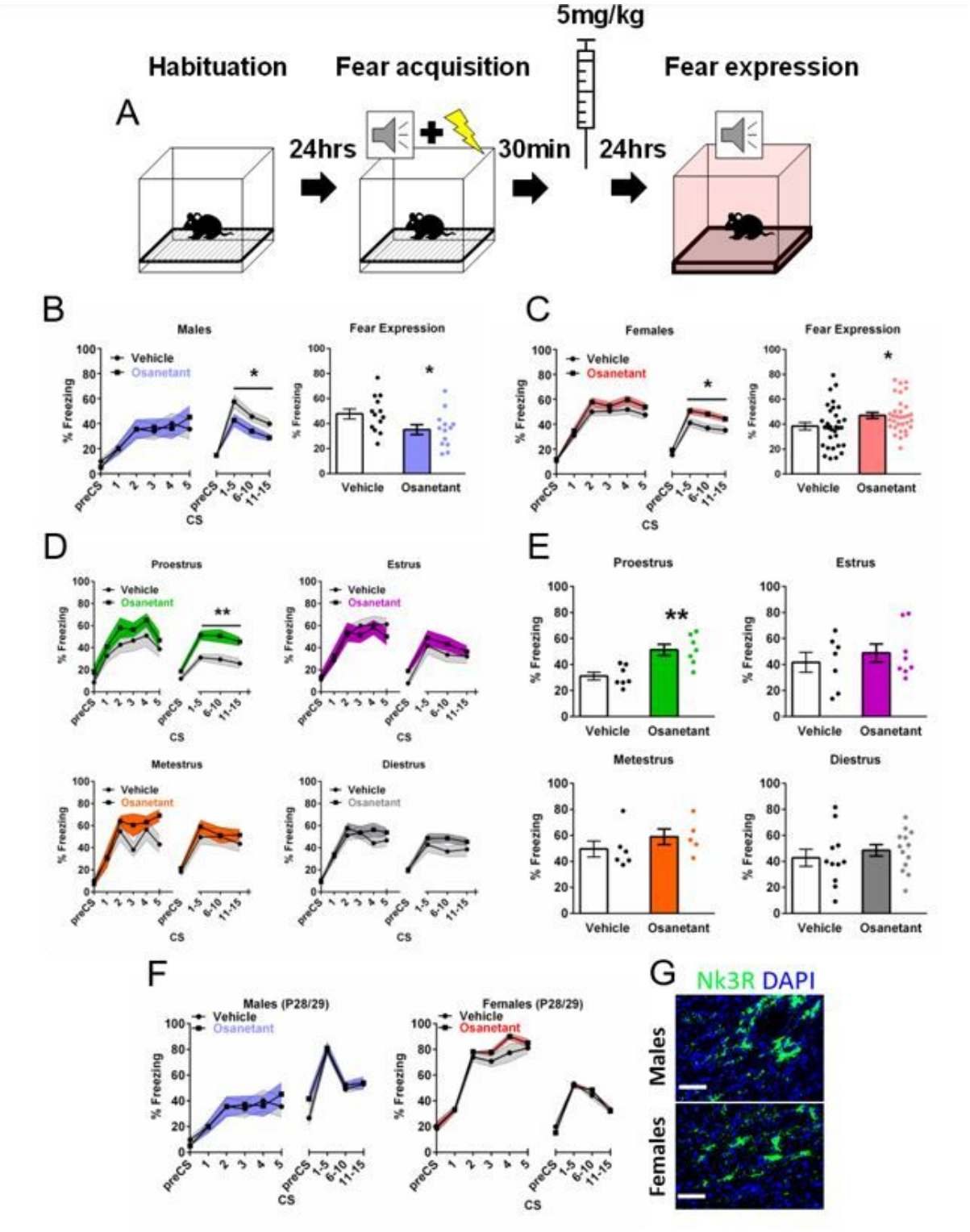

Figure 1

A systemic injection of osanetant impairs memory in males and enhances it in females. Fear memory consolidation procedure (A). Effect in the fear memory test of osanetant (5mg/kg, ip) or vehicle administration 30 min after de FA and 24 hours before FE in adult males (B) and females (C). Fear procedure in females divided by the estrous cycle during FA (D-E). Same fear procedure in prepubertal mice on P28 the FA day (F). Representative confocal images of the Nk3R in the CeA of prepubertal mice

in males and females. Scale bar = 60µm (G). Data are means ± SEM. *p≤0.05 **p≤0.01 vs vehicle. Asterisks above a line indicate treatment effect in repeated measures ANOVA.

