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Silu Wang 3/27/2023

An Investigation into Orbitofrontal Cortex's Role in Driving Compulsive-Like Behavior

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Abstract

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Compulsions involve the preservation of behavior in the face of adverse consequences. In human patients with obsessive compulsive disorder (OCD), the orbitofrontal cortex (OFC) is hyperactive. We have demonstrated in mice bred experimentally to display compulsive-like behavior that chemogenetic inhibition of excitatory neurons in the OFC or a single dose of ketamine can reduce their compulsive-like grooming, suggesting that the OFC drives compulsive-like behavior. Ketamine has been shown to change dendritic spine morphology in the prefrontal cortex and rapidly reduce symptoms of OCD in human patients and compulsive-like behavior in animal models for studying compulsion. In addition to synaptic contacts within the OFC as being a potential mechanism that leads to compulsive-like behavior, evidence suggests that melanocortin 4 receptor (MC4R) could also play a key role. Here we hypothesized that ketamine rescues compulsive-like grooming in experimentally-bred mice through changing dendritic spines, which house the majority of excitatory synapses in the brain, and further, that hyperactive MC4R+ neurons in the OFC drive compulsive-like behavior. Mushroom to thin spine ratios appeared to have a wide dendrite-to-dendrite variance in the experimentally-bred mice that was normalized by ketamine treatment, though further investigation is needed. Meanwhile, chemogenetic stimulation of MC4R+ neurons in the OFC induced compulsive-like grooming. These results serve as stepping stones for our future efforts in investigating ketamine's anti-compulsive properties mediated via OFC dendritic spines, as well as molecular factors such as MC4R.

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Table of Contents

Abstract

Compulsions involve the preservation of behavior in the face of adverse consequences. In human patients with obsessive compulsive disorder (OCD), the orbitofrontal cortex (OFC) is hyperactive. We have demonstrated in mice bred experimentally to display compulsive-like behavior that chemogenetic inhibition of excitatory neurons in the OFC or a single dose of ketamine can reduce their compulsive-like grooming, suggesting that the OFC drives compulsive-like behavior. Ketamine has been shown to change dendritic spine morphology in the prefrontal cortex and rapidly reduce symptoms of OCD in human patients and compulsive-like behavior in animal models for studying compulsion. In addition to synaptic contacts within the OFC as being a potential mechanism that leads to compulsive-like behavior, evidence suggests that melanocortin 4 receptor (MC4R) could also play a key role. Here we hypothesized that ketamine rescues compulsive-like grooming in experimentally-bred mice through changing dendritic spines, which house the majority of excitatory synapses in the brain, and further, that hyperactive MC4R+ neurons in the OFC drive compulsive-like behavior. Mushroom to thin spine ratios appeared to have a wide dendrite-to-dendrite variance in the experimentally-bred mice that was normalized by ketamine treatment, though further investigation is needed. Meanwhile, chemogenetic stimulation of MC4R+ neurons in the OFC induced compulsive-like grooming. These results serve as stepping stones for our future efforts in investigating ketamine's anti-compulsive properties mediated via OFC dendritic spines, as well as molecular factors such as MC4R.

Introduction

Compulsions are defined in part by the preservation of behavior in the face of adverse consequences (Koob, 2014). Such compulsivity is thought to be a fundamental building block of drug and alcohol addiction, negatively impacting numerous lives in the modern society. It has been reported in human patients with obsessive compulsive disorder (OCD) that pathological changes occur in the orbitofrontal cortex (OFC) (Volkow and Fowler, 2000), and OFC hyperactivity was found during symptom provocation (Simon et al., 2010). The intensity of cocaine craving was also found to be positively correlated with the metabolism in the OFC during early withdrawal (Volkow and Fowler, 2000). Moreover, enhanced activity in the OFC is associated with compulsive cocaine self-administration, while the inhibition of OFC reduces compulsive-like reinforcement (Pascoli et al., 2018). Repeated hyperactivation of the OFC generated aberrant grooming in mice (Ahmari et

al., 2013). This evidence together suggests that the OFC plays a critical role in driving compulsive behavior.

