# Predator odor increases glutamatergic synaptic transmission in the prelimbic cortex via corticotropin-releasing factor receptor 1 signaling



**Running title**

Predator odor increases PL synaptic transmission via CRF-R1

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**ABSTRACT**

Acute exposure to a salient stressor, such as in post-traumatic stress disorder, can have lasting impacts upon an individual and society. To study stress in rodents, some naturalistic methods have included acute exposure to a predator odor, such as the synthetized fox odor 2,4,5,trimethyl-3-thiazoline (TMT). These experiments explore the stress-related behaviors and cortical activity induced by TMT exposure in adult male C57BL/6J mice and the influence of the stress neuropeptide, corticotropin-releasing factor (CRF) on these responses. Compared to $H_2O$, mice exposed to TMT in the home cage showed increased avoidance and defensive burying indicative of evident stress responses. Consistent with stress-induced activation of the medial prefrontal cortex (mPFC), we found that the prelimbic (PL) and infralimbic (IL) subregions of the mPFC had elevated *c-Fos* immunolabeling after TMT compared to $H_2O$. Slice physiology recordings were performed in layers 2/3 and 5 of the PL and IL, following TMT or $H_2O$ exposure. In TMT mice, PL layers 2/3 showed heightened spontaneous excitatory post-synaptic currents and synaptic drive, suggesting TMT enhanced excitatory transmission. Synaptic drive in PL was increased in both TMT and $H_2O$ mice following bath application of CRF, and systemic pretreatment with the CRF-R1 antagonist CP154526 reduced excitatory transmission in TMT mice, but not $H_2O$ mice. CP154526 also reduced stress-reactive behaviors induced by TMT.

## INTRODUCTION

Although stress can divert resources for optimal performance on the ascending phase of the dose-effect curve, it is the descending limb of distress that can lead to disorder (Karasek and Theorell 1990). Not only can cognitive and behavioral perturbations occur after chronic stress, but also acute exposure to an extremely stressful or traumatic event to which an individual responds with fear, helplessness, or horror can develop into post-traumatic stress disorder (PTSD; Yehuda 2002). Therefore, it is imperative to understand how acute stress can cause immediate neuroadaptations in vulnerable brain areas by studying preclinical models.

There are several proposed rodent models of PTSD, most involving a single exposure to a stressor leading to prolonged symptomatology (Yamamoto et al. 2009). The predator odor, 2,3,5-trimethyl-3-thiazoline (TMT), is a synthetic single-molecule component of fox feces with most robust effects on avoidance (Fendt and Endres 2008). TMT is thought to be an unconditioned threatening stimulus because naïve, laboratory bred, and raised rats and mice display fear-like responses on their first exposure to TMT (Rosen et al. 2015) as well as corticosterone secretion (Day et al. 2004). Unsurprisingly, TMT activates specific brain regions involved in stress, anxiety, and fear (Janitsky et al. 2015).

One well-known brain region impaired by both acute and chronic stress is the medial prefrontal cortex (mPFC), where neuroadaptations at the cellular level can cause executive dysfunction (Holmes and Wellman 2009). In the mPFC, the prelimbic (PL) and infralimbic (IL) subregions can regulate differential hypothalamic responses to stress (Radley et al. 2006). Dendritic remodeling occurs in PL pyramidal neurons in layer 2/3 after 3 weeks of restraint stress or corticosterone administration (Wellman 2001; Radley et al. 2004). However, stress can decrease serotonin-mediated glutamatergic currents in layer 5 of the mPFC (Liu and Aghajanian 2008) and can cause dendritic retraction and spine loss in IL layer 5 (Goldwater et al. 2009). These long-term effects of chronic stress are also observed after an acute episode of inescapable footshock stress, which enhances glutamate transmission in the PFC for 24 hr (Treccani et al. 2014, Musazzi et al. 2017).

A probable central mechanism for how prefrontal reorganization can occur is through surges in the stress neuropeptide corticotropin-releasing factor (CRF). CRF acts mainly through stimulation of the CRF type-1 receptor (CRF-R1), which is expressed throughout cortical areas in the brain, including the mPFC (Steckler and Holsboer 1999). Past studies have linked the role of forebrain CRF-R1-expressing neurons in emotional adaptation to stress (Refojo et al. 2011). For example, avoidance of stimuli paired with bobcat urine is modulated by mPFC CRF-CRF1 signaling in rats (Schreiber et al. 2017). In an elegant series of studies, Uribe-Mariño et al.

(2016) demonstrate that acute social defeat stress affects mPFC-mediated behavioral flexibility, CRF modulates the impairment, and intra-mPFC CRF-R1 deletion abolishes the stress-induced executive dysfunction.

This set of experiments builds upon existing literature by exploring the role of specific cortical layers in the CRF/CRF-R1 system in response to an acute stressor in mice, exposure to TMT predator odor. First, we observed prototypical stress and avoidance responses to TMT in the home cage. After determining which subregions of the mPFC had the highest activation after TMT exposure, we measured synaptic transmission in layers 2/3 and 5 in the PL and IL of stressed and non-stressed mice. Lastly, we determined a modulatory role of CRF and CRF-R1 using a CRF-R1 antagonist to affect stress responses in behavior and in slice. These experiments demonstrate that a single exposure to predator odor can result in an increase in acute excitatory transmission to specific cortical subregions, and further support TMT as a potent and effective stressor for mice.



## MATERIALS AND METHODS

### *Animals*

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) arrived at 6 weeks of age and were group-housed with littermates in a temperature-controlled vivarium maintained on a reversed light/dark cycle with lights off at 0700 hours. After one week in the vivarium, mice were single housed in fresh cages lined with corn cob pellet bedding and a nestlet. They had an additional week of single-housing, so mice were 8 weeks old during the tests. Mice had access to standard rodent chow and water at all times except during testing.