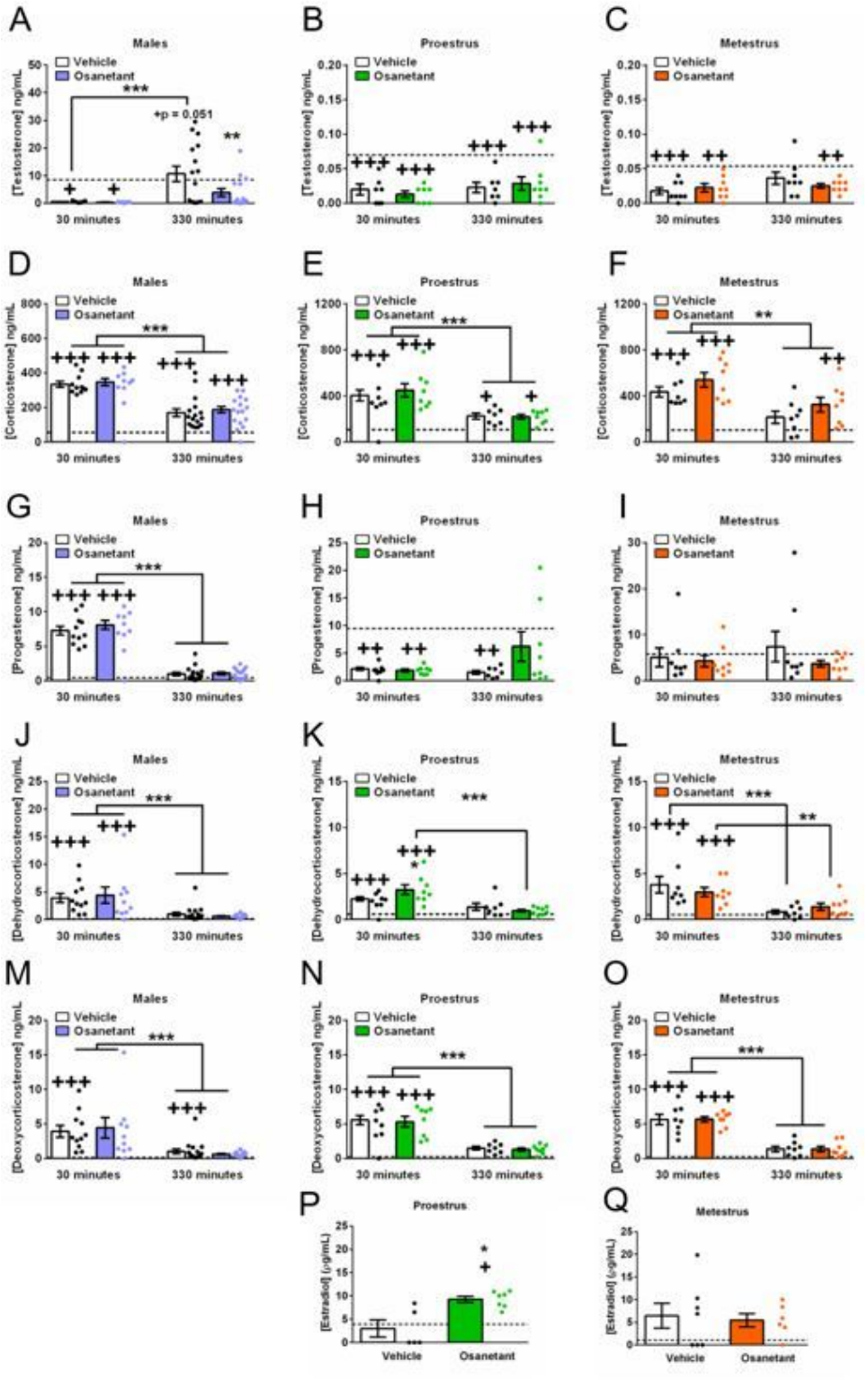

Figure 2

Testosterone (A-C), corticosterone (D-F), progesterone (G-I), dihydrocorticosterone (J-L), deoxycorticosterone (M-O) and Estradiol (P-Q) concentrations in plasma after fear acquisition. All mice received fear acquisition, osanetant or vehicle 30 min later and trunk blood was collected either 30 min or

