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The therapeutic effects of projection-specific optogenetic stimulation in an animal model of social anxiety disorder

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By

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ABSTRACT OF THE THESIS

The therapeutic effects of projection-specific optogenetic stimulation in an animal model of social anxiety disorder

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Professor Byungkook Lim, Chair

Social anxiety disorder (SAD), also known as social phobia, is a major physical and mental health concern with a high lifetime prevalence. Despite being the most common anxiety disorder, current treatment options available are unsatisfactory among SAD patients. Therefore, we adapted a social fear conditioning (SFC) paradigm to mimic the main behavioral symptoms of SAD of humans in mice. In this protocol, social fear is induced by administering foot shocks during social interaction with an unknown conspecific. This form of conditioning did not induce any other behavioral alterations such as general anxiety. The negative social experience inducing social fear in this animal model and SAD patients satisfies construct validity, whereas the similarity in behavioral phenotypes satisfies face validity. After establishing that our model of social fear has face and construct validity, we aim to assess 1) whether this model has predictive validity, and 2) test the therapeutic efficacies of three novel therapeutic strategies: cognitive behavioral therapy, pharmacological selective-serotonin reuptake inhibitor, and targeted brain stimulation.

Introduction

Since social behaviors are essential to the well-being and survival of a social species, social deficit disorders can be highly detrimental. Social anxiety is an emotional trait characterized by persistent fear and avoidance of social situations in response to the evaluation of others (Toth et al, 2012). When social anxiety reaches a high degree of severity such that functioning is impaired, the emotional trait is referred to as Social Anxiety Disorder (SAD) (Morrison et al, 2013). Social anxiety disorder, also known as social phobia, is epidemiologically, the third most common psychiatric disorder with a 12-month and lifetime prevalence of 6.8% and 12.1%, respectively (Kessler et al, 2005a, 2005b). The debilitating nature of SAD is associated with negative consequences such as developing depression, substance abuse, impaired academic performance, social isolation, and unemployment (Reus et al, 2014). The fear and avoidance behavior play an important role in reinforcing social anxiety and preventing social fear extinction (American Psychiatric Association, 1994; Strangier et al, 2006). Despite posing as one of the largest threats to global mental health and the most common of all anxiety disorders, little is known about the underlying neurobiology of SAD to develop better treatments (Reus et al, 2014). Since using animal models can be effective for studying the mechanisms of disease as well as developing and testing novel treatments, it is necessary to develop an appropriate model for social anxiety disorder. For an animal model to be considered valid, the model must satisfy three criteria: face, construct, and predictive validity. To have face validity, the model must recapitulate the symptoms that are observed in humans such as demonstrating social aversion or anxiety-like responses to social situations. To meet construct validity, the

model must have etiological similarities to the human disease such as a substantially negative social experience. Lastly, to satisfy predictive validity, treatments used in patients must also similarly affect the animal.

Since patients with social anxiety disorder report dramatically negative social experiences, we adapted a social fear conditioning (SFC) protocol to induce social aversion in mice (Toth et al., 2012). Based on the principles of operant conditioning, animals associate a voluntary behavior with its negative consequence, and thus, the likelihood that the response will occur decreases. In this protocol, social fear is induced by administering foot shocks during the investigation of an unknown conspecific and mice learn to avoid subsequent social stimuli (Figure 1A – 1C). Importantly, the shocked mice do not exhibit other markers of fear and anxiety – highlighting the specificity of this approach. The etiological similarity between this model and patient reports suggest that this model satisfies the requirements for construct validity, whereas the similarity in behavioral phenotypes meets the requirements for face validity. After establishing that our model does indeed satisfy the face and construct validity requirements for animal models of disease, we sought to test 1) whether this model has predictive validity, and 2) test the effects of novel therapeutic strategies. In this thesis, we discuss three different approaches to treating our mouse model of social anxiety disorder: cognitive behavioral therapy, pharmacological selective serotonin reuptake inhibitor, and targeted brain stimulation.

Methods

Animals

All animal housing and handling were conducted under the ethical standards of University of California Institutional Animal Care and Use Committee (IACUC). Adult male C57BL/6J wild-type mice were obtained from Jackson Laboratory. Mice were grouped-housed prior to experiments and maintained on a 12-hr light/dark cycle (lights on 7:00). Animals were randomly assigned to either control or experimental groups. Surgeries were performed between 10-12 weeks of age.

Surgeries and Viral Vectors

Virus generation

AAV vector plasmids were constructed using standard molecular cloning methods.

Stereotaxic injections and optical fiber/cannula implantations

Adult (8-12 week old) male C57BL/6J (wild-type) animals were grouped housed in a 12-hr (07:00-14:00) light-dark cycle prior to surgery. Mice were anesthetized intraperitoneally (i.p.) with ketamine (100mg/kg) and dexmedetomidine (1 mg/kg) cocktail and fixed in a stereotaxic apparatus (Kopf Instruments). Temperature was regulated with a heat pad (Physitemp). Holes were drilled (Kopf Instruments) into the skull. Capillary glass tubing (outer diameter = 1.20 mm, inner diameter = 0.94 mm, length = 10 cm, Warner Instruments) were formed into pipettes using DMZ Zeitz-Puller, loaded with virus, lowered into the brain, and pressure-infused (Harvard Apparatus)

unilaterally. After viral administration, the pipette was left in place for ten minutes to minimize leakage from pipette removal. After the pipette was slowly removed, the scalp was sutured and resealed with tissue adhesive (Vetbond, 3M). Carprofen (5.0 mg/kg) was administered i.p. for analgesia. Anti-sedan (150mg/kg) was administered subcutaneously. The animals were monitored on a heat pad (Harvard Apparatus) during recovery from anesthesia.

All injections were verified histologically. All tracing experiments were conducted in male C57BL/6J and vGlut1-Cre adult mice (8-12 weeks). Stereotactic coordinates were derived from Paxinos and Franklin *The Mouse Brain in Stereotaxic Coordinates* (2^{nd} edition) atlas and empirically adjusted. Empirically determined coordinates were on average shifted 0.4mm anterior relative to atlas.

For experiments examining excitatory downstream targets of vHPC neurons, 350 nL of Cre-recombinase-dependent AAV-DIO-mRuby-T2A-Synaptophysin-eGFP was infused into the vHPC in vGlut1-Cre mice at -3.0mm anteroposterior, +/-3.2mm mediolateral, and -3.5mm dorsoventral from dura relative to bregma.

For examining vHPC inputs to mPFC and LS, 300 nL of RV-mRuby2 and RV-eGFP virus were pressure-injected unilaterally into mPFC and LS, respectively.

To label collaterals the vHPC make in LS and mPFC, 350 nl of Cre-recombinase dependent virus (AAV-DIO-Synaptophysin-eGFP) was pressure-injected into the vHPC and 350 nL of EIAV-Cre into the LS or mPFC.

For optogenetic behavioral experiments, mice were bilaterally injected with 350 nL AAV-hsyn-ChR2-eYFP and AAV-hsyn-eYFP into vHPC of experimental and control

groups, respectively. For vHPC terminal stimulation in the LS and mPFC, mice received bilateral chronic dual optical fiber cannula (200 um, 0.22 NA; Doriclenses, Canada) implanted above the LS or mPFC at +0.85 mm anteroposterior, +/-0.4 mm mediolateral, and -2.4 mm dorsoventral from dura relative to bregma; or at +2.0 mm anteroposterior, +/-0.4 mm mediolateral, and -2.6 mm dorsoventral from dura relative to bregma, respectively. One layer of adhesive cement (C&B Metabond; Parkell) was used to secure implant to skull. Absorbable sutures (Oasis) and sterile tissue adhesive (Vetbond, 3M) were used to seal head incision. Four weeks were given for adequate viral expression in the terminals before behavioral testing commenced. Upon completion of behavioral experiments, viral injections and fiber placement were verified histologically.

Behavior

Behavioral Assays

Mice were housed individually and were given *ad libitum* access to food and water. They were maintained on a 12-hr (7:00 to 14:00) light-dark cycle in a room kept at 21°C. All tests were conducted during the dark cycle. Mice were habituated to handling and transportation from the colony room to the behavioral room for 5 days prior to behavioral tests. Mice were given 1 hour to habituate in the behavioral room before the start of experiment. Position and locomotion of freely moving mice were recorded by a web camera (Logitech C922 Pro Steam). The behavior apparatus was cleaned with 10% bleach followed by odor remover (Nature's Miracle) between animals. Animals in all behavioral experiments were monitored and analyzed with the BIOBSERVE video-tracking software.

Social Interaction Test (SIT)

To assess the display of social fear, social investigation was assessed using a 3-chamber social approach/avoid task called the 3-chamber social interaction test (SIT) (60.96 cm x 59.69 cm x 30.48 cm). The behavior apparatus consisted of 3 chambers designated for social interaction or aversion; the third chamber is a neutral zone. The social interaction and aversion chambers each contained a small empty tube that serve as non-social stimuli (15.24 cm x 14.73 cm; 7.62 cm radius). The non-social stimulus in the social interaction chamber gets replaced with a social stimulus which consists of an unknown male conspecific housed in an identical tube to the non-social stimulus. Freely moving pre-conditioned and conditioned mice were placed in the neutral chamber and have the choice to explore the other two chambers. In three 3-min epochs, the mice were exposed to: 1) non-social stimuli (without social stimulus), 2) a social stimulus, 3) non-social stimuli (without social stimulus). The time mice spent investigating the social stimulus (social interaction or SI), defined by the time spent in the chamber containing the social stimulus, was measured and analyzed using the BIOBSERVE software.