Ketamine is an NMDA receptor (NMDAR) antagonist that is used as an anesthetic agent. It also has antidepressant properties that are hypothesized to act through selective disinhibition of excitatory glutamatergic neurons or inhibition of spontaneously activated NMDARs — both are potential mechanisms that lead to an increase in synaptic strength of excitatory circuits (Zanos et al., 2023). Imbalances in glutamatergic neurotransmission are suspected to be a key component contributing to OCD (Pittenger, 2015). Ketamine was found to have the ability to rapidly improve OCD symptoms in humans and decrease compulsive grooming behavior in synapse-associated protein 90/postsynaptic density protein 95-associated protein 3 (SAPAP3)-null mice, which is an animal model for compulsive behavior (Martinotti et al., 2021) (Davis et al., 2021).

We have developed an experimentally-bred mouse line that displays compulsive-like behavior. Further, a single dose of ketamine, or repeated inhibition of excitatory neurons in the OFC using inhibitory (Gi-coupled) designer receptors exclusively activated by designer drugs (DREADDs) rescues compulsive-like behavior in our experimentally-bred mouse line. As either treatment worked to reduce compulsive-like behavior, our current research aims to investigate how potential treatments reduce compulsive-like behavior.

A theory of the mechanism of action of ketamine posits that it rapidly modifies dendritic spine morphology to achieve the antidepressant effect. To be more specific, the spine density as well as the number of mushroom spines increase was visible as soon as 24 hours after a low dose of ketamine administration, indicating more spine maturation and stronger synapses (Li et al., 2010) (Liu et al., 2013). Dendritic spines are protrusions on dendrites and are the active sites that communicate with neighboring neurons. They are susceptible to changes in response to neuronal signals and are classified into three types based on their morphology: mushroom spines, which are mature; thin spines, which are young; and stubby spines, which are dying off. We have investigated the spine changes following the ketamine injection in the experimentally-bred mice. We found high variability of mushroom to thin spine ratio of the experimentally-bred mice which appeared to be normalized by a single dose of ketamine injection. Due to the complexity of spine growth and pruning, we decide to additionally identify molecular factors within the OFC that drive compulsive-like behavior to understand the specific neuronal populations targeted by the ketamine's anti-compulsive property.

One such likely candidate for driving compulsive-like behavior is the melanocortin 4 receptor (MC4R). MC4R is a type of G-protein coupled receptor typically thought to regulate energy homeostasis (Tao, 2014) and is widely expressed throughout the human brain (The Human Protein Atlas, n.d.). Previous work by Alvaro et al. in 2003 showed that stimulation of MC4R resulted in compulsive-like grooming behavior in rats. Deletion of the gene coding for MC4R in the OFC and the nucleus accumbens shell of the ventral striatum, along with the pharmacological inhibition of MC4R signaling, normalized compulsive-like grooming as well as ventral striatal synaptic transmission in SAPAP3-null mice (Xu et al., 2013). Additionally, transcriptomic analysis of the experimentally-bred mice in our lab showed an increased level of MC4R (Allen et al., 2022). These data have provided strong support for MC4R's role in driving compulsive behavior, but there is a gap in the literature regarding how the MC4R+ neuron populations in the OFC could contribute to compulsive-like behavior, which we intend to fill with this project.

We now want to investigate whether the MC4R is a key component in the OFC's role in driving compulsive-like behavior and interrogate how ketamine treatment can rescue compulsive-like behavior in our model of compulsive-like behavior. We predict that repeated stimulation of the OFC MC4R+ neurons will lead to compulsive-like grooming behavior in mice. A single dose of ketamine will lead to changes in dendritic spine morphology, specifically in spine density and volume.

Methods

Animals

Experimentally-bred mice treated with ketamine for studying dendritic spine morphology were *Thy1-YFP-H* transgenic mice (H line, stock #003782; Feng et al., 2000), bred as described in Allen et al. (2022). The control mice used for spine analysis were the same strain of mice that were not experimentally-bred. Mice used to study MC4R's role in compulsive-like behavior were *Mc4r-2a-Cre* knock-in mice (stock #030759). Equal numbers of males and females were used. All mice were group-housed on a normal 12-hour light cycle and provided food and water ad libitum unless otherwise noted.