330 min after the injection. Estradiol measures were only determined 330 min after the injection. The dashed lines are the mean baseline levels obtained from a different group of animals. Data are means ± SEM. *p≤0.05 **p≤0.01 ***p≤0.001 vs its vehicle. When with lines, it indicates specific comparisons. +p≤0.05 ++p≤0.01 +++p≤0.001 vs baseline. Statistic used was Wald's 2 with pairwise comparisons between groups.

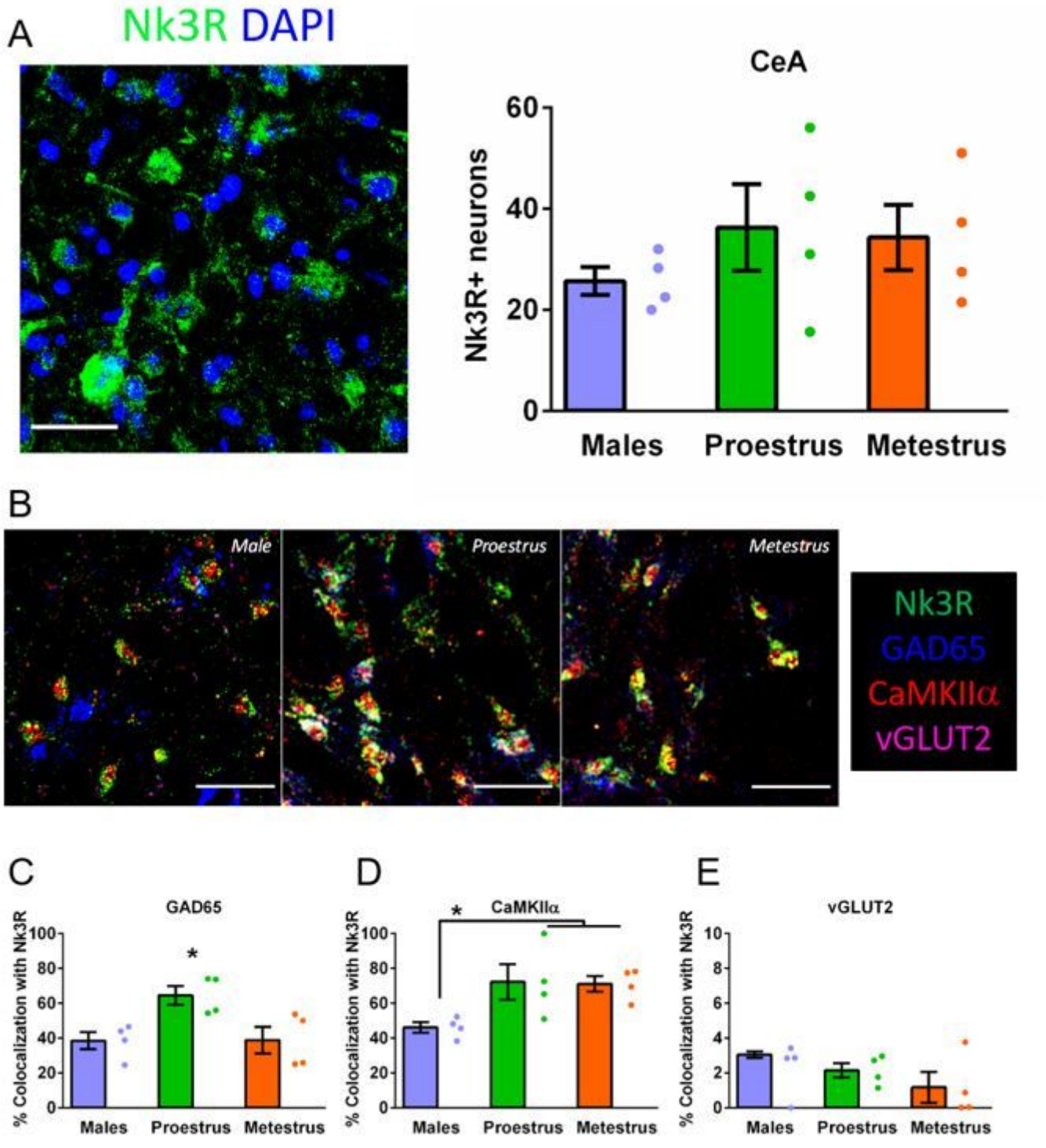

Figure 3

Confocal immunohistochemistry showing Nk3R expression and colocalization with GAD65/CamKIIα/vGLUT2 in the CeA. Samples were obtained at baseline without treatment in males, females during proestrus and during metestrus. Confocal image of Nk3R in the CeA indicate no differences between males and females across the cycle (A). Confocal images of the CeA showing colocalization of Nk3R with GAD65, CaMII and vGLUT2. White pixels indicate colocalization of Nk3R with GAD65. Yellow pixels indicate colocalization of Nk3R with CaMKII. Scale bar = 50 m. (B). Mander's colocalization coefficient between Nk3R and GAD65 (C), CaMKII (D) and vGLUT2 (E) was used as a measure of colocalization. Data are means ± SEM. *p≤0.05. Asterisks indicate one-way ANOVA with pairwise comparisons.