### *Predator Odor Exposure*

During the 10-minute pre-test, mice were habituated to a shortened cotton tip applicator held vertically with a plastic stand, referred to as the 'object,' which was placed in one corner of the rectangular home cage. For the post-test period, 2.5 µl of TMT (Part 300000368, Scotts Canada Ltd.) or distilled water ($H_2O$) was applied to the cotton tip, and mice were monitored for an additional 10 min. All trials were video recorded for hand-scoring by a blind observer or for locomotor analysis in Ethovision XT13 (Noldus, The Netherlands). The following behaviors were assessed in the baseline and trial tests: time spent contacting the object, time spent in the far corners of the cage, duration of defensive burying, and distance traveled. Object contact includes sniffing, biting, touching, or physically exploring the cotton tip apparatus. Defensive burying is the vigorous treading of bedding with the forepaws or nose. All mice were tested for

behavioral analyses (n=22) while approximately half were used for *c-Fos* immunohistochemistry (n=12), and half were used for electrophysiological recordings (n=10). For experiments exploring the role of CRF-R1 on behavior and physiology, mice were first habituated to handling and i.p. injections. On the test day, mice received a 10 ml/kg injection of saline or 10 mg/kg CP154526 (Tocris, Minneapolis, MN) 20 minutes before the pre-test trial. Saline was the vehicle for CP154526. This dose was chosen based on previous studies demonstrating it reduced anxiety-like and stress-related behaviors in mice (Griebel et al. 1998). There is empirical evidence that CP154526 is still present in cortical structures by the time of recordings (Keller et al. 2002). Again, all mice were tested for behavioral analysis (n=32), while half were used for electrophysiological recordings (n=16).



***c-Fos immunohistochemistry***

Ninety minutes after TMT or $H_2O$ exposure in the home cage, mice were anesthetized with a mixture of 2,2,2-tribromoethanol and 2-methyl-2-butanol in saline, then intracardially perfused with a chilled 0.01 mM phosphate buffer solution (PBS), followed by 4% paraformaldehyde (PFA) in PBS. Brain tissue was collected, post-fixed in 4% PFA overnight, then transferred to 30% sucrose/PBS and stored at 4°C before coronal sections were obtained on a vibratome (Leica VT1000S). Free-floating brain sections (45 um thick) were washed in PBS, and then incubated in 50% methanol for 30 min followed by 3% $H_2O_2$ for 5 min. Tissue was then blocked in 0.3% Triton X-100 and 1% bovine serum albumin for 60 min before a 48 hr incubation at 4°C in the blocking buffer containing a rabbit anti-*c-Fos* antibody (1:3000, ABE457 Millipore). After the primary step, slices were washed in TNT buffer for 10 min then TNB buffer for 30 min. Slices were next incubated in a goat anti-rabbit horse radish peroxidase-conjugated IgG (1:200, NEF812001EA, PerkinElmer, Waltham, MA) for 30 min then washed in TNT for four 5-min washes. For amplification of the signal, tissue was then processed using a tyramine signal amplification (TSA) kit with a Cy3-tyramide (1:50, PerkinElmer, Waltham, MA) for 10 min. Four serial sections per animal were mounted on slides, sealed with a mounting medium containing DAPI (VectaShield), then coverslipped. Slides were imaged on a confocal laser-scanning microscope (LSM 800, Zeiss Microscopy, Thornwood, NY) and *c-Fos* immunoreactive cells were quantified using ZEN imaging software (Zeiss Microscopy, Thornwood, NY). Each subregion was analyzed over four serial slices, and the single mean was used per animal (n=6 $H_2O$, n=6 TMT). PL and IL regions and layers 2/3 and 5 were classified according to Franklin and Paxinos 2008, Pittaras et al. 2016, and Stewart and Plenz 2006.

***Slice Electrophysiology***

Ninety minutes following TMT or $H_2O$ exposure in the home cage, mice were sacrificed via deep isoflurane anesthesia, and acutely-prepared coronal brain slices containing the mPFC were collected according to standard laboratory protocols (Pleil et al. 2015). Whole-cell voltage-clamp electrophysiological recordings were performed in pyramidal neurons in layers 2/3 and 5 in the PL and IL regions of the mPFC based on landmarks in the Allen Mouse Brain Atlas [**Fig 2a**]. The effect of TMT on basal synaptic transmission was assessed in voltage clamp by adjusting the membrane potential and using a cesium methanesulfonate-based intracellular solution (135 mM cesium methanesulfonate, 10 mM KCl, 10mM HEPES, 1 mM MgCl2, 0.2 mM EGTA, 4 mM MgATP, 0.3 mM GTP, 20 mM phosphocreatine, pH 7.3, 285-290 mOsmol). Lidocaine n-ethyl bromide (1 mg/ml) was included in the intracellular solution to block postsynaptic sodium currents. Neurons were held at -55 mV, the reversal potential for chloride, to assess glutamatergic synaptic transmission. In the same cell, neurons were held at +10 mV, the reversal potential for sodium, to assess GABAergic synaptic transmission. Fluctuations in current were used to determine spontaneous post-synaptic current (sEPSC or sIPSC) frequency and amplitude, as well as to calculate sEPSC/sIPSC ratios and synaptic drive (sEPSC frequency X amplitude / sIPSC frequency X amplitude). To look at CRF-gated plasticity in the PL, 1µM CRF was bath applied at a rate of 2 ml/min for 10 min after a stable baseline (Kash and Winder 2006) in voltage clamp. Synaptic transmission experiments in PL layer 2/3 were also performed in animals that received either 10 mg/kg CP154526 or saline prior to TMT or $H_2O$ exposure. For all experiments, (n=2-4) cells were collected for each animal, given the four different subregions. Each condition includes at least (n=3) mice/group. During CRF bath application experiments, baseline synaptic transmission was also included as cells for region-specific recordings. Electrophysiological recordings were then analyzed using Clampfit 10.7 software (Molecular Devices, Sunnyvale, CA).