Surgery

MC4R-2a-Cre mice were infused with recombinant adeno-associated virus (AAV) encoding an excitatory Gq-coupled DREADD

(pAAV8-hsyn-DIO-hM3D(Gq)-mcherry) or control viral vector (pAAV8-hsyn-DIO-mcherry) into the OFC using previously established intracranial coordinates (ML±1.5, AP+3.0, DV-3.1). Mice would be allowed 3 weeks for recovery and viral vector expression before behavioral testing.

Stimulus Elicited Grooming Assay

Mice were set in the testing room with dim light and cage coverage for 30 minutes before the spray test commences. A large empty box without bedding was wiped down with water. The mice were suspended momentarily by the tail to receive six sprays of clean water. For the baseline test, the mice were exposed to the same apparatus except the water spritzes. One mouse was placed per box and instances of grooming and wet dog shakes were recorded over the next 6 minutes, with 30 seconds of interval between each observation. After the test, mice were returned to their home cage. The boxes were then cleaned and wiped with water for the next round of experiment.

Experimental Timeline-MC4R Mice

Clozapine N-oxide (CNO) (1mg/kg *i.p.*) was administered for 7 days. Compulsive-like behavior was measured by the difference score calculated using grooming incidence during the preliminary testing on days 3 and 4 and during a final spray test on day 9. A single dose of ketamine (30mg/kg *i.p.*) would be administered on day 8.

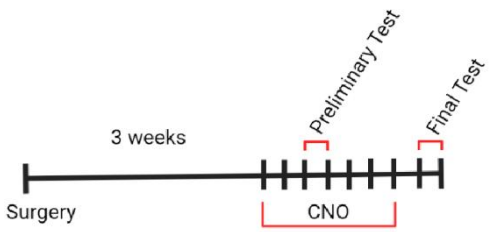

Figure 1. Timeline for Spray Test and Injections

Experimental Timeline-Experimentally-Bred Mice

The experimentally-bred mice were given ketamine (30mg/kg *i.p.*) or saline injection, waited 24 hours and tested in spray test on the following day. Counterbalanced spray and baseline tests were administered in the morning and evening. After one week of washout period, the mice were given a dose of ketamine (30mg/kg *i.p.*) and then sacrificed after 24 hours.

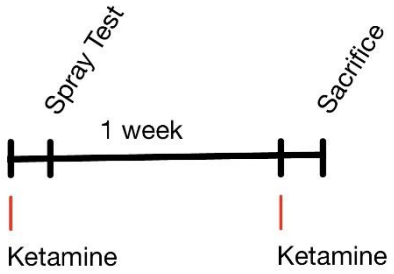

Figure 2. Timeline for Experimentally-bred Mice's Spray Test and Injection

Tissue Preparation

Mice were first anesthetized with isoflurane and then euthanized by rapid decapitation following the behavioral testing. Experimentally-bred mice were euthanized 24 hours after a final single dose of ketamine (30 mg/kg i.p). Brains are soaked in 4% paraformaldehyde for 48 hours then transferred to 30% weight by volume sucrose solution and sectioned into 50 µm thick sections on a freezing microtome. MC4R brains were observed on Keyence BZ-X710 to confirm viral vector placement.

Dendritic Spine Analysis

Dendritic spine data in the OFC of the experimentally-bred mice were collected and analyzed using the IMARIS software. These mice expressed *Thy1*-driven YFP on the layer 5 pyramidal neurons. The tissues were imaged using Leica DM5500B spinning disk confocal microscope. We collected 6-8 dendrites per animal. The dendritic spines were counted and classified into stubby, mushroom, and thin types. Spine length, head diameter, and head volume for mushroom and thin type spines were analyzed.

Statistical Analysis

For the experiments involving different groups (experimentally-bred or control) or drug treatments (ketamine or saline), 2-factor mixed analysis of variance (ANOVA) was used to compared between groups. Post-hoc analysis was conducted following interaction effect. For the experiment investigating OFC MC4R+ neurons' role in driving compulsive-like behavior, 2-tailed unpaired t-test was used.