SFC paradigm

To investigate whether conditioned mice displayed social fear, we adapted and modified a social fear conditioning (SFC) paradigm (Toth et al, 2012). On day 1, freely moving mice were subjected to the SIT test as described previously to achieve a baseline social interaction score.

On days 2 and 3, mice were conditioned to associate shock-induced pain with the investigation of a social stimulus in a fear-conditioning chamber (45 x 22 x 40 cm; transparent Perspex box with a stainless steel grid floor). Mice were subjected to SFC for 35 minutes: 1) 5 minutes without social stimulus, 2) 10 minutes with social stimulus A and foot shock, 3) 5 minutes without social stimulus, 4) 10 minutes with social stimulus B and foot shock, 5) 5 minutes without social stimulus. An unknown male conspecific was used as a social stimulus to prevent mice from associating fear with a specific individual as well as to maintain high interest between the experimental mouse and social stimulus. Repeated exposure to the same conspecific has been shown to decrease social interest in both mice (Ferguson et al, 2002; Choleris et al, 2009). The social stimuli were counterbalanced to prevent mice from associating the shock-induced pain with one side of the chamber. Each time the mice investigated the social stimulus via direct contact, the mice were given a 1-s electric foot shock (0.3 mA, pulsed current). Mice received between 2-5 foot shocks. Mice were returned to their home cage with no further social contact.

1 day and after SFC, conditioned mice were subjected to the SIT. The time mice spent investigating non-social stimuli was considered a measure of non-social anxiety, whereas approach or aversion towards social stimuli was considered a measure of social interest and social fear, respectively. Social interaction time was calculated as the percentage of time spent in the social interaction chamber from the total time spent in the chamber. Successful social fear conditioning was indicated by a reduction in social investigation and increased aversion towards the social stimulus. Other

indications of successful social fear conditioning include freezing, tail rattling, and stretched approaches when a social stimulus is present.

Open Field Test (OFT)

To determine whether SFC affects locomotion and induces general, non-social anxiety, conditioned mice were assessed via the open field test. For non-optogenetic experiments, freely moving mice were allowed to explore the open field arena (44 x 44 x 44 cm) for 5 minutes. Position and locomotion were measured in one 5-min session. The time spent in the center during the 5-min period was calculated as the percentage from the total time spent on the OFT, normalized to pre-conditioning time. Increased time spent on the edges of the OFT during the 5-min testing period, calculated as the percentage from the total time spent on the OFT indicated general, non-social anxiety. The distance travelled on the OFT during the 5-min testing period indicated locomotor activity.

Elevated Plus Maze (EPM)

To verify the specificity of the induced social fear, conditioned mice were assessed on the elevated plus maze (EPM). Freely moving mice were placed in the center of a plus-shaped maze elevated 30.5 cm off the ground. Each arm (30 x 6.35 cm) protruded from the center of the maze. Two enclosed arms facing each other had walls (15.24 cm high) flanking the platform while the other two exposed had railings (2 cm high) flanking the platform. For non-optogenetic experiments, time spent in the enclosed and exposed arms, excluding the middle neutral area, were measured in one 5-min session. The time spent in the open arms during the 5-min period was calculated

as the percentage from the total time spent on the EPM, normalized to pre-conditioning time.

In-vivo optogenetics

For ChR2-mediated stimulation experiments, optical fibers (Doric) were connected to a 473 nm blue laser diode (OEM Laser Systems). Laser power was measured (Thorlabs) before each animal and measured to be 11-15 mW.

To investigate whether conditioned mice displayed social fear and whether this fear could be extinguished, freely moving conditioned mice were assessed in a 12-min SIT: 1) 3 minutes of light OFF without social stimulus, 2) 3 minutes of light OFF with social stimulus, 3) 3 minutes of light ON (473 nm phasic illumination: 20 Hz for 10 sec and 0 Hz for 20 sec) with social stimulus, 4) 3 minutes of light ON (473 nm tonic illumination: 5 Hz continuously) with social stimulus. Position and locomotion of freely moving mice were recorded with a web camera. Mice were returned to their home cage with no further social contact. The time mice spent in the social interaction chamber was measured to generate a social interaction index (SI Index): ($SI\ time_{ON} - SI\ time_{OFF}$) / ($SI\ time_{ON} + SI\ time_{OFF}$).

In-vivo pharmacology

To determine whether the effects of SFC on social fear could be reversed by medication prescribed to patients with SAD, we assessed the effects of fluoxetine (SSRI). Separate groups of mice were subjected to SFC or no SFC. Social investigation was assessed 1 day later with the SIT. On the same day, mice were injected i.p. with vehicle (saline; SAL) or with fluoxetine (20 mg/kg; FLX) daily for at

least two weeks. Every 7 days later, the time the mice spent socially interacting with an unknown conspecific in the SIT was measured until extinction was observed.

Histology

Mice were anaesthetized with isoflurane gas and perfused with 0.9% NaCl solution, followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The brain was fixed overnight in 4% PFA in PBS at 4°C. 60 µm coronal sections were sliced on a Leica VT1000 vibratome and mounted on Superfrost plus microscope slides with DAPI Fluoromount-G.

Imaging

Images were subjected to fluorescent microscopy using the Olympus VS120 slide scanner.

Results

An animal model of social anxiety

Since mice are intrinsically a social species, it is necessary to artificially pair novel social interactions with an aversive foot shock. Moreover, to evaluate the validity of this model of social fear / anxiety, it is necessary to measure individual mouse preference for social interaction. In brief, we place a social stimulus animal in a transparent tube on one side of the 3-chambered box and measure the amount of time an experimental mouse spends in each of the three chambers: the social interaction chamber, the neutral chamber, and the avoidance chamber (Figure 1B). Each test was

15 minutes long and consisted of three epochs: 1) 5 minutes without social stimulus, 2) 5 minutes with social stimulus, 3) 5 minutes without social stimulus.

Our preliminary data suggests that male mice will consistently interact with female mice despite being shocked dozens of times – likely reflecting some innate sexual drive, so our experiments were performed on male-male interactions (data not shown). During epochs with no social stimuli, mice did not significantly prefer to spend time in one chamber or the other. Surprisingly, during epochs with the social stimulus, while mice showed a modest preference for the chamber with the social stimulus mouse, the average time spent between the two chambers did not drastically differ.

The following two days, we randomized mice into two groups such that the baseline SI index between groups remained balanced: experimental group (n = 7) and control group (n = 5). The experimental group was conditioned, whereas the control group was exposed to the same social stimulus and context but not given a shock (Figure 1A). On average, mice received less than half a dozen shocks within 20 minutes. During this process, mice explored more in the absence of a stimulus mouse, and only rarely approached the conspecific mouse (Figure 1C). Interestingly, after a few bouts of shocks, mice exhibited classical fear responses only in the presence of a conspecific mouse: freezing, tail rattle, and aversion.

Subsequent social interaction tests revealed that despite not being shocked anymore, conditioned mice avoided the chamber that contained the conspecific mouse – despite randomly switching the conspecific chamber. Similar to conditioning, mice exhibited increased freezing response and tail rattling only in the presence of the conspecific mouse.

An important potential confound to our experiments would be that the mice become injured as a result of the fear conditioning paradigm. We assume that significant physical injuries would result in substantial impairments in gross locomotion or paw use. Therefore, we placed animals in a large open-field arena and tracked the distance each animal moved in five minutes (data not shown). Moreover, since previous studies have demonstrated that mice prefer spending their time in the edges and corners of an open field arena, we classified the mouse position at a given time as "edge" or "center" (Figure 1F). Then, taking the ratio between the time the mouse spends in the center before conditioning and the time spent in the center after conditioning, we determine that social fear conditioning does not substantially alter the mouse's preference for edges – a proxy for general anxiety (Figure 1H).

Interestingly, since this protocol seems to induce a fear / anxiety response specific to social contexts and not a form of general social fear / anxiety, we assessed the animals on the well-established elevated plus maze paradigm (EPM) (Figure G). In brief, the mice are placed in an elevated plus-shaped apparatus with 2 of the 4 arms shielded by walls. Mice naturally prefer to spend most of their time in the enclosed arms, only rarely exploring on un-enclosed (open) arms. Similar to the social interaction assay, individual differences predominate an individual mouse's preference for the un-enclosed arm. Therefore, it is necessary to measure each mouse prior to the social fear conditioning, and compare its preference (measured by time spent) on the open arms before and after fear conditioning. Unlike regular fear conditioning paradigms, our social fear conditioning protocol does not seem to induce significant changes in the preference for exposed arms between experimental and control mice (Figure 1I). Taken together,

this suggests that our animal model of social anxiety disorder has both face and construct validity.