***Statistical Analyses***

Behavioral data including time spent in contact with the object, time spent in the far corners, defensive burying, and distance traveled were analyzed using mixed factor ANOVA to assess within-subjects pre-test versus post-test phases and between-group stress exposure (TMT vs. $H_2O$). For drug effects, behavioral data were analyzed with two-way ANOVAs for between stress group and drug effect (saline vs. CP154526) differences. Separate two-way ANOVAs were run for the pre-test phase for assessing drug effects on baseline behavior. *C-Fos* data were analyzed by repeated measures two-way ANOVA, and mean *c-Fos* positive nuclei were compared between mPFC subregion (PL vs. IL) and stress exposure group.

Electrophysiological data were analyzed using two-way ANOVA for mPFC subregion and stress group. The effect of CRF bath application was also analyzed with a repeated measures two-way ANOVA for baseline versus drug application in TMT and $H_2O$ mice. All significant main effects were then followed up by multiple comparisons using Holm-Sidak post hoc tests. All values presented are mean ± standard error of the mean (SEM), and $\alpha$=0.05.



## RESULTS

### TMT elicits active stress behaviors in C57BL/6J mice

Adult male C57BL/6J mice were exposed to $H_2O$ or TMT in the home cage (n=11 $H_2O$, n=11 TMT), and contact with the object, time spent in the far corners of the cage, and defensive burying were observed [**Fig 1a**]. Two-way repeated measures ANOVA found an interaction between testing phase and stress exposure for contact with object [$F(1,20)=9.69$, $p<0.01$]. TMT-exposed mice had less contact with the object compared to $H_2O$-exposed mice [$t(20)=3.20$, $p<0.01$], which was also less than the baseline condition [$t(20)=3.54$, $p<0.01$]. There was also an interaction between test phase and stress for time spent in the far corners of the cage [$F(1,20)=19.10$, $p<0.001$]. Compared to baseline testing, $H_2O$ mice spent less time in the far corners [$t(20)=2.56$, $p<0.05$], but TMT mice spent more time in the far corners [$t(20)=3.63$, $p<0.01$]. We also found an interaction for total time defensive burying [$F(1,20)=7.56$, $p<0.05$] and a main effect on test phase[$F(1,20)=17.34$, $p<0.001$]. TMT-exposed mice buried significantly more than $H_2O$-exposed mice [$t(20)=3.23$, $p<0.01$] and compared to their baseline burying [$t(20)=4.89$, $p<0.01$]. Finally, distance traveled was quantified [**Fig 1c**] where there was a main effect of time [$F(1,20)=28.24$, $p<0.0001$]. Both $H_2O$ [$t(20)=3.24$, $p<0.01$] and TMT mice [$t(20)=4.27$, $p<0.001$] decreased distance traveled compared to their baseline, but there was no effect of TMT. Representative heat maps of total time spent in the home cage are shown after $H_2O$ or TMT exposure [**Fig 1d**].

### TMT produces neuronal activation in the medial prefrontal cortex

As a previous study from the Gilpin lab suggested the PFC as a critical site of action for predator odor-induced learning in rats (Schreiber et al. 2017), we first examined how acute TMT exposure could increase expression of the immediate early gene *c-Fos* in the mPFC. The areas of interest in the mPFC, the PL and IL, are illustrated in **Fig 2a**. We show example confocal images of fluorescent *c-Fos* staining after $H_2O$ (n=6) or TMT (n=6) in the home cage with overlaid regions of interest [**Fig 2b**]. Further, close-up images of *c-Fos* immunohistochemistry overlaid with DAPI in the PL are displayed in **Fig 2c**. Active neuronal populations were quantified [**Fig 2d**], where we found subregion differences [$F(1,10)=50.35$, $p<0.0001$] in addition

to a stress effect [F(1,10)=16.59, p<0.01]. TMT increased *c-Fos* cell counts in both the IL [t(10)=3.00, p<0.05] and the PL [t(10)=4.30, p<0.05]. PL had higher *c-Fos* counts than IL in both $H_2O$ [t(10)=3.98, p<0.01] and TMT mice [t(10)=6.05, p<0.001].

**Increased synaptic drive of mPFC PL 2/3 after TMT exposure**

We next used slice electrophysiology following acute exposure to TMT to determine if there was a change in neuronal function in the PL and IL. Synaptic transmission was recorded in PL and IL, in both layers 2/3 and 5. Of the total n=10 mice, sample sizes were n=6-10 total cells/region, and n=5 mice/group. Representative traces for sEPSC in PL 2/3 after $H_2O$ and TMT are shown in **Fig 3a** and sIPSC in **Fig 3b**. A two-way ANOVA revealed a main effect of stress on sEPSC frequency [**Fig 3c**, F(1,59)=19.91, p<0.0001], specifically in PL layer 2/3, where TMT exposed mice had higher sEPSC frequency than $H_2O$ mice [t(59)=3.60, p<0.01]. There were no subregion differences or stress effect on sIPSC frequency [**Fig 3d**]. Consequently, we found an effect of TMT on the E/I ratio [**Fig 3e**, F(1,59)=7.39, p<0.01], located in PL 2/3 [t(59)=2.73, p<0.05]. There were no main effects or an interaction on sEPSC amplitude [**Fig 3f**]. However, there was a trend towards a main effect of stress on decreasing amplitude [F(1,59)=3.97, p=0.051]. TMT affected sIPSC amplitude [**Fig 3g**, F(1,59)=6.19, p<0.05], specifically in PL layer 5 [t(59)=3.04, p<0.05]. Similarly, TMT increased synaptic drive [**Fig 3h**, F(1,59)=4.94, p<0.05], specifically in the PL 2/3 cells [t(59)=2.64, p<0.05].