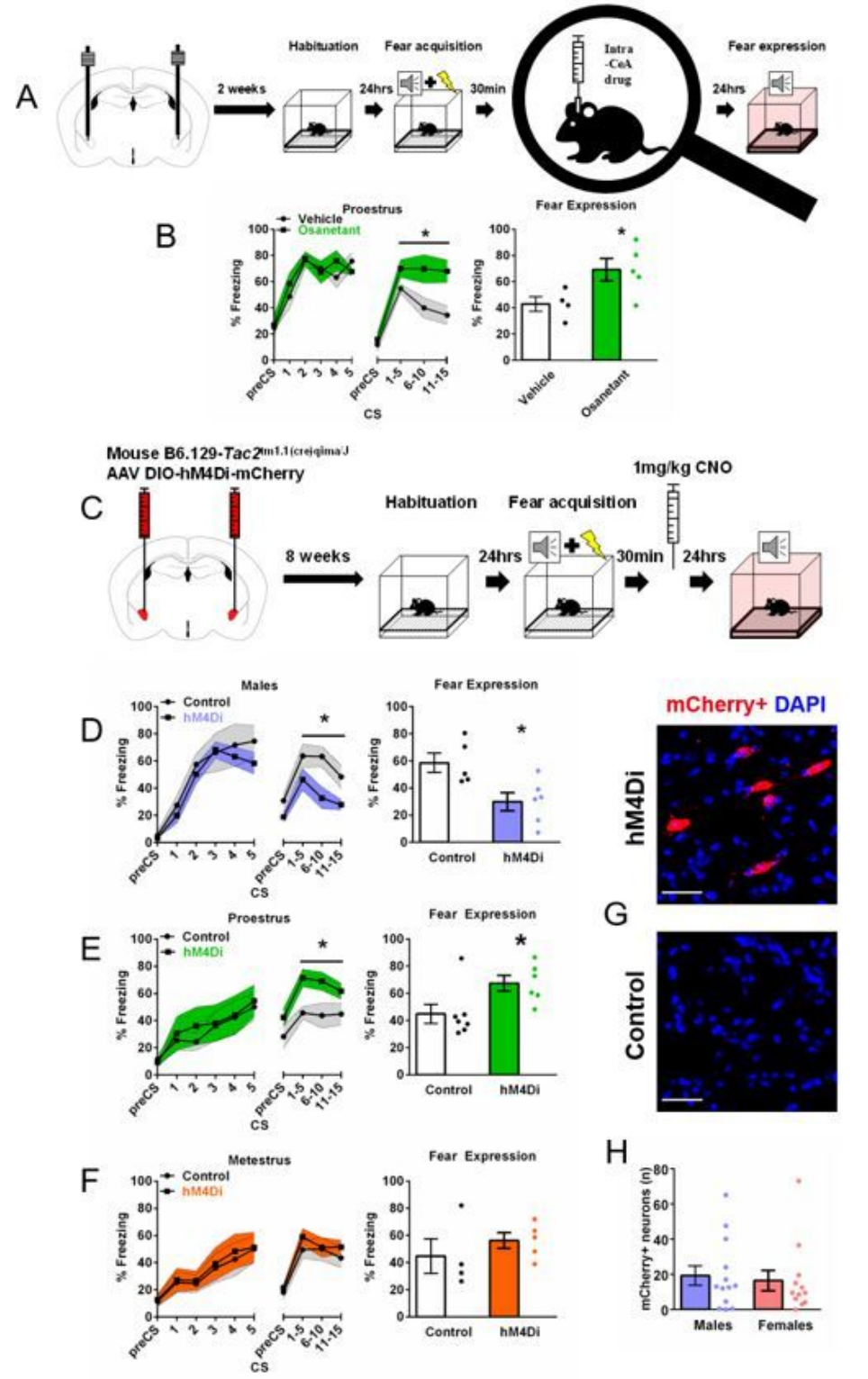

Figure 4

Temporal silencing of the Tac2-CeA neurons impairs fear memory consolidation in males and enhances it in females during proestrus. (A) Intracerebral injections of osanetant in the CeA after fear acquisition enhances fear memory consolidation in females proestrus (B). (C) Schematic and fear procedure in males (D), females in proestrus on the acquisition day (E) and females in metestrus (F). (G-H) Confocal images showing mCherry positive neurons in heterozygous Tac2-Cre mice (hM4Di) but not in wild-type

(control). Scale bar = 30µm. (G) males and females present similar levels of Tac2-CeA mCherry positive neurons. Data are means ± SEM. *p≤0.05 vs vehicle. Asterisks above a line indicate treatment effect in ANOVA repeated measures. Mann-Whitney's U was used for (H).