An animal model of cognitive behavioral therapy

One particularly effective therapy for treating patients with social anxiety disorder focuses on identifying and changing the cognitive factors that maintain the anxious association (Gould et al, 2007, Federof and Taylor, 2001; Hoffman et al, 2007). This "cognitive behavioral therapy" (CBT) is loosely based on principles of classical conditioning in which a learned (conditioned) stimulus-outcome association can become unlearned or forgotten. In other words, through gradual and repeated re-exposures or simulations of stressful stimulus in a neutral environment, patients will become less fearful over time. In the case of social anxiety disorder, this may involve exposing patients to social situations or public speaking. We believe that this process can be recapitulated in our animal model in order to demonstrate the predictive validity of our model.

Our exposure/extinction paradigm is similar to the repeated exposure therapy during CBT where patients are repeatedly exposed to social situations. In brief, after fear conditioning, we re-expose animals to social stimulus without shocking the animals. Our study shows that extinction occurs as a function of the number of exposures rather than time since the social fear phenotype persists over the course of a month without social stimulus, whereas immediate re-exposure can quickly lead to fear extinction (Figure 1E). Interestingly, the rate of extinction seems to follow a non-linear pattern with animals remaining completely fearful for 6 bouts, and rapidly interacting more over the

course of 2 additional bouts (data not shown). This susceptibility to extinction over the course of several bouts demonstrates the predictive validity of our model.

Treating our model of social anxiety disorder with Selective Serotonin Reuptake Inhibitors

Despite the successes of CBT, a high percentage of social anxiety patients fail to respond to this treatment option. Instead, combinations of CBT with pharmacological drugs seem to induce better response rates (Liebowitz et al, 1992; Baldwin et al, 1999; Van Amerigen et al, 2001). One especially effective class of drugs are selective serotonin reuptake inhibitors (SSRI). The serotonin molecule modulates neuronal activity by binding to serotonin receptors: G-Protein coupled receptors on post-synaptic neurons. Serotonin is rapidly removed from the synapse through selective serotonin reuptake transporters on the presynaptic terminals to be recycled into vesicles. Therefore, SSRI blocks serotonin reuptake and increases serotonin signaling at the synapse. Paradoxically, acute administration of SSRI induces anxiety-like phenotypes, whereas chronic administration of SSRI reduces anxiety phenotypes. This phenomenon remains largely unsolved, but highlights the importance of molecular and cellular plasticity due to chronic serotoninergic signaling or the importance of precise serotonergic modulation.

A previous study has demonstrated that the very potent SSRI, paroxetine, is capable of increasing social fear extinction (Toth et al, 2012). Clinically, paroxetine is used to treat social anxiety, however, the potency of paroxetine generates powerful unwanted side effects as well as a prominent discontinuation effect. Therefore, we sought to test whether a weaker SSRI, fluoxetine, can treat social anxiety by reversing

the social avoidance phenotype. Unlike paroxetine, fluoxetine is used to treat chronic depression and social anxiety as an off-label indication.

Therefore, to determine whether chronic fluoxetine administration can reverse the social avoidance phenotype, we split mice into four groups using 2 factors and 2 levels in our experimental design. Since acute doses of SSRI can induce anxiety, it is necessary to measure the effects of SSRI administration on non-conditioned mice. Moreover, since delivering SSRI through an intraperitoneal injection, which requires animal handling, can stress the animals, we sought to also inject saline into un-conditioned animals. Thus, we have four groups: 1) conditioned mice with fluoxetine, 2) conditioned mice with saline, 3) unconditioned mice with fluoxetine, 4) unconditioned mice with saline. After inducing social fear conditioning, we administered fluoxetine or saline daily in the afternoon and evaluated mice using SIT weekly after conditioning (Figure 2A). As expected, we observed no changes in SI index of unconditioned mice regardless of whether they received saline or fluoxetine which demonstrates that fluoxetine does not induce any observable changes in social preference/avoidance (Figure 2B). Unexpectedly, both conditioned groups that received saline or fluoxetine had similar extinction rates which suggests that fluoxetine may not be sufficient to mediate social fear extinction (Figure 2B). Furthermore, evaluations on the EPM or OFT indicated no change between treatment or control groups which suggests that chronic fluoxetine does not induce any unwanted anxiolytic or anxiogenic side effects (Figure 2C and 2D).

While pharmacological therapies can potentially target specific receptors, this approach lacks the necessary spatiotemporal resolution necessary to precisely

modulate neuronal signaling. Since serotonin receptors are abundantly expressed throughout the brain, flooding the brain with SSRI can induce both desired and undesired effects. Furthermore, since different SSRIs such as paroxetine and fluoxetine have decay constants that differ by orders of magnitude, the effects of SSRI is confounded by the ambiguous time window of action. Therefore, we sought to use brain stimulation since it has a high degree of spatiotemporal precision.

Targeted brain stimulation of the ventral hippocampus

Brain stimulation can activate or inhibit neuronal activity with high spatiotemporal resolution. However, a major drawback to this approach lies in the lack of cellular specificity. Indeed, findings from our lab and others have demonstrated that neurons in the same brain region may mediate completely different behavioral responses. Therefore, indiscriminately activating every neuron in a given region may give rise to unwanted side effects. To overcome this problem, we leveraged advances in viral-targeted optogenetic stimulation by expressing the light activated cation channel, Channelrhodopsin2 (ChR2) in distinct neuronal populations based on their anatomical connectivity. Thus, we can activate specific subsets of neurons using 470nm light and very precisely stimulate specific brain areas to determine whether brain stimulation can also serve to treat our model of social anxiety disorder.

The ventral hippocampus has been implicated in social behaviors; in particular, the CA2 seems to play a role in storing social memories and this information is passed down to downstream brain areas to modulate behaviors (Hitti et al, 2014). A recent study shows that the ventral hippocampus sends axonal projections to the nucleus accumbens, olfactory bulb, and basolateral amygdala. Our viral tracing experiments

also show that vGLUT1 neurons of the ventral hippocampus makes synaptic connections with the medial prefrontal cortex and the lateral septum – two regions that have been implicated in anxiety and social behaviors (Figure 3B – 3E). In brief, we injected an adeno-associated virus (AAV) that expresses synaptophysin-fused GFP along with mRuby2.0 (also known as SynapTag) into the ventral hippocampus of vGLUT1-Cre transgenic mice (Figure 3A). These mice express Cre-recombinase specifically in neurons that express vGLUT1. Neurons that are infected with AAV and express Cre-recombinase expresses SynapTag. The mRuby fills the neuronal cell bodies which can be used to visualize the soma as well as axons; synaptophysin preferentially targets to the presynaptic terminals and therefore can be used as a marker of a synaptic connection. Given the roles of the lateral septum and the medial prefrontal cortex, we hypothesized that stimulating the ventral hippocampus projections to the LS and mPFC can impair the social avoidance phenotype in our model.

To investigate the potential of ventral hippocampal inputs to the lateral septum or the medial prefrontal cortex to treat our model of social anxiety disorder, we expressed ChR2 and GFP in the ventral hippocampus using AAV under the control of hSyn1 promotor (Figure 4B). Control groups were transfected with GFP instead of ChR2. Since we hypothesized that stimulating ventral hippocampal projections to different targets can differentially modulate social fear, we implanted optical fibers in the output structures: either the lateral septum or the medial prefrontal cortex. In total, we had four groups that we exposed to social fear conditioning:

Table 1. Summary of experimental (ChR2) and control (eGFP) groups with chronic bilateral optical fiber implants in LS or mPFC.

vHPC virus	Optical fibers in LS	Optical fibers mPFC
eGFP	Group 1 (n = 6)	Group 2 (n = 2)
ChR2	Group 3 (n = 8)	Group 4 (n = 4)

By delivering 473nm light through the optical fibers, we could selectively activate ventral hippocampal inputs to either the lateral septum or the medial prefrontal cortex. Furthermore, several studies have demonstrated that the effects of optogenetic stimulation on animal behavior may intensely correlate with the stimulation protocol, thus, we tested the effects of two paradigms: phasic and tonic stimulation (see methods). We modified our social interaction test to accommodate for testing multiple stimulation protocols. In brief, we tested mice in the 3-chambered box for 12 minutes: 1) 3 minutes of light OFF without social stimulus, 2) 3 minutes of light OFF with social stimulus, 3) 3 minutes of light ON (phasic) with social stimulus, 4) 3 minutes of light ON (tonic) with social stimulus (Figure 4A). Additionally, we modified our social interaction index to accommodate for comparisons between light epochs.

Table 2. Summary of epochs of different optogenetic stimulation parameters and social conditions in the SIT.

	Epoch 1	Epoch 2	Epoch 3	Epoch 4
Light	OFF	OFF	ON (phasic)	ON (tonic)
Social stimulus	NONE	WITH	WITH	WITH

To account for the different light conditions, we generated a Social Interaction (SI) index to evaluate the changes in social interaction time. The SI index is calculated as ($SI\ time_{ON} - SI\ time_{OFF}$) / ($SI\ time_{ON} + SI\ time_{OFF}$). A SI index of 0 indicates no preference for either chamber; a SI index of 1 indicates a preference for social

interactions; a SI index of -1 indicates an avoidance of social interactions. Therefore, successful social fear conditioning should generate a SI index of -1.