**CRF-R1 antagonist improves responses to stress**

We further examined the role of CRF-R1 signaling in the behavioral response to TMT. The CRF-R1 antagonist CP154526 or saline was administered before $H_2O$ or TMT exposure (n=8/group). During the pre-test trials, there were no differences between saline and CP154526 treatment for contact with the object, time in far corners, or defensive burying [**Fig 4a-c**]. There was a group effect of TMT on distance traveled [**Fig 4d**, F(1,20)=4.60, p<0.05], but no main effect of drug or significant post-hoc differences. During the post-test, TMT affected contact with object [**Fig 4e**, F(1,28)=10.96, p<0.01] where TMT decreased contact with object during saline trials [t(28)=3.45, p<0.01] but not after CP154526. There was no significant effect of drug or TMT on time spent in the far corners [**Fig 4f**]. A two-way ANOVA revealed an interaction for defensive burying [**Fig 4g**, F(1,28)=14.67, p<0.001], a drug effect [F(1,28)=8.061, p<0.01], and stress effect [F(1,28)=6.49, p<0.05]. Like non-drug results, TMT increased defensive burying in the saline condition [t(28)=4.72, p<0.001]. However, pretreatment with CP154526 decreased TMT-induced defensive burying [t(28)=4.51, p<0.001]. Overall there was a stress effect on distance traveled [**Fig 4h**, F(1,28)=10.74, p<0.01], in which TMT increased distance traveled,

but no main effect of drug. Representative heat maps of total time spent in the home cage after saline or CP154526 are shown after TMT exposure [**Fig 4i**].

**CRF mimics TMT in slice, and CRF-R1 antagonism blocks TMT effects**

Exposure to TMT had the most robust effects on synaptic function on layer 2/3 neurons in the PL, we next explored how CRF signaling could alter function in the mPFC. Initially, we examined how bath application of 1 µM CRF altered synaptic function in these neurons in both control and TMT-exposed mice. The same mice from the region-specific experiments were used (n=10), and n=8-10 total cells/group were collected from n=4 mice/group. Two-way repeated measures ANOVA revealed a significant drug effect [**Fig 5a**, $F(1,16)=31.53$, $p<0.0001$], where CRF increased sEPSC frequency in both $H_2O$ [$t(16)=3.71$, $p<0.01$] and TMT mice [$t(16)=4.26$, $p<0.01$]. There was no effect on sIPSC frequency [**Fig 5b**]. In line with these data, there was a drug effect on E/I ratio [**Fig 5c**, $F(1,16)=30.63$, $p<0.0001$], where CRF increased the E/I ratio in both $H_2O$ [$t(16)=3.70$, $p<0.01$] and TMT-exposed mice [$t(16)=4.17$, $p<0.01$]. There was an effect of TMT on sEPSC amplitude [**Fig 5d**, $F(1,16)=9.81$, $p<0.01$], where TMT-exposed mice had reduced sEPSC amplitude throughout both baseline [$t(16)=2.72$, $p<0.05$] and CRF bath application [$t(16)=3.04$, $p<0.01$]. We also found an interaction for sIPSC amplitude [**Fig 5e**, $F(1,16)=9.27$, $p<0.01$] and a CRF effect [$F(1,16)=18.98$, $p<0.001$]. In the TMT mice, sIPSC amplitude decreased with CRF application [$t(16)=2.35$, $p<0.05$], which was different from the $H_2O$ mice [$t(16)=5.55$, $p<0.001$]. These total changes were reflected in a drug effect on synaptic drive [**Fig 5f**, $F(1,16)=25.58$, $p<0.001$], where CRF increased synaptic drive in both $H_2O$ [$t(16)=3.12$, $p<0.05$] and TMT-exposed mice [$t(16)=4.10$, $p<0.01$].

To test that CRF-R1 is engaged to alter PL 2/3 function following TMT exposure, we tested synaptic transmission in mice pretreated with a CRF-R1 antagonist or saline. Of the total n=16 mice used, n=10-12 total cells/group were collected from n=4 mice/group. Two-way ANOVA showed a stress effect on sEPSC frequency [**Fig 5g**, $F(1,41)=3.61$, $p<0.05$], where TMT increased sEPSC compared to $H_2O$ in the saline group [$t(41)=2.88$, $p<0.05$]. In contrast, CP154526 decreased sEPSC frequency in the TMT group [$t(41)=2.63$, $p<0.05$]. There were no significant drug effects on sIPSC frequency [**Fig 5h**]. Comparing E/I frequency, there was a subsequent significant interaction for E/I ratio [**Fig 5i**, $F(1,41)=4.80$, $p<0.05$]. TMT increased E/I ratio after saline injection [$t(41)=2.50$, $p<0.05$], and CP154526 reduced E/I ratio in TMT-exposed mice [$t(41)=2.70$, $p<0.05$]. There were no drug effects for sEPSC amplitude [**Fig 5j**] or sIPSC amplitude [**Fig 5k**]. Overall, analysis of synaptic drive also showed a significant interaction [**Fig 5l**, $F(1,41)=4.95$, $p<0.05$]. TMT increased E/I ratio after saline injection [$t(41)=2.95$, $p<0.05$], and CP154526 reduced E/I ratio in TMT-exposed mice [$t(41)=2.46$, $p<0.05$].

## DISCUSSION

The current set of studies demonstrate a role for CRF-R1 as a potent modulator of stress responses and synaptic transmission in the mPFC. We exposed mice to a fox feces-derived predator odor, TMT, and observed robust stress-related behaviors including avoidance and defensive burying. Acute TMT exposure caused increased *c-Fos* activation in both the PL and IL subregions of the mPFC. Consistent with this, we saw increased sEPSC and synaptic drive in PL layer 2/3 of TMT mice compared to $H_2O$, suggesting enhanced glutamatergic activity. When we pretreated mice with a systemic CRF-R1 antagonist, mice spent greater time contacting the TMT and less time defensive burying. We also show that CRF-R1 antagonist can block the TMT effect on synaptic function, normalizing PL 2/3 cells to non-stressed transmission.