Results

Ketamine reduces compulsive-like grooming in experimentally-bred mice

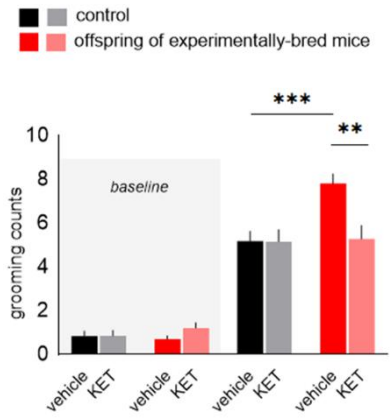

Figure 3. Ketamine Suppresses Compulsive-like Grooming in Experimentally-bred Mice

*A single dose of ketamine (30mg/kg i.p.) was given to control mice or experimentally-bred mice 24 hrs before testing. The experimentally-bred mice groomed more than the control animals. Ketamine reduced grooming counts in experimentally-bred mice. Bars represent means + SEMs. ***p=0.006, **p=0.0038.*

We then confirmed that the experimentally-bred mice exhibit higher grooming counts and proceeded to test if a single dose of ketamine would work to lower the grooming counts on the same mice. Ketamine significantly lowered the grooming counts of experimentally-bred mice [interaction $F_{(1,62)} = 7.368$].

Ketamine's impact on dendritic spines on orbitofrontal cortical neurons

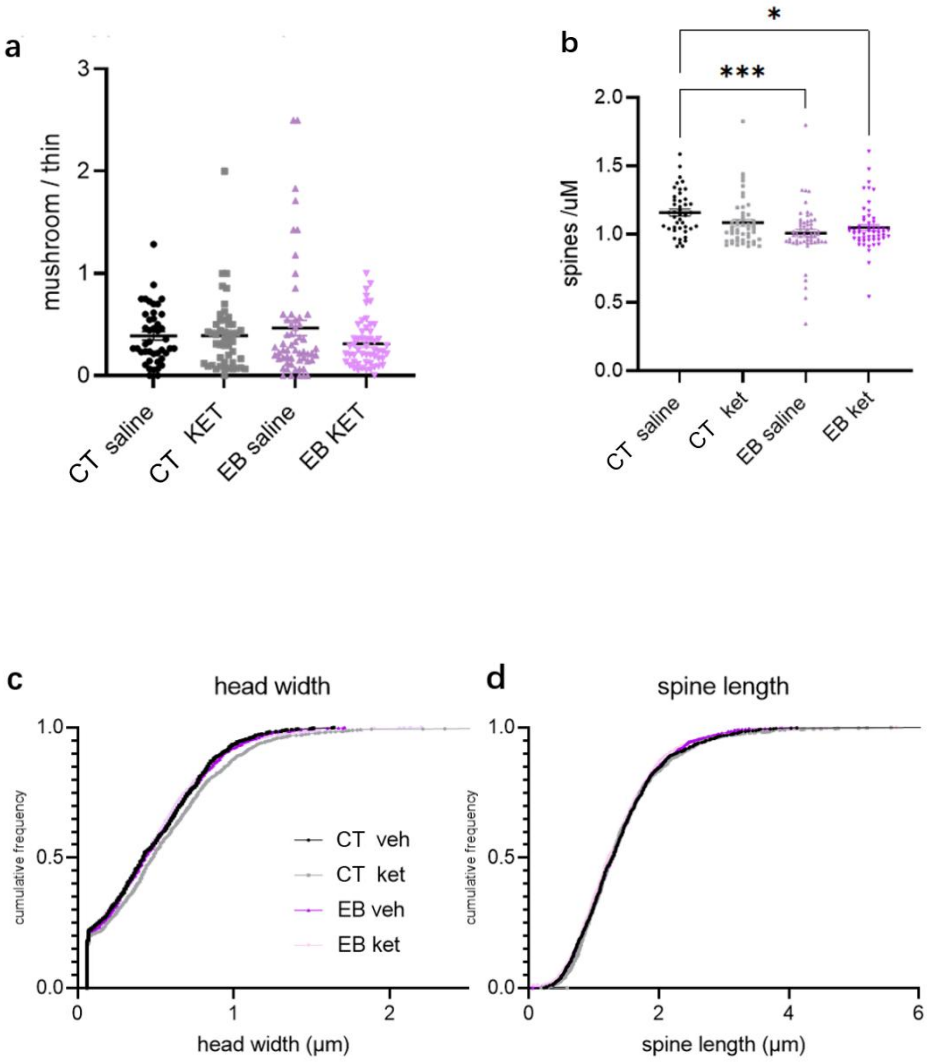

Figure 4. The Dendritic Spine Morphological Changes in Experimentally-bred Mice and in Ketamine Treatment Group