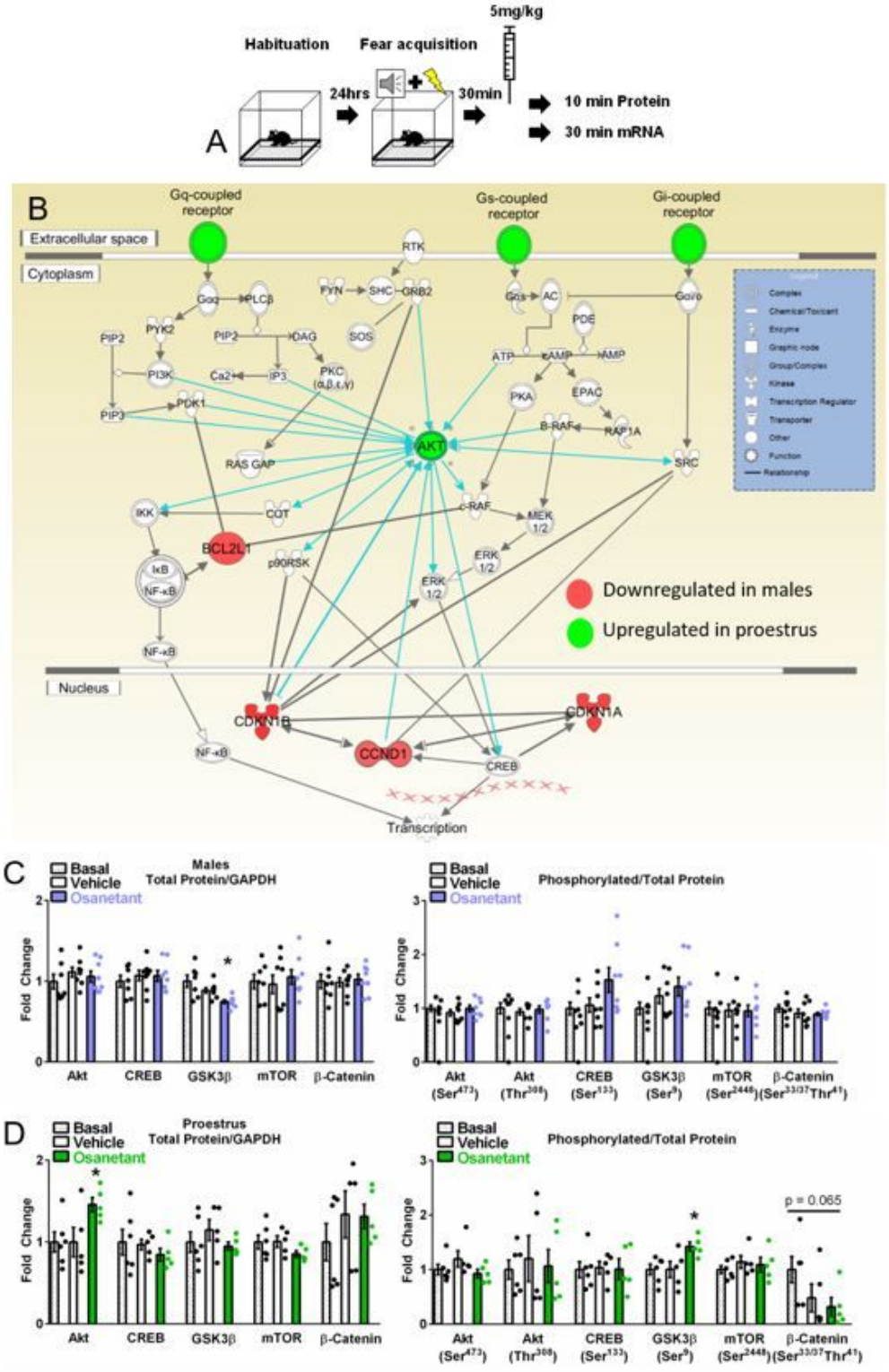

Figure 5

Osanetant reduces Akt/GSK3β/β-Catenin pathway after fear acquisition in males and it enhances it in proestral females in the amygdala. (A) Fear procedure to obtain mRNA 30 min after receiving osanetant

(B) and protein 10 min after receiving osanetant (C) by amygdala micropunches. (B) The GCPR qPCR array results analyzed by bioinformatics with the IPA software. Red indicates downregulation and green upregulation (C-D) Western blot analysis of Akt and its downstream signaling paths. Data are means ± SEM. *p≤0.05 vs all the other groups. For qPCR analysis, t-test or Man-Whitney's U test. Western blots were analyzed with one-way ANOVA or Kruskal-Wallis' H test. Pairwise comparisons are indicated when appropriate.