Prior to social fear conditioning, we found that stimulating either population of neurons (lateral septum-projecting, or prefrontal cortex-projecting) had no effect on the amount of time spent in the social interaction chamber (Figure 4C). On the other hand, after social fear conditioning, we found that activating ventral hippocampal terminals in the lateral septum, but not the medial prefrontal cortex, significantly increased the amount of time spent in the social interaction chamber (Figure 4D). Interestingly, we only observed the effect of light stimulation under the phasic stimulation protocol, not the tonic stimulation protocol. Moreover, since the tonic protocol follows the phasic protocol, the effect we see is also not due to an extinction response caused by extended exposures to social stimuli. Taken together, we demonstrate that stimulating the ventral hippocampus neurons that project to the lateral septum, but not the medial prefrontal cortex, may serve as a potential therapy for social anxiety disorder.

A potential circuit explanation for specific stimulation effects

Since activating ventral hippocampal projections to the lateral septum, but not to the medial prefrontal cortex, can rescue the social avoidance phenotype, we wondered why activating terminals from the same area can yield drastically different results. One possibility is that the ventral hippocampus contains two separate populations of neurons: one that projects to the medial prefrontal cortex and the other to the lateral septum; thus, activating one population is independent of the other. The other possibility is that the effects of stimulation do not actually depend on the presynaptic neuron (ventral hippocampus neurons), but instead on the post-synaptic partner. Indeed, recent

evidence has demonstrated that stimulating the medial prefrontal cortex or the lateral septum directly can decrease fear response or increases social interactions. Therefore, to dissect out these two possibilities, we decided to use retrograde viral tracing to label the neurons that project to the lateral septum and/or the medial prefrontal cortex.

We injected modified rabies virus that express mRuby2 or eGFP into the lateral septum and medial prefrontal cortex, respectively (Figure 5A). By sectioning the mouse brains and imaging the ventral hippocampus, we were able to identify eGFP-expressing neurons, mRuby-expressing neurons, and neurons that express both eGFP and mRuby2 (Figure 5B – 5E). This suggests that potentially 3 populations of neurons exist: one that projects to either the lateral septum and the medial prefrontal cortex, and one that projects to both lateral septum and the medial prefrontal cortex. However, only a small fraction of these neurons co-expressed mRuby and eGFP.

To discount the effects of rabies viral tropism, we sought a complementary approach to determine whether ventral hippocampal neurons form axonal collaterals to the medial prefrontal cortex and the lateral septum. Therefore, we injected Cre-recombinase dependent AAV under the control of hSyn1 promoter expressing eGFP at the synaptic terminals (AAV-hSyn1-DIO-Synaptophysin-eGFP) into the ventral hippocampus and a corresponding modified retrograde equine infectious anemia virus (RG-EIAV) expressing Cre-recombinase into each of the target areas (Figure 5F). As expected, when we injected RG-EIAV into the lateral septum, we found green puncta indicative of synaptic terminals in the lateral septum, whereas RG-EIAV injections into the medial prefrontal cortex yielded green puncta in the medial prefrontal cortex. Importantly, in each of the two conditions, we found green puncta in the other brain regions (Figure 5G,

5H). For example, injecting RG-EIAV into the medial prefrontal cortex, we found green puncta in the lateral septum as well. Taken together, this suggests that ventral hippocampal neurons form axonal collaterals to the lateral septum and the medial prefrontal cortex.

Figures

Figure 1. The effects of social fear conditioning. (**A**) Experimental schematic of social fear conditioning (SFC). Social stimuli were counterbalanced. Conditioned mice received a foot shock when socially interacting with the social stimulus. Unconditioned animals were exposed to the same social stimulus as conditioned mice but did not receive a foot shock. (**B**) Experimental schematic of 3-chamber social interaction test (SIT). (**C**) Heat map of a conditioned mouse in SIT exposed to non-social stimuli and social stimuli. (**D**) Experimental timeline for conditioned and unconditioned mice. (**E**) Extinction curve of social fear illustrating the change in social interaction time of conditioned mice against the number of exposures to social stimuli. (**F**) Representative animal track on OFT after SFC. (**G**) Representative animal track on EPM after SFC. (**H**) Comparison of the ratio of the time spent in the center of the OFT after SFC normalized to the time spent in the center before SFC of conditioned and unconditioned mice. (**I**) Comparison of the ratio of the time spent in the open arms of the EPM after SFC normalized to the time spent in the open arms before SFC of conditioned and unconditioned animals.

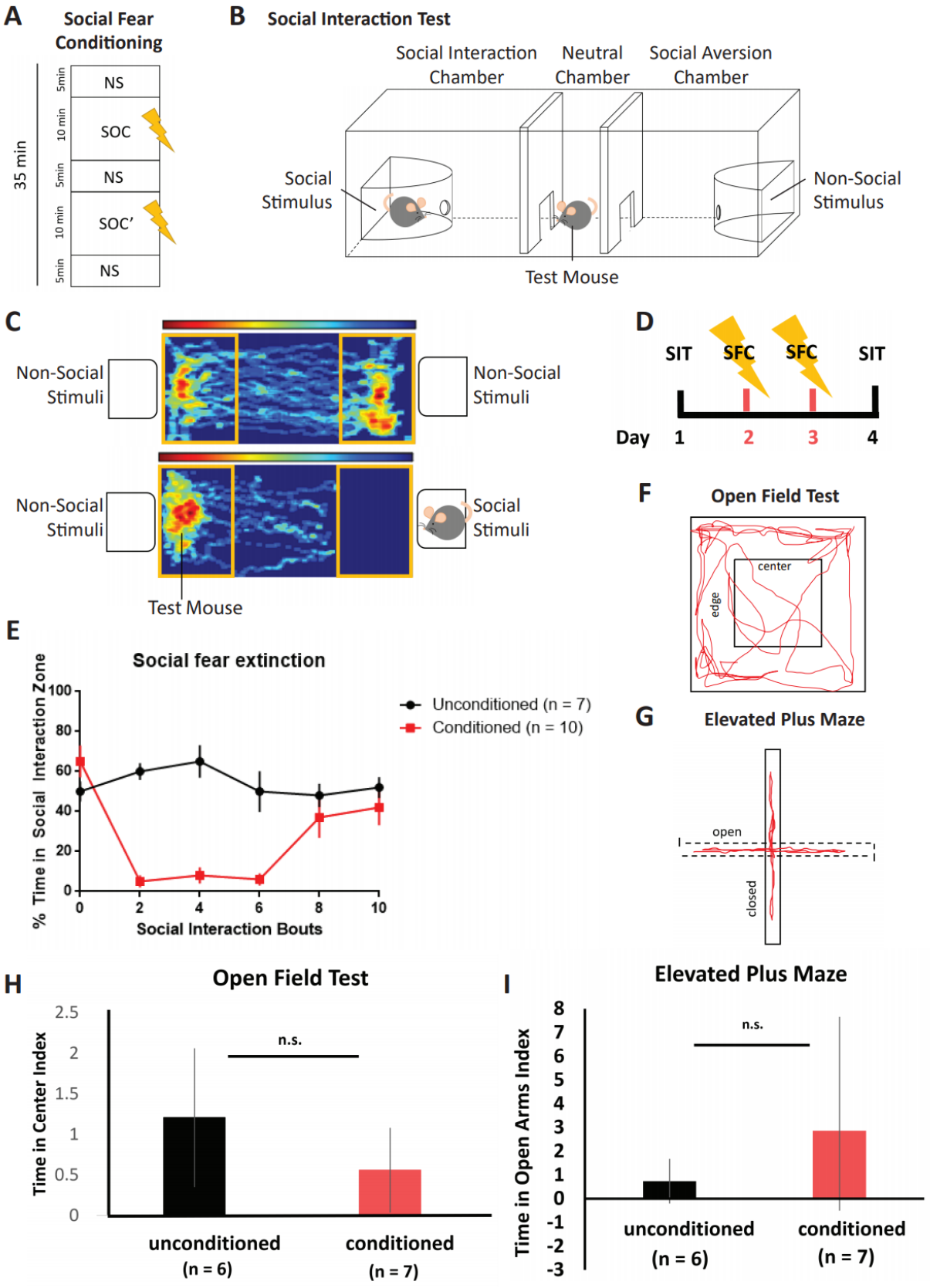
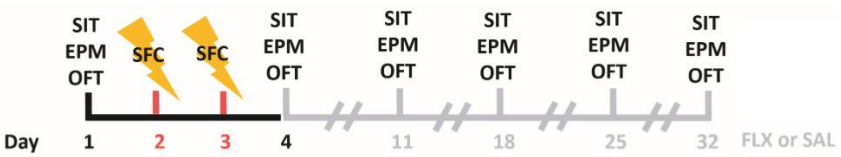
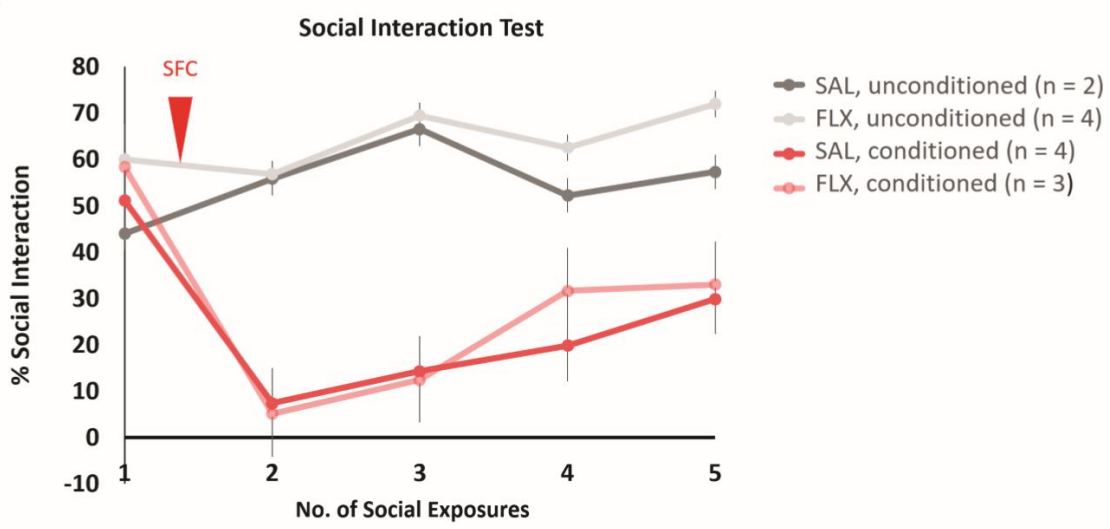