In our studies, TMT increased avoidance behavior and defensive burying, suggestive of stress reactions to the aversive olfactory stimulus. Others have similarly reported decreases in object zone, increases in far corners, and robust defensive burying in response to TMT, but not other predator odors like cat fur odor and β-phenylethalamine, in the home cage in C57BL/6J mice (Pérez-Gómez et al. 2015). Exposure to TMT can alter the spatial deployment of specific behavioral modules like avoidance and freezing for the generation of adaptive behavior, as characterized by a 3D autoregressive mixed model (Wiltschko et al. 2015). Like fleeing and freezing, defensive burying is among the behavioral repertoire of innate unconditioned, species-specific defensive actions (De Boer and Koolhaas 2003; Rosen et al. 2015). This defensive behavior was originally observed forty years ago in rats using the shock prod in the home cage (Pinel and Treit 1978) but has also been observed with noxious smells (Silverman 1978) and predator odor (Holmes and Galea 2002). Interestingly, we did not observe freezing behavior in the C57BL/6J mice, as other groups have reported (Yang et al. 2016). It may be that freezing occurs in an unfamiliar open field while the active burying response occurs with substantial bedding such as in the home cage. The 2.5 ul dose of TMT is also a low dose of the predator odor (Fendt and Endres 2008). These behavioral strategies can also be present in combination in some species and represent different adaptive strategies with individual differences, which is an avenue for future study.

There exist some inconsistencies in comparing stress behaviors between the initial, behavioral descriptions versus the later saline/CRF-R1 antagonist trials, such as in locomotor activity. The initial experiments demonstrate a reduction in activity between pre-test and post-test difference across both $H_2O$ and TMT groups while the later drug testing shows TMT-

induced increase in activity. It is possible that the reduction of locomotor behavior in non-handled mice occurs over time because of novelty-induced increase in activity during the pre-trial, and habituation during the post-trial. For the TMT-induced increase in locomotor activity in the drug experiments, this may be caused by a stress-enhanced handing effect combined with TMT exposure that was not revealed during the former conditions. We also observed longer durations of defensive burying in the saline/CRF-R1 antagonist-injected mice during the $H_2O$ pre-trial and post-trial. In this manner, the time in the far corners of the home cage was trending towards an increase in the saline-TMT group, in the same direction as the non-injected group, but this may be directly related to the increased locomotor activity spent around the home cage. These minor irregularities between cohorts of mice convey that handling stress could be a confound despite habituation to ip injections. Even so, TMT still produced robust decreases in contact with the TMT and increased defensive burying across experiments.

We demonstrate that TMT exposure generates active stress behaviors and increased synaptic transmission, CRF similarly increases excitatory synaptic transmission, and CP154526 treatment suppresses defensive burying and reduces synaptic transmission. Central CRF infusions can potentiate autonomic and behavioral responses during a shock-prod defensive burying test in rats and defensive behaviors in mice (Diamant et al. 1992, Yang et al. 2006). Our experiments confirm previous studies examining the CRF/CRF-R1 system in the mPFC after acute stressors. Specifically, recent investigations show that CRF in the vmPFC, consisting of the lower PL and IL, mediates conditioned avoidance to bobcat urine in rats (Schreiber et al. 2017). In addition, a single episode of social defeat causes mPFC deficits in working memory through CRF/CRF-R1 microcircuits (Uribe-Mariño et al. 2016). In both these studies, CRF infusions into the mPFC had similar effects as the stressor, and either intra-mPFC CRF-R1 deletion or CRF-R1 antagonist infusions ameliorated the stress-induced behavioral impairments. An important caveat of the present study is that we did not perform a local CRF-R1 manipulation in the PL As such, it is possible that CRF-R1 signaling could drive network dependent effects that results in changes in glutamatergic transmission in the PFC. However, we were able to identify CRF-R1-dependent changes in neural transmission in a specific layer and subregion after systemic CP154526 treatment, suggestive of TMT driving local CRF-R1 activation.

We additionally show increased *c-Fos* activation in the PL and IL after TMT like others (Hebb et al. 2004, but see Asok et al. 2013), which could very well be in *Crhr1*-containing cells, as PL and IL cells have increased *Crhr1* mRNA after acute social defeat (Uribe-Mariño et al. 2016). It is unlikely that these effects are CRF-R2-mediated since there is minimal CRF-R2 in

the vmPFC (Chalmers et al. 1995). Although, it is possible that CRF-R2 may be recruited to the plasma membrane in other brain sites after chronic, repeated TMT, as repeated social defeat promotes CRF-R1 internalization in the dorsal raphe (Wood et al. 2013). These effects of CRF acting on CRF-R1 can affect downstream signaling pathways such as protein kinase A after stress (Miguel et al. 2014, Uribe-Mariño et al. 2016). CRF-R1 activity may also act by modulating other neurotransmitters in the mPFC such as 5-HT (Hwa et al. 2016). Exposure to rat odor substantially increases in extracellular 5-HT, dopamine, and acetylcholine in the PFC of mice (Smith et al. 2006). It would be an interesting future direction to examine how CRF-R1 and 5-HT interact in response to TMT.