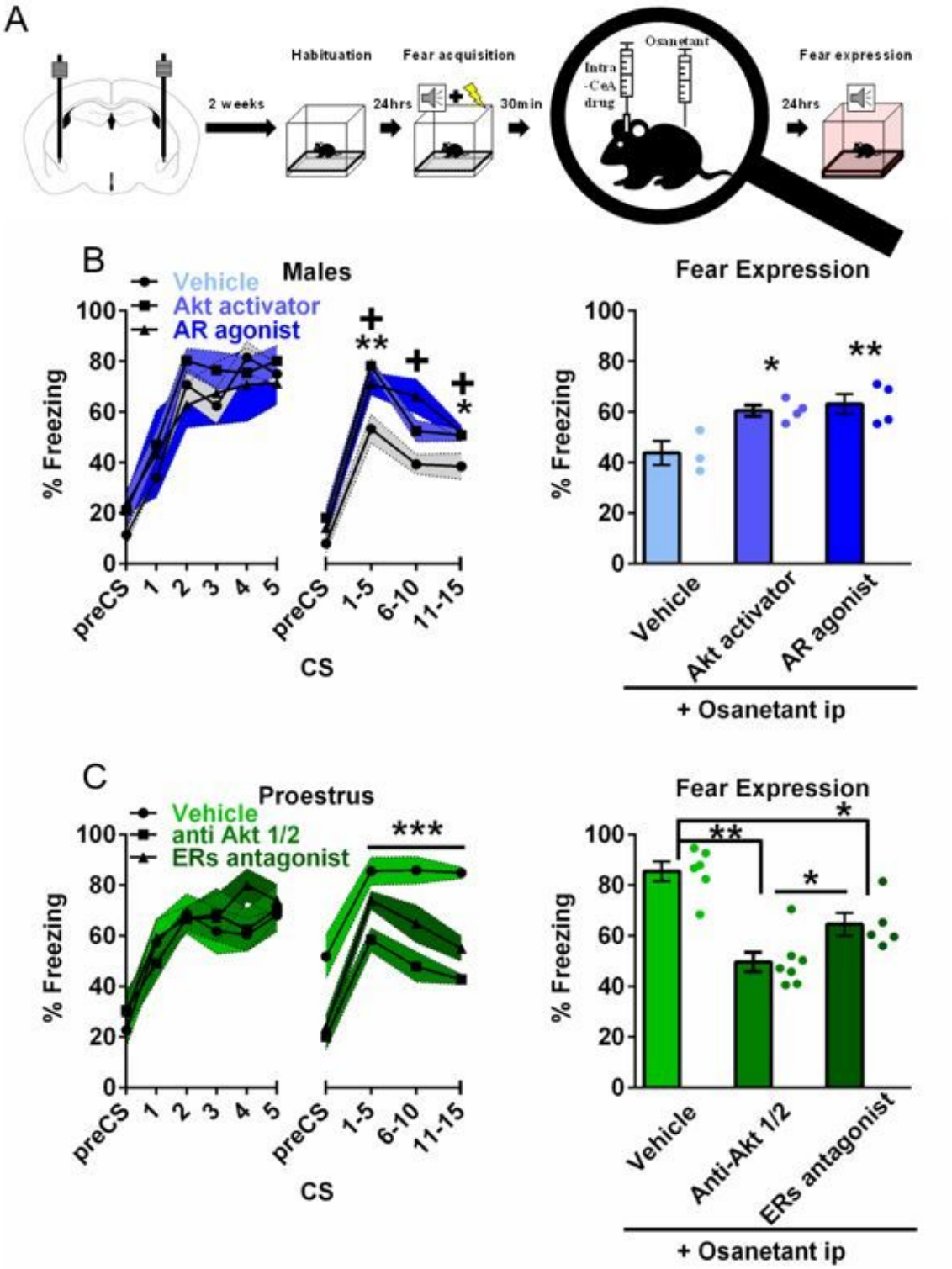

Figure 6

CeA-Akt and testosterone in males and CeA-Akt and estradiol in females proestrus are necessary for the systemic effect of osanetant in memory. (A) Schematic and intra-CeA infusion of drugs in males (B) and females proestrus at the time of fear acquisition (C). Data are means ± SEM. (B) *p≤0.05 **p≤0.01 vs vehicle. +p≤0.05 AR antagonist