*The mushroom and thin spines were counted along with their head widths, lengths, and density on the dendrites. Each data point in figure 4a and 4b represented a dendrite. The large variance of mushroom-to-thin type spine ratio of experimentally-bred mice appeared to be reduced after ketamine treatment. The spine density was lower in the experimentally-bred mice, and ketamine did not restore it. The spine head width increased after ketamine treatment in control mice, while no change was observed in spine lengths. *p=0.0136, ***p=0.0002*

After observing the behavioral effect of ketamine, we decided to investigate the underlying morphological changes in the neurons that could be associated with the behavioral change. First, we looked at the mushroom-to-thin type dendritic spine ratio.

The variance of mushroom-to-thin spine ratio of experimentally-bred mice appears larger, but this trend appears to be normalized by ketamine treatment (figure 4a).

We then looked at the overall spine density across each group. The spine density of the experimentally-bred mice treated with saline decreased, and ketamine did not block this reduction (figure 4b). The increase in spine head width in the control mice treated with ketamine compared to other treatment groups (figure 4c) echoed the results found in previous literature regarding ketamine's effect on spines, despite no change in spine length (figure 4d).

Hyperactivation of MC4R+ neurons in the OFC led to aberrant grooming

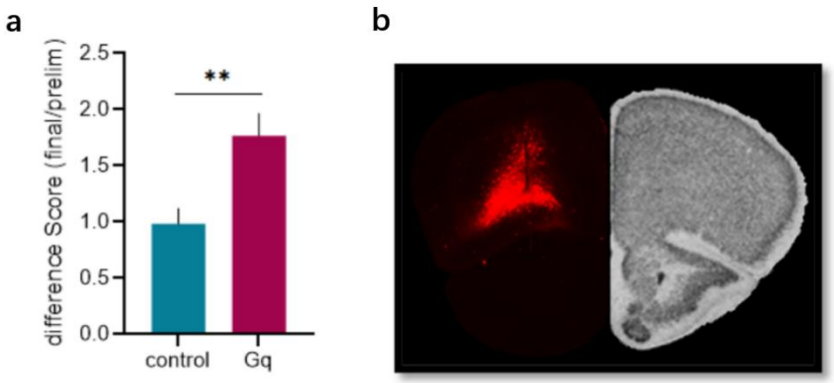

Figure 5. Gq-DREADD Activation of OFC MC4R+ Neurons Led to Compulsive-like Grooming

*Mice infused with Gq-DREADDs in their OFC neurons expressing MC4R had elevated grooming behavior upon repeated activation of the MC4R+ neurons. **p = 0.007.*

Mice with Gq-DREADDs or control viral vectors expressed in their MC4R+ neurons were injected with CNO for 7 days. We conducted the preliminary stimulus elicited grooming assay on day 3 or 4 to get the mice's baseline grooming behavior. On day 8, the mice either received a single dose of ketamine or saline injection, then underwent the final spray test 24 hours later. The compulsivity-like behavior of the mice was quantified by a difference score calculated by dividing the final assay's grooming count over the preliminary assay's grooming count. Their behavior reported in the form of raw grooming counts is included in the supplementary section. Repeated activation of Gq-DREADDs in MC4R+ neurons in the OFC significantly increased the compulsive-like grooming behavior [$t(19) = 3.020$, $p = 0.007$] (figure 5a). Figure 5b shows the OFC viral vector delivery confirmation.

Discussion

With previous research suggesting that the abnormal activation of the OFC is an integral part of the circuit driving compulsive behavior (Volkow and Fowler, 2000), we aimed to identify morphological substrates underlying behavior in an animal model of compulsive-like behavior. Further, we interrogated the role of a molecularly defined neuron population potentially responsible for this phenotype.