Our slice recordings found that TMT exposure produced the most robust effects in PL 2/3 compared to PL 5, IL 2/3, and IL 5. This corroborates the original Wellman (2001) study and others showing that pyramidal neurons in layer 2/3 of the PL and anterior cingulate have reorganized dendrites in response to stress (Radley et al. 2004). More recently, others have shown that stress exposure during the first postnatal week hinders dendritic development in layers 2/3 and 5 pyramidal neurons in the PL and cingulate of neonatal mice, an effect reversed by antalarmin, a CRF-R1 antagonist (Yang et al. 2015). Specifically, we saw an increase in sEPSC frequency, sEPSC/sIPSC ratio, and synaptic drive after TMT stress, with no effect on sIPSC frequency, indicating escalated glutamatergic activity in the PL 2/3. As we also confirm, it is well known that application of CRF excites the frontal cortex (Eberly et al. 1983). The decrease in excitatory synaptic transmission we observed after CRF-R1 antagonist pretreatment implies that the CRF-R1 antagonist may suppress glutamate signaling in the PL of TMT-exposed mice. It will be a crucial next step to acutely apply CP154526 before CRF on mPFC slices of $H_2O$ and TMT mice to directly assess if local antagonism can drive similar changes in the E/I ratio. Others have also blocked stress-enhanced excitatory mPFC transmission and dendritic remodeling with ionotropic glutamate receptor antagonists (Li et al. 2011, Martin and Wellman 2011). Again, it is important to note that while prefrontal reorganization can occur after chronic stress, a single episode of inescapable footshock can enhance glutamate transmission in the PFC for up to 24 hours (Musazzi et al. 2017), which may also occur with TMT exposure. This acute stressor induces rapid enhancement of depolarization-evoked glutamate overflow though increased readily releasable pool of glutamate vesicles in synaptic terminals of the PFC, which then stimulates corticosterone levels downstream (Treccani et al. 2014, Musazzi et al. 2010). In our study of PL 2/3 synaptic transmission, we observed some significant reductions in sEPSC and sIPSC amplitude after TMT exposure and with CRF bath application. Some have shown increases in NMDAR-EPSC

and AMPAR-EPSC amplitude after acute forced swim in mPFC layer 5 (Yuen et al. 2009), but others have also reported decreased sEPSC amplitude after adolescent social defeat in mPFC layer 2/3 (Caruso et al. 2018; Urban and Valentino 2018). Our effects on sEPSC/sIPSC amplitude appear minor since the stress effect on sEPSC/sIPSC frequency dominates the increased synaptic drive. Liu and Aghajanian (2008) notably find changes in layer 5 sEPSC after repeated restraint stress, suggesting that acute stress could affect PL 2/3 initially but then later develop changes throughout other mPFC layers with repeated stress.

In future studies, it will be important to characterize both the upstream and downstream nuclei impacted by TMT stress exposure. The current study focused on mPFC layers and TMT stress, but there is a large body of literature studying the diverse olfactory systems that respond to TMT (Takahashi et al. 2014; Pereira and Moita 2016). Known circuits for the detection of threatening chemical cues start with the olfactory subsystem leading to several amygdala subnuclei and the lateral hypothalamus (Blomeley et al. 2018) with involvement of the laterodorsal tegmentum and lateral habenula (Yang et al. 2016), interpeduncular nucleus and periaqueductal grey (PAG). One potentially important downstream site of action for TMT is the extended amygdala. Neurons in the extended amygdala, including the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA), are involved in innate fear, anxiety, and stress. TMT induces *c-Fos* expression in the BNST of rats and C57BL/6J mice (Day et al. 2004; Asok et al. 2013; Janitzky et al. 2015), and inactivation of the BNST blocks TMT-induced freezing (Fendt et al. 2003). However, to date there has not been an investigation of the cell-types or signaling mechanisms in the BNST that drive these TMT-related behaviors. A recent paper demonstrated that 5-HT2A receptors in the central amygdala can have opposing effects on innate fear, via TMT, and learned fear, via footshock, with separate processing through the dorsal PAG and ventral PAG (Isosaka et al. 2015). Altogether, we will be interested in how the PL connects to these extended amygdala circuits to regulate TMT processing of stress, threat, and innate fear.

In conclusion, TMT exposure in the homecage may be a suitable model for a simple ethological stressor, as we are able to observe robust stress and defensive behaviors and mPFC plasticity in C57BL/6J mice. Additionally, this study confirms the important role of the CRF/CRF-R1 system in regulating stress behaviors and excitatory signaling in the mPFC. We identify that PL 2/3, among other mPFC layers, receives enhanced excitatory transmission after acute TMT via CRF-R1 signaling. In the future, it will be important to compare the acute neuroadaptations as in the current study with long-term adaptations after chronic stress.

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## FIGURE LEGENDS

**Fig 1.** After a 10-minute pre-test with a cotton tip apparatus in the home cage, mice were exposed to either 2.5 µl of water ($H_2O$, n=11) or the fox-derived predator odor, TMT (n=11), for 10 minutes post-test. Pre-tests are in white bars, post-tests are in colored bars, $H_2O$ as grey, TMT as red. a) Duration (sec) of contact with the apparatus/object, time spent in the far corners, b) defensive burying, and c) Distance traveled (cm) in the home cage. d) Representative heat maps of time and location of mice after $H_2O$ and TMT. The white triangle indicates the location of the stimulus. The legend indicates blue for minimum and red for maximum. **p<0.01 vs. $H_2O$, #p<0.05 vs. BL, ##p<0.01 vs. BL.

**Fig 2.** a) Coronal section of the mPFC subregions, the prelimbic (PL) and infralimbic (IL) regions, where *c-Fos* was quantified based on the mouse Allen Brain Atlas. b) Representative images of *c-Fos* immunostaining (pseudocolored yellow) after $H_2O$ and TMT with PL and IL regions outlined. The scale bar indicates 200 µm. c) Close-up of the PL region after $H_2O$ and TMT with *c-Fos* immunostaining (yellow) and DAPI (blue). The scale bar indicates 100 µm. d) Mean *c-Fos* positive nuclei (per $mm^2$) in the IL and PL after $H_2O$ (grey) and TMT (red). *p<0.05 vs. $H_2O$, **p<0.01 vs. $H_2O$, #p<0.05 vs. IL. ##p<0.01 vs. IL. Mean *c-Fos* puncta for each subregion were obtained across four serial sections for n=6/group.