group vs vehicle. (C) *p≤0.05 **p≤0.01 ***p≤0.001 interaction group x block of CS. Blocks of CS and CSxTreatment interaction were analyzed with repeated measures ANOVA. Pairwise comparisons are indicated when appropriate.

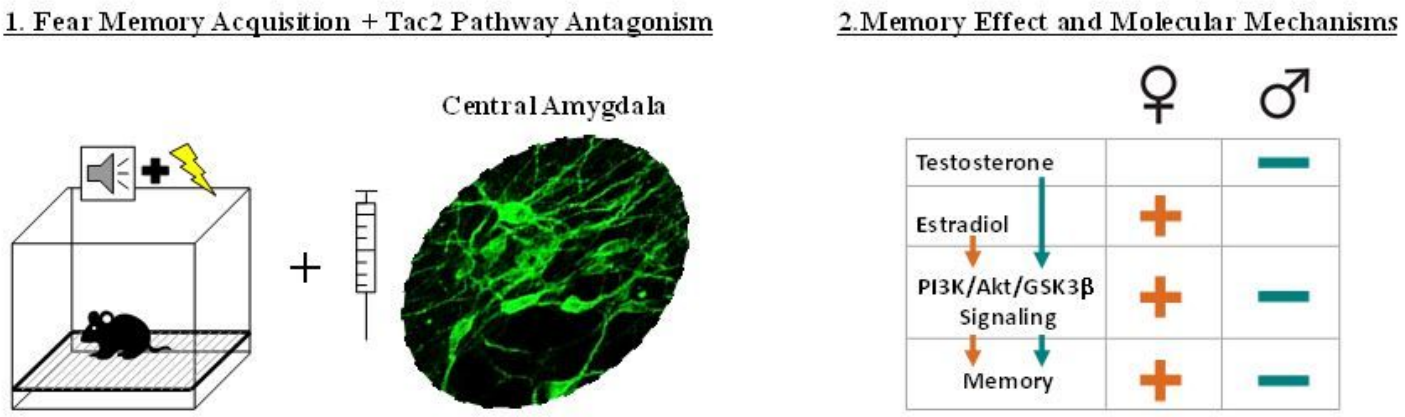

Figure 7

Graphical summary of the study.

Supplementary Files

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