It has been shown here that ketamine successfully reduced the elevated grooming behavior of the mice bred experimentally to demonstrate compulsive-like behavior, which reflected the clinical efficacy demonstrated previously (Martinotti et al., 2021). The mechanism through which ketamine works may be mediated by dendritic spines (Wu et al., 2021). The dendritic spines of the experimentally-bred mice appeared to have a wider variance in spine type ratios compared to control counterparts, and this variability seemed to be reduced by ketamine treatment (figure 4a). Additionally, there was an increase in spine head width in the control mice treated with ketamine. The trend of ketamine modifying mushroom-to-thin spine ratio and increasing spine head width have both been reported in previous literature regarding ketamine's anti-depressant effect (Liu et al., 2013). Overall, these data provide further support for the notion that compulsive-like behavior may stem from aberrant morphology, or potential circuit connectivity, within the OFC. However, it does not necessarily prove a causative role for dendritic spine morphology in compulsive-like phenotypes of our animal model.

The MC4R receptor is expressed in various brain regions including the OFC. Its expression in the brain modified the compulsive behavior in the *SAPAP3-null* mice model, leading us to hypothesize that MC4R in the OFC is a molecular driver to compulsive-like behavior. In this study, we identified that activation of neurons expressing MC4R receptors in the OFC generates compulsive-like grooming in mice. Our results serve as preliminary evidence that MC4R+ neurons in the OFC are capable of driving compulsive-like behavior, while ketamine is effective at treating such behavior by changing neuronal morphology in the OFC.

While the results look promising, there are limitations to the study that need to be addressed. We have collected a wide range of dendritic spine morphological parameters. We are still in the process of isolating the characteristics being changed by the ketamine treatment. Despite our results from the ketamine treatment group showing correspondence with previous literature, the analysis of spine length and head diameter showed no significant difference in distribution between the

ketamine-treated mice and the control. We are still in the process of isolating specific spine parameters that could reflect ketamine's anti-compulsive property.

An immediate next step of this study will be to see if ketamine could reduce the compulsive-like behavior driven by the hyperactivation of MC4R+ neurons in the OFC. We have used ketamine treatment on the current cohort that showed increased compulsive-like behavior and observed a trend in which ketamine indeed reduced aberrant grooming. We need to replicate this experiment for confirmation.

Since the hyperactivation of the OFC MC4R+ neurons led to an increase in compulsive-like behavior, we are interested in mapping out the circuit driving this behavioral change. The experimentally-bred mice demonstrating high compulsive-like behavior show decreased levels of post-synaptic density protein 95 (PSD95) in the striatum, possibly indicating aberrant corticostriatal connectivity (Allen et al., 2022), leading us to suspect that the corticostriatal network may be a driver of compulsive-like behavior. We plan to process the striatum samples of the Gq-DREADD animals that underwent the spray test by western blot to measure PSD95 to examine presence of excitatory synapses in the striatum.

Further, it will be interesting to see what neuron populations are active after the stimulus spray. By sacrificing the experimentally-bred animals after they received stimulus spray, we can identify if the MC4R+ neurons were active by testing for c-Fos RNA transcripts using RNAScope (figure 7a). We did not identify obvious support for this hypothesis, but interestingly, we observed a slight trend showing that control mice have more MC4R- populations active after receiving sprays [$t(6) = 0.9727$, $p = 0.01814$] (figure 7b). This could indicate that the non-MC4R neurons in the OFC of the control mice are recruited to create a "stop signal" and inhibit the compulsive-like grooming. A prominent idea of the OFC's involvement in OCD is its hyperactivation reflects the need to inhibit compulsive behaviors (Maia et al., 2008) (Evans et al., 2004). The higher activation of non-MC4R neurons in the control group could reflect the inhibitory effect of OFC activation, thus supporting what we have observed.