A
Social Fear Conditioning
35 min
5min
NS
10 min
SOC
5min
NS
10 min
SOC'
5min
NS
B
Social Interaction Test
Social Interaction Chamber
Neutral Chamber
Social Aversion Chamber
Social Stimulus
Non-Social Stimulus
Test Mouse
C
Non-Social Stimuli
Non-Social Stimuli
Non-Social Stimuli
Social Stimuli
Test Mouse
D
SIT
SFC
SFC
SIT
Day 1 2 3 4
E
Social fear extinction
% Time in Social Interaction Zone
Social Interaction Bouts
Unconditioned (n = 7)
Conditioned (n = 10)
F
Open Field Test
center
edge
G
Elevated Plus Maze
open
closed
H
Open Field Test
Time in Center Index
n.s.
unconditioned (n = 6)
conditioned (n = 7)
I
Elevated Plus Maze
Time in Open Arms Index
n.s.
unconditioned (n = 6)
conditioned (n = 7)

Figure 2. Acute and chronic administration of fluoxetine does not rescue social fear. (**A**) Experimental timeline for unconditioned and conditioned mice receiving acute and chronic saline or fluoxetine i.p. (**B**) Comparison of changes in social interaction time between unconditioned mice with vehicle (saline) (n=2), unconditioned mice with fluoxetine (n=4), conditioned mice with vehicle (n=4), and conditioned mice with fluoxetine (n=3). (**C**) Comparison of the ratio of the time spent in the center of the OFT after SFC normalized to the time spent in the center before SFC of conditioned and unconditioned mice. (**D**) Comparison of the ratio of the time spent in the open arms of the EPM after SFC normalized to the time spent in the open arms before SFC of conditioned and unconditioned animals.

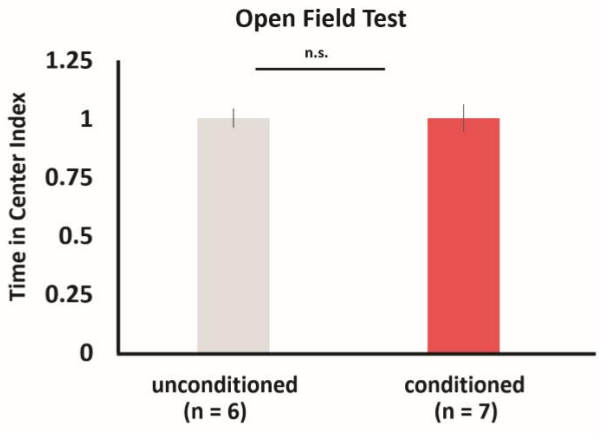
A


SIT
EPM
OFT
SFC
SFC
SIT
EPM
OFT
SIT
EPM
OFT
SIT
EPM
OFT
SIT
EPM
OFT
SIT
EPM
OFT
Day
1
2
3
4
11
18
25
32
FLX or SAL

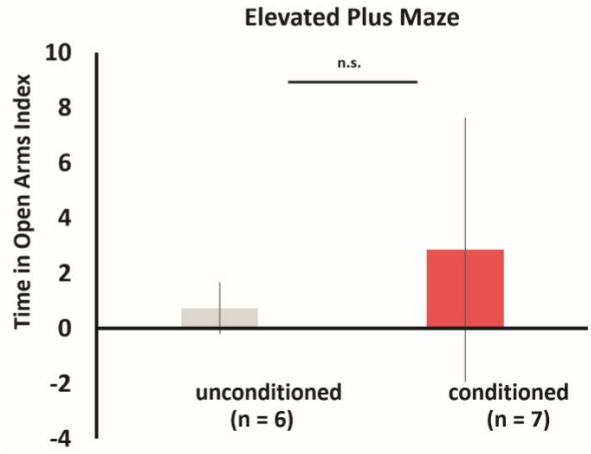
B


Social Interaction Test
SFC
% Social Interaction
80
70
60
50
40
30
20
10
0
-10
1
2
3
4
5
No. of Social Exposures
SAL, unconditioned (n = 2)
FLX, unconditioned (n = 4)
SAL, conditioned (n = 4)
FLX, conditioned (n = 3)

C


Open Field Test
n.s.
Time in Center Index
1.25
1
0.75
0.5
0.25
0
unconditioned
(n = 6)
conditioned
(n = 7)

D


Elevated Plus Maze
n.s.
Time in Open Arms Index
10
8
6
4
2
0
-2
-4
unconditioned
(n = 6)
conditioned
(n = 7)