**Fig 3.** Synaptic transmission recordings in PL layer 2/3, PL layer 5, IL layer 2/3, and IL layer 5 in mice exposed to $H_2O$ (grey) or TMT (red) featuring: a) representative PL 2/3 sEPSC traces in $H_2O$ and TMT, b) representative PL 2/3 sIPSC traces, c) spontaneous excitatory postsynaptic current (sEPSC) frequency (Hz), d) spontaneous inhibitory postsynaptic current (sIPSC) frequency (Hz), e) sEPSC/sIPSC (E/I) frequency ratio, f) sEPSC amplitude (pA), g) sIPSC amplitude (pA), and h) synaptic drive defined as sEPSC frequency X amplitude divided by sIPSC frequency X amplitude. *p<0.05 vs. $H_2O$, **p<0.01 vs. $H_2O$. Of the total n=10 mice, sample sizes were n=6-10 total cells/region, and n=5 mice/group.

**Fig 4**. Mice were given i.p. 10 mg/kg CP154526, a CRF-R1 antagonist (striped), or saline (non-striped) before exposure to TMT or $H_2O$ (n=8/group). Pre-tests are in white bars, post-tests are in colored bars, $H_2O$ as grey, TMT as red. Behavioral measures assessed during the pre-test phase were: a) contact with the object (s), b) time spent in the far corners (s), c) defensive burying (s), and d) distance traveled (cm). These same variables were measured during the post-test: e) contact with object, f) time spent in the far corners, g) defensive burying, and h) distance traveled. i) Representative heat maps of time and location of mice after saline and 10

mg/kg CP154526 pretreatment and TMT exposure. The white triangle indicates the location of the stimulus.The legend indicates blue for minimum and red for maximum. **p<0.01 vs. $H_2O$, ##p<0.01 vs. saline.

**Fig 5**. Bath application of 1 μM CRF affected synaptic transmission in $H_2O$- (grey) and TMT-exposed (red) PL cells as indicated by a) sEPSC frequency (Hz), b) sIPSC frequency (Hz), c) sEPSC/sIPSC (E/I) ratio, d) sEPSC amplitude (pA), e) sIPSC amplitude (pA), and f) synaptic drive. *p<0.05 vs. BL, **p<0.01 vs. BL. #p<0.05 vs. $H_2O$. The same mice from the region-specific experiments were used (n=10), and n=8-10 total cells/group were collected from n=4 mice/group. Separate mice were pretreated with 10 mg/kg CP154526 (striped) or saline (non-striped) before $H_2O$ or TMT. Electrophysiological recordings of g) sEPSC frequency (Hz), h) sIPSC frequency (Hz), i) E/I ratio, j) sEPSC amplitude (pA), k) sIPSC amplitude (pA) and l) synaptic drive *p<0.05 vs. saline. #p<0.05 vs. $H_2O$. Of the n=16 mice used, n=10-12 total cells/group were collected from n=4 mice/group.

Figure 1.

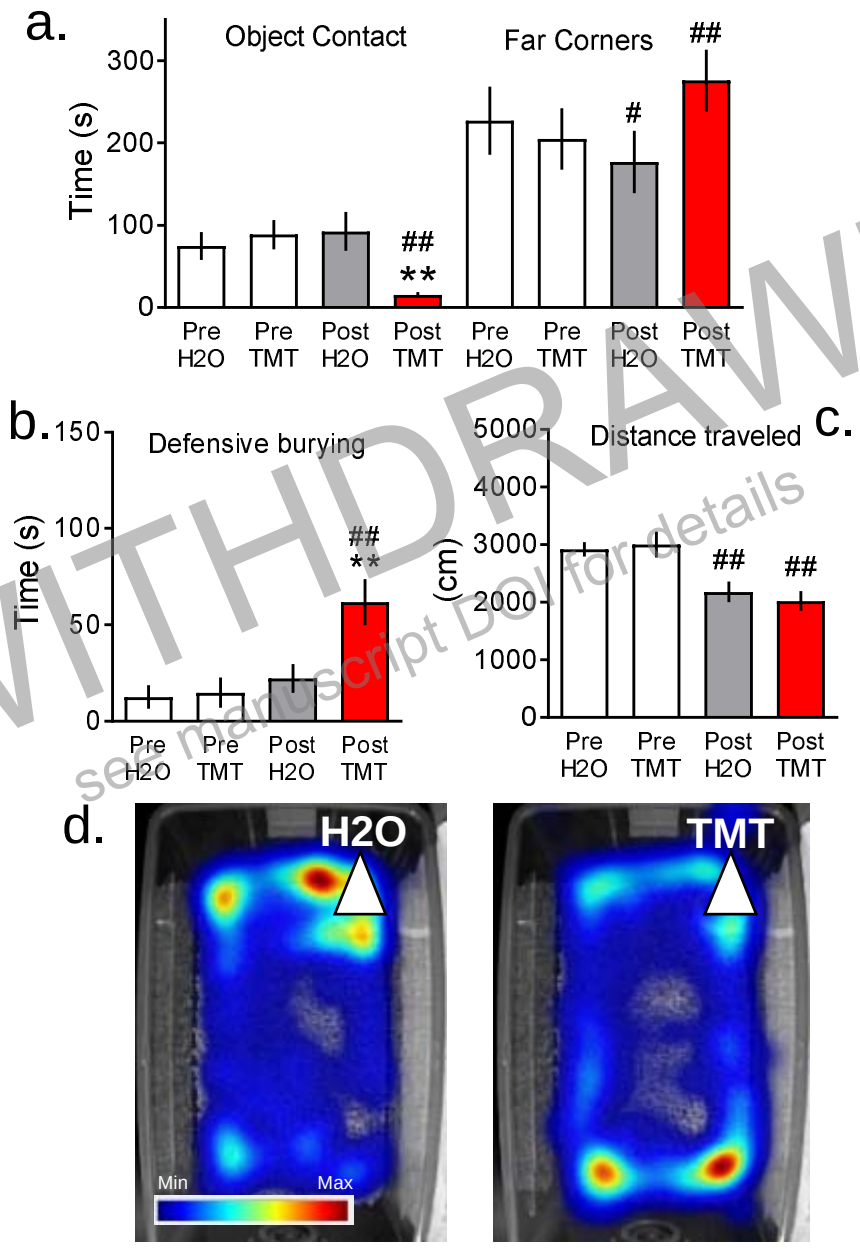

Figure 2.