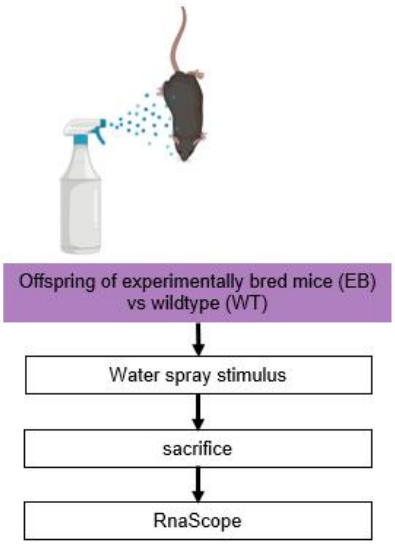

Figure 6. Flowchart of Behavioral Experiment and RNAScope

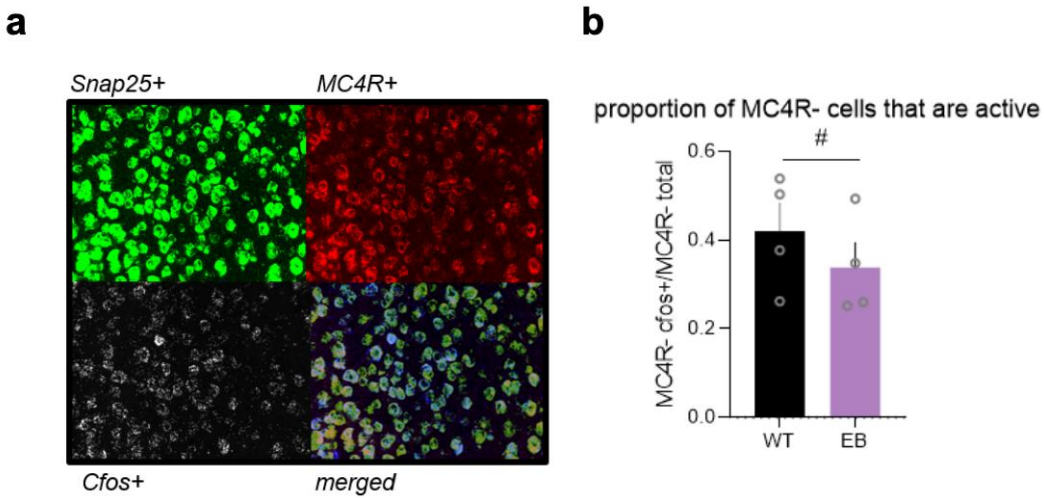

Figure 7. RNAScope Showing Trend of More Non-MC4R Neuron Activation Following Water Spray Stimulus

Snap25 RNA transcript was visualized to mark the excitatory neurons; c-Fos RNA transcript was visualized to mark neuronal activation. By labeling for MC4R RNA and merging the images together, experimentally-bred mice show a trend of having less MC4R- neurons activated than their control counterparts.

Conclusion

Here we report that ketamine induces changes in OFC dendritic spine morphology. However, the link between the spine morphology changes and ketamine's anti-compulsive-like property remains unclear. Hyperactivation of the MC4R+ neurons in the OFC alone can lead to compulsive-like behavior. Based on data from the spray test and preliminary result of RNAScope, we suspect that while MC4R+ neurons drive compulsive-like behavior, MC4R- neurons may inhibit such behavior.

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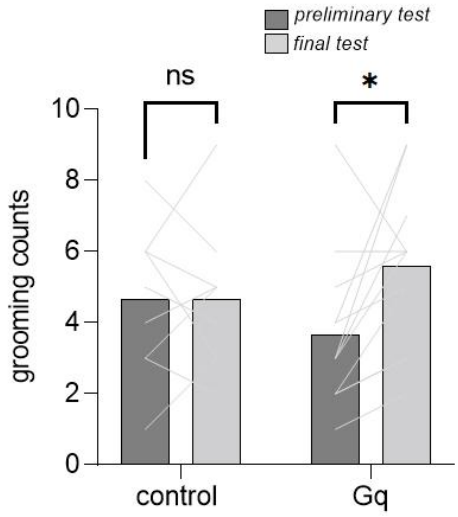
Supplementary Data

The two-way ANOVA test of the spray test raw data of MC4R mice in supplementary table 1 showed that the p-value of the interaction effect was trending but not significant (p = 0.0720).

Despite the interaction effect being not significant, we conducted post-hoc testing to see if the Gq-DREADD activation significantly changed the grooming counts of mice, and the result was positive. The exploratory post-hoc showed that stimulation of MC4R+ neurons increased grooming in the final spray test.

Source of Variation	% of Total Variation	P-value	P-value Summary	Significant?
group x test	4.780	0.0720	ns	No
group	0.009035	0.9592	ns	No
test	4.780	0.0720	ns	No

Supplementary Table 1. Two-way ANOVA Test for Stimulus Elicited Grooming Assay Data of MC4R Mice



Supplementary Figure 1. Gq-DREADD Mice Showed Elevated Grooming Counts After Activation of OFC MC4R+ Neurons