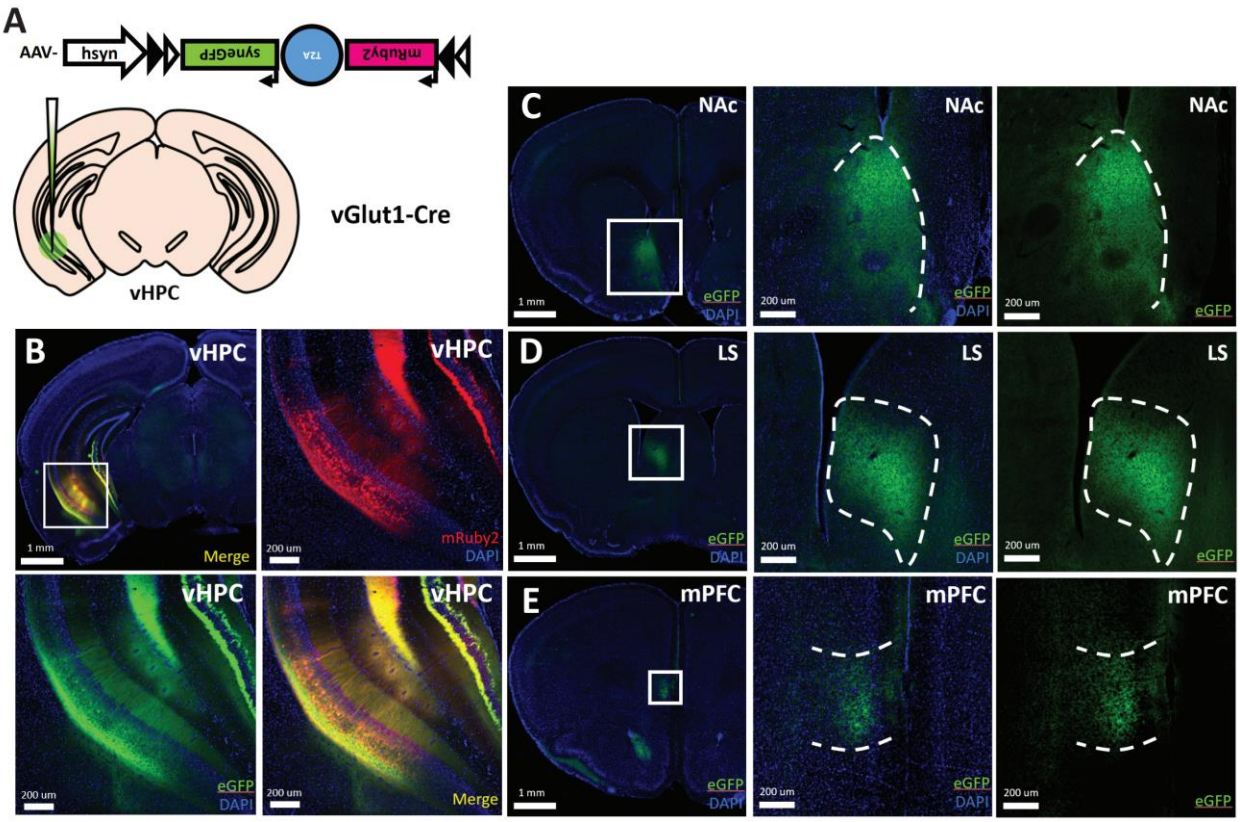
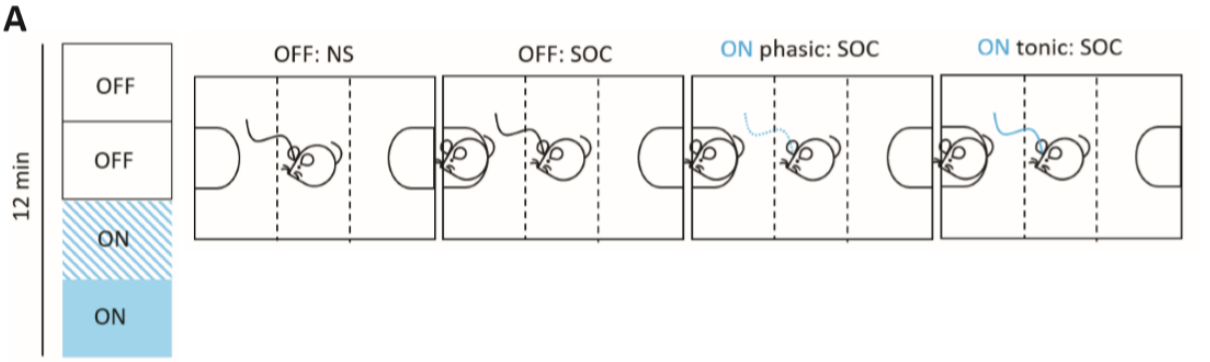
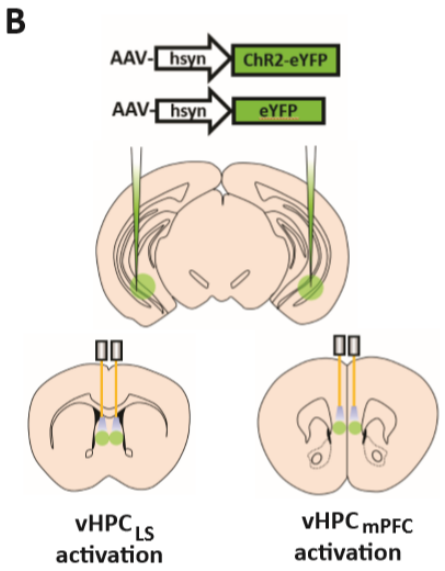
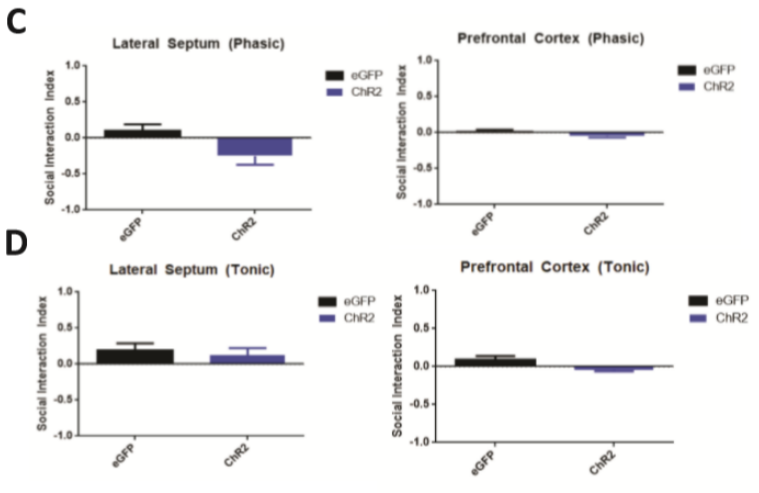


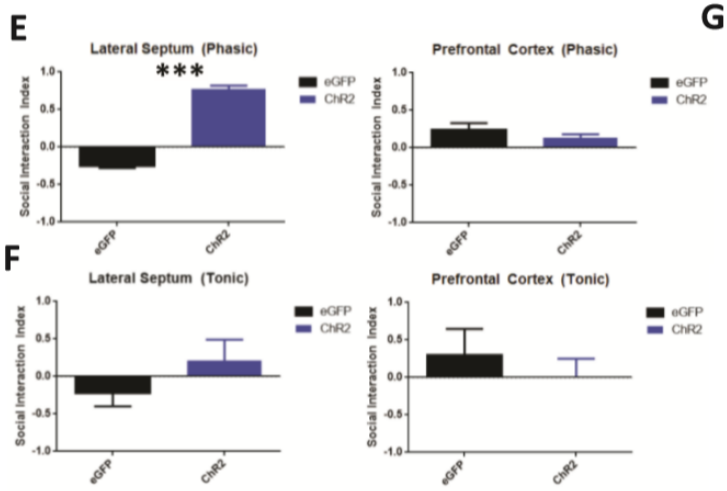
Figure 3. Anatomical characterization of vHPC. (**A**) Experimental schematic of Cre-mediated transgene expression in the vHPC of vGlut1-Cre mice. RV-eGFP (green) is injected into LS and RV-mRuby2 (red) is injected into mPFC. (**B**) Coronal vHPC sections of vGlut1-Cre mouse injected with AAV-hsyn-DIO-mRuby2-T2A-synpGFP in vHPC. Cell bodies are labeled with mRuby2 (red); synaptic terminals are labeled with eGFP (green). Boxed area in (B) is magnified. (**C** to **E**) Coronal sections of eGFP-labelled vHPC synapses in NAc (mSh) (C), LS (D), mPFC (E). Boxed areas in (C), (D), and (E) are magnified.

Figure 4. Phasic optogenetic activation of LS-projecting vHPC neurons rescues social fear. (**A**) Experimental schematic of SIT over the course of 12 minutes: 1) 3 minutes of light OFF without social stimulus, 2) 3 minutes of light OFF with social stimulus, 3) 3 minutes of light ON (phasic 470 nm illumination: 20 hz for 10 seconds and 0 hz for 20 seconds) with social stimulus, 4) 3 minutes of light ON (tonic 470 nm illumination: 5 hz continuously) with social stimulus. (**B**) Experimental schematic of wild-type mouse indicating the site of viral injections (vHPC) and chronic implants (LS and mPFC). vHPC neurons were transduced with ChR2 or eGFP. (**C**) Comparing social interaction indexes between mice with eGFP or ChR2 subjected to phasic and tonic stimulation of $vHPC_{LS}$ or $vHPC_{mPFC}$ before SFC. Significance for multiple comparisons: paired t-test, $*P < 0.05$; $**P < 0.01$; ***P < 0.008, n.s., not significant. (**D**) Comparing social interaction indexes between mice with eGFP or ChR2 subjected to phasic and tonic stimulation of $vHPC_{LS}$ or $vHPC_{mPFC}$ after SFC. Significance for multiple comparisons: paired t-test, $*P < 0.05$; $**P < 0.01$; ***P < 0.008, n.s., not significant. (**E**)Comparing the percent of time spent in the social interaction zone 24 hours after social fear has been reversed by optogenetic stimulation. Significance for multiple comparisons: paired t-test, $*P < 0.05$; $**P < 0.01$; ***P < 0.008, n.s., not significant.


A
12 min
OFF
OFF
ON
ON
OFF: NS
OFF: SOC
ON phasic: SOC
ON tonic: SOC


B
AAV- hsyn ChR2-eYFP
AAV- hsyn eYFP
vHPC LS activation
vHPC mPFC activation


C
Lateral Septum (Phasic)
Prefrontal Cortex (Phasic)
D
Lateral Septum (Tonic)
Prefrontal Cortex (Tonic)
Social Interaction Index
eGFP
ChR2


E
Lateral Septum (Phasic)
***
Prefrontal Cortex (Phasic)
F
Lateral Septum (Tonic)
Prefrontal Cortex (Tonic)
Social Interaction Index
eGFP
ChR2

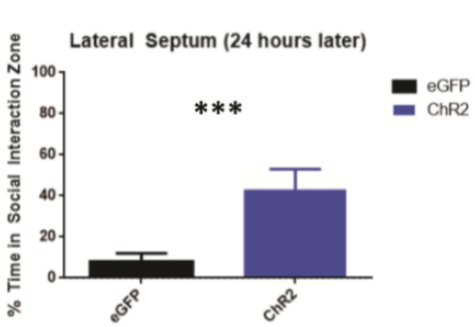
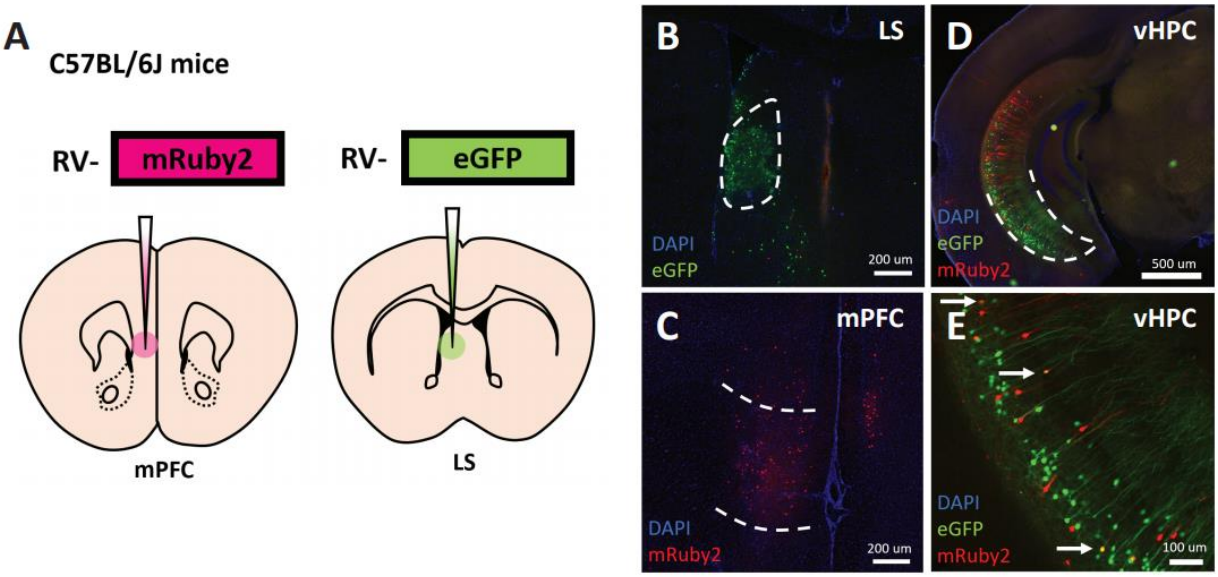
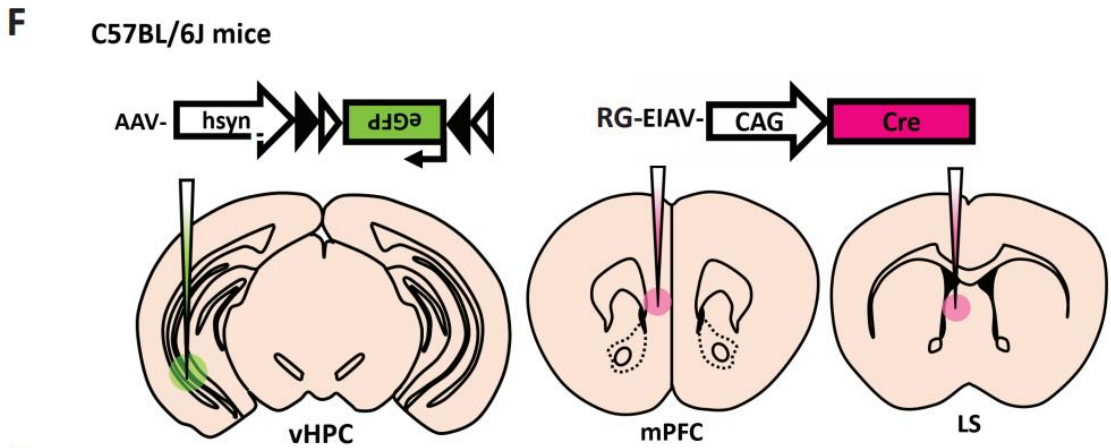
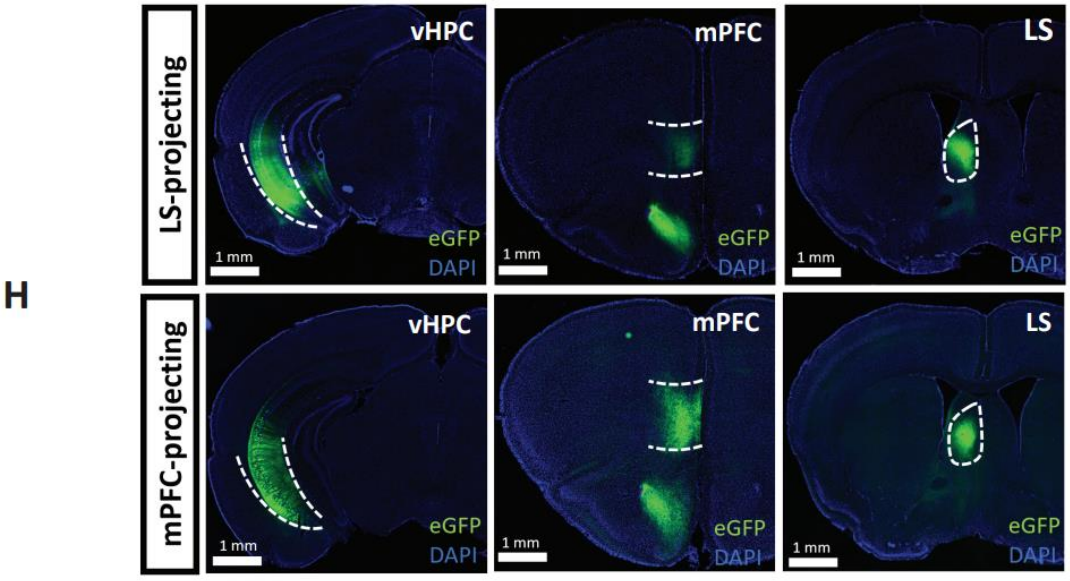

G
Lateral Septum (24 hours later)
***
% Time in Social Interaction Zone
eGFP
ChR2

Figure 5. vHPC makes collateralizing inputs to LS and mPFC. (**A**) Experimental schematic of retrograde rabies viral labelling of vHPC inputs to LS and mPFC in wild-type mice. (**B**) Coronal LS section of injection site with rabies virus expressing eGFP. (**C**) Coronal mPFC section of injection site with rabies virus expressing mRuby2. (**D**) Coronal section of vHPC cell bodies retrogradely-labelled with eGFP (green), mRuby2 (red), and both eGFP and mRuby2 (yellow). (**E**) Magnified coronal section of vHPC cell bodies in (D) retrogradely-labeled with eGFP (green) and mRuby2 (red). White arrows indicate retrogradely-labelled vHPC cell bodies co-expressing eGFP and mRuby(yellow). (**F**) Schematic of Cre-mediated transgene expression using Cre-dependent virus AAV-hysn-DIO-eGFP injected into the vHPC and retrograde (RG) EIAV-Cre injected into the LS or mPFC in wild-type mice. (**G**) eGFP-labeled vHPC neurons in LS and mPFC, LS-projecting vHPC neurons and LS-projecting neurons forming collaterals in mPFC are both labeled with eGFP (green). (**H**) eGFP-labeled vHPC neurons in LS and mPFC, mPFC-projecting vHPC neurons and mPFC-projecting neurons forming collaterals in LS are both labeled with eGFP (green).


A
C57BL/6J mice
RV-
mRuby2
RV-
eGFP
mPFC
LS
B
LS
DAPI
eGFP
200 um
D
vHPC
DAPI
eGFP
mRuby2
500 um
C
mPFC
DAPI
mRuby2
200 um
E
vHPC
DAPI
eGFP
mRuby2
100 um


F
C57BL/6J mice
AAV-
hsyn
eGFP
RG-EIAV-
CAG
Cre
vHPC
mPFC
LS


G
H
LS-projecting
mPFC-projecting
vHPC
mPFC
LS
eGFP
DAPI
1 mm

Discussion

On Social Anxiety Disorders

Like so many neuropsychiatric diseases, social anxiety disorder is the result of maladaptive gene-environment interactions. While many socially anxious patients attribute their dysfunction due to significantly negative social experiences, some patients do not report such experiences. On the other hand, while genome wide association studies have identified several genetic similarities in patients with social anxiety disorder, these features are unable to predict whether an individually is or will become socially anxious. The lack of a precise mechanism is likely due to the fact that many different non-linear combinations of factors can cause social anxiety; however, this knowledge deficit complicates preclinical work trying to establish an appropriate model of social anxiety disorder in mice.

To simplify the problem of generating a mouse model of social anxiety disorder, we decided to rely on using C57BL6 mice – a strain with a homogenous genetic background - to focus on the environmental components. Since patients attribute negative social interactions to their disorder, we sought to induce negative bouts of social interaction between mice. The social defeat stress is one model that uses aggressive CD1 mice to attack C57BL6 mice; however, this model also generates other phenotypes that resemble major depression rather than social anxiety disorder. Therefore, we sought to induce negative social interactions by pairing bouts of social interaction with foot shock.

This “social fear conditioning” paradigm is loosely inspired by previous attempts in the literature to generate a model of social anxiety disorder. We were able to independently verify that this model can induce social aversion in mice and that these symptoms seem specific to the social context and not generalized fear / anxiety since mice only displayed fear responses in the presence of a conspecific social stimuli, and did not exhibit fear / anxiety responses on well-established paradigms that measure anxiety: elevated plus maze and open field test. One potential caveat of this approach is the lack of human-relatability. In other words, humans don’t get foot shocked during social interactions. The artificial nature of this protocol can potentially resemble more like other fear conditioning paradigms such as cue- and context- fear conditioning. However, since cue and context-based fear conditioning are mediated through completely different neural circuitry, it is possible that our social fear conditioning is also facilitated by a novel neural circuit. In support of this idea, recent studies have suggested that social memories are predominantly stored in the ventral hippocampus which may differ from context- and cue-based fear conditioning.

Nevertheless, our social fear conditioning shares similar features to cue- and context based conditioning. In particular, all three forms of conditioning can be resolved through re-exposing the fearful stimuli in a neutral context. This fear extinction seems evolutionarily conserved since cognitive behavioral therapy is effective at treating fear-related psychiatric disorders such as post-traumatic stress disorder or even social anxiety disorder. Indeed, the fear-avoidance response, under the right context, does have significant evolutionary advantages which suggests that these types of mental health disorders reflect an intrinsic inability to adapt after a negative experience. Taken

together, this supports the idea that social anxiety disorder is likely mediated by maladaptive gene-environment interactions.

A putative regulation of social anxiety through limbic circuitry

We propose two possible models for generating social anxiety through negative social interactions: 1) powerful negative social memories elicit a stress response, 2) powerful negative social memories generalizes broadly and struggles to adapt in the face of non-threatening social stimuli. It is important to note that these models may not be mutually exclusive. A central node of this premise is the notion of a “social memory” or a neural representation of individual bouts of social interaction or social partners. Susumu Tonegawa’s group has recently shown that social memories seem to be stored in the ventral, not dorsal, hippocampus. And in line with our models, the information regarding these social memories must be passed on to downstream areas. From basic anatomical tracing experiments, we identified the lateral septum and the medial prefrontal cortex as receiving axonal projections from the ventral hippocampus. The medial prefrontal cortex is believed to be involved in top-down control of anxiety and fear responses. Our group has recently shown that the lateral septum projects to the hypothalamus – the main output of the limbic system and modulator of neuroendocrine responses such as stress.

While we do not have critical evidence to support either models of generating social anxiety disorder, our experiments do demonstrate that artificial stimulation of ventral hippocampus neurons projecting to the lateral septum, but not the prefrontal cortex can reduce social avoidance. This suggests that perhaps social anxiety is not a problem of fear generalization, but instead, a problem of eliciting a stress response in

the absence of an immediate stressor (foot shock), but instead in the face of a perceived stressor (conspecific social stimulus). In support of this evidence, our anatomical tracing experiments show that ventral hippocampal neurons that project to the lateral septum may also form collaterals to the prefrontal cortex. This finding suggests that ventral hippocampal changes due to stimulation is irrelevant to reversing social avoidance, but instead, relies more on the post-synaptic target. However, this notion assumes that every neuron forms collaterals, but instead, ventral hippocampal neurons may exist in three populations: ones that project to lateral septum, ones that project to prefrontal cortex, and a third population that projects to both areas.

Distinct neuronal populations

One approach to specifically label prefrontal cortex-projecting or lateral septum-projecting neurons is to take advantage of combinatorial retrograde viral tracing using Boolean logic. For example, in our experiments, we rely on Cre-recombinase acting on double-inverted-open reading frame (DIO) sequences to selectively express our transgene in specific populations. However, we could also use another popular recombinase, Flp, which acts on fDIO sites and not DIO sites. Thus, we can exclusively express ChR2 in neurons that project to both the lateral septum and prefrontal cortex by injecting RG-EIAV-Cre and RG-EIAV-Flp into the lateral septum and prefrontal cortex, respectively. And by expressing ChR2 in a Cre and Flp manner, we can selectively label neurons that project to both areas. Moreover, by injecting a virus that expresses ChR2 in a Cre and not Flp-dependent manner, we can selectively label just the population that projects to one area but not the other.

The ground-truth method to determine whether three populations of ventral hippocampal neurons actually exist is to label and trace every single neuron to determine whether any do not form collaterals. However, at the moment, this approach is very time consuming and may be improved using automated neuron tracing technologies in intact brain preparations such as using the CLARITY technique. At best, this knowledge would not improve our quest to identify how social anxiety is generated or how stimulation can reduce social avoidance, but instead, only satisfy my curiosity.

Serotonin and beyond

The function of serotonin is hotly contested in contemporary neuroscience. On the cellular level, the effects of this molecule seem heavily dependent on which of the seven receptor subtypes is expressed. On the circuit level, the effects of this molecule is dependent on where in the brain it binds to since different cell types and brain areas respond differently to serotonin. Lastly, on the behavioral level, the effects of serotonin seem to depend on how much serotonin there is in the system. Importantly, most of these studies rely on using selective serotonin reuptake inhibitors rather than direct delivery of serotonin. For example, evidence from humans and rodents show that acute doses of serotonin (through SSRI) can induce anxiogenic effects whereas chronic doses of SSRI induces anxiolytic effects. These effects may reflect neuronal plasticity as a result of serotonergic modulation rather than directly of serotonin itself.

Interestingly, serotonin signaling seems linked with social anxiety. For example, genome wide association studies have implicated many genes involved in serotonergic signaling, whereas SSRI is one of the main methods of treating social anxiety disorder. Moreover, modifying serotonin receptor expression directly effects social behavior. In

our animal model, we found that fluoxetine does not substantially reduce social avoidance than saline controls; however, this experiment may simply be confounded by the fact that we regularly assessed social interaction (thereby inducing social fear extinction) that interferes with studies of the effects of the drug alone. Another explanation is that fluoxetine simply does not affect social anxiety disorder since, despite being used as an off-label treatment for social anxiety, it still has yet to pass clinical trials. On the other hand, another SSRI, paroxetine, is used clinically to treat social anxiety disorder and has been shown to reduce social avoidance in a similar model of social fear conditioning. This suggests that the effects of SSRI may depend on the differences between fluoxetine and paroxetine.

A careful literature search of their pharmacokinetic and pharmacodynamics indicates that fluoxetine is far less potent than paroxetine and has a far longer half-life. Since the effects of SSRI seem to be mediated through chronic administration, it could be that different subtypes of serotonin receptors (which have different binding affinities for serotonin) are activated by different levels of serotonin – similar to how dopaminergic receptors are activated by different dopamine concentrations. Moreover, a possible reason may be due to the anxiogenic and anxiolytic properties of 5-HT_{2C} receptor (5-HT_{2C}R), a common 5-HTR paroxetine and fluoxetine acts upon. 5-HT_{2C}R activation has been shown to have anxiogenic effects. Paroxetine indirectly activates 5-HT_{2C}R through SERT inhibition. Acute 5-HT_{2C}R activation through paroxetine may explain the initial anxiogenic effects reported by SAD patients; however, homeostatic changes in 5-HT_{2C}R expression may occur in response to chronic exposure to paroxetine, thus resulting in anxiolytic effects. Despite fluoxetine's therapeutic effects be attributed

primarily to its inhibition of SERT like paroxetine; interestingly, fluoxetine has been demonstrated have antagonistic effects on 5-HT_{2C}R by inhibiting membrane current responses mediated by 5-HT_{2C}R (Ni et al, 1997). The therapeutic effects of fluoxetine may be a consequence of fluoxetine blocking 5-HT from binding to 5-HT_{2C}R (Ni et al, 1997). Fluoxetine also has a weak affinity for 5-HT_{2C}R, and through chronic use, fluoxetine accumulates and block these receptors, which prevent 5-HT_{2C}R activation, yielding anxiolytic effects. However, despite chronically administering fluoxetine to the social fear conditioned mice, we did not see these anxiolytic effects. However, adult 5-HT_{2C}R knock out mice has been shown to exhibit social deficits, which implicates 5-HT_{2C}R may be necessary for normal social behavior (Ni et al, 1997). The opposing literature suggests that drug interactions with 5-HTR and 5-HT signaling in social behavior are poorly understood. Although our findings cannot be explained mechanistically through 5-HT, this does not completely rule out 5-HT being a key player in social anxiety disorder. The contradictory findings across the primary literature may be due to different underlying mechanisms (i.e. homeostatic neuroplasticity, oxytocin signaling, etc.) or inconsistent use of behavioral assays, animal models, and drugs used (Švob Štracet al, 2016). Given fluoxetine's elusive therapeutic effects in a social context, it is important to study in more detail the action of fluoxetine on 5-HT receptors. Dissecting apart these confounds is not trivial and requires significant expertise in pharmacology, biochemistry, and neuroscience.

In summary, we show that the projection-specific optogenetic stimulation of $vHPC_{LS}$ abolishes SFC-induced social fear. The SFC-induced fear was not accompanied by any confounding behavioral alterations. The reversal of social fear in

male SFC mice is mediated by phasic stimulation and not tonic stimulation. We hope that this information points towards an updated conceptual model for the dysfunctional neural circuitry underlying SAD in humans, which will lead to a better future therapeutic development in these patients.

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