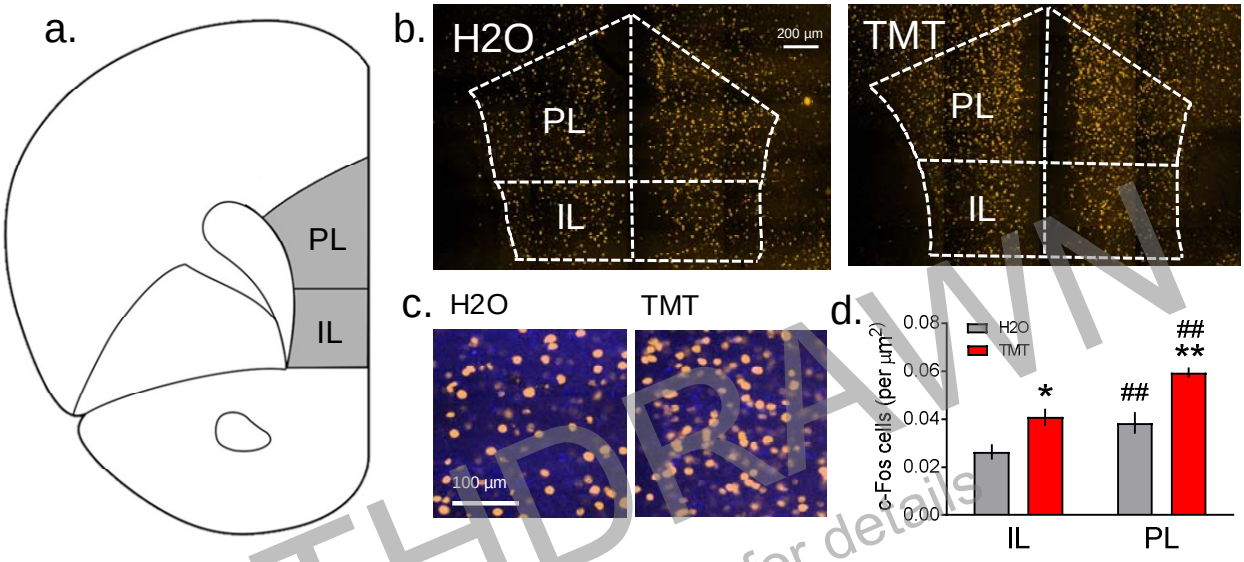

Figure 3.

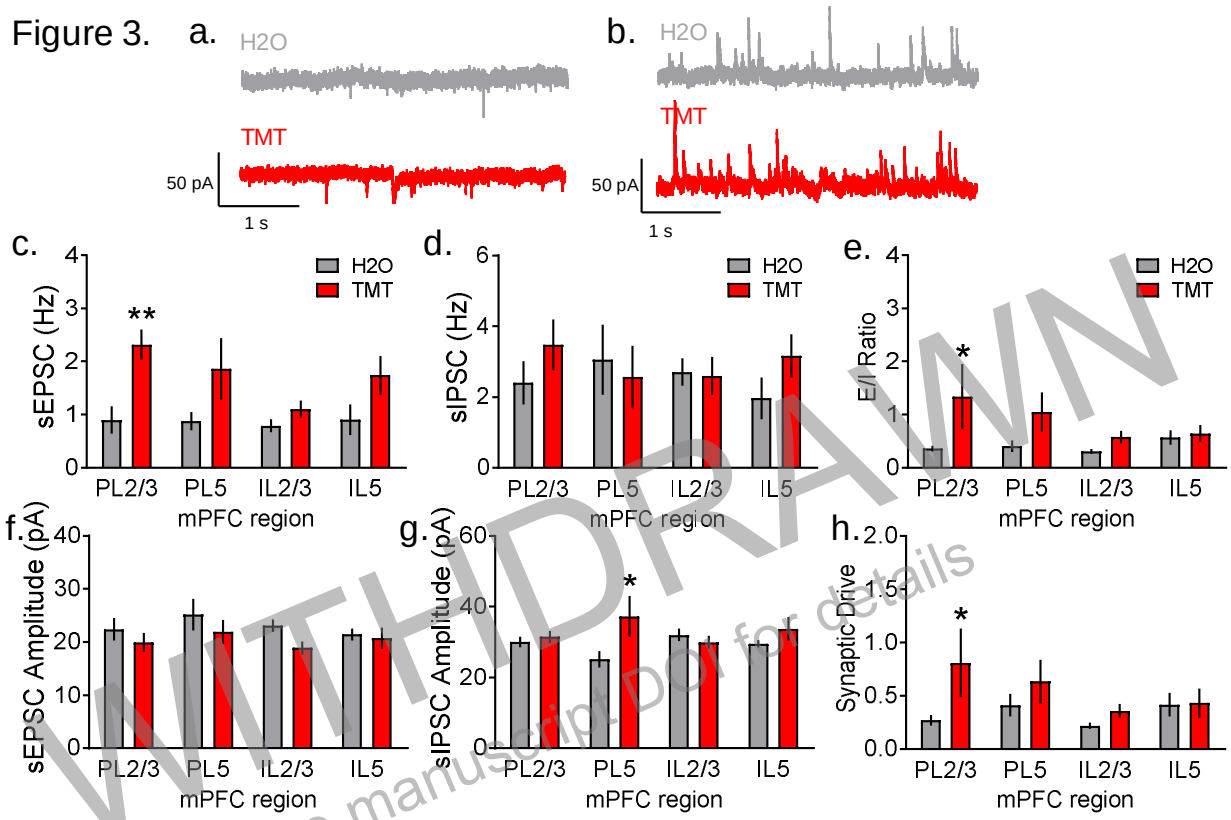

Figure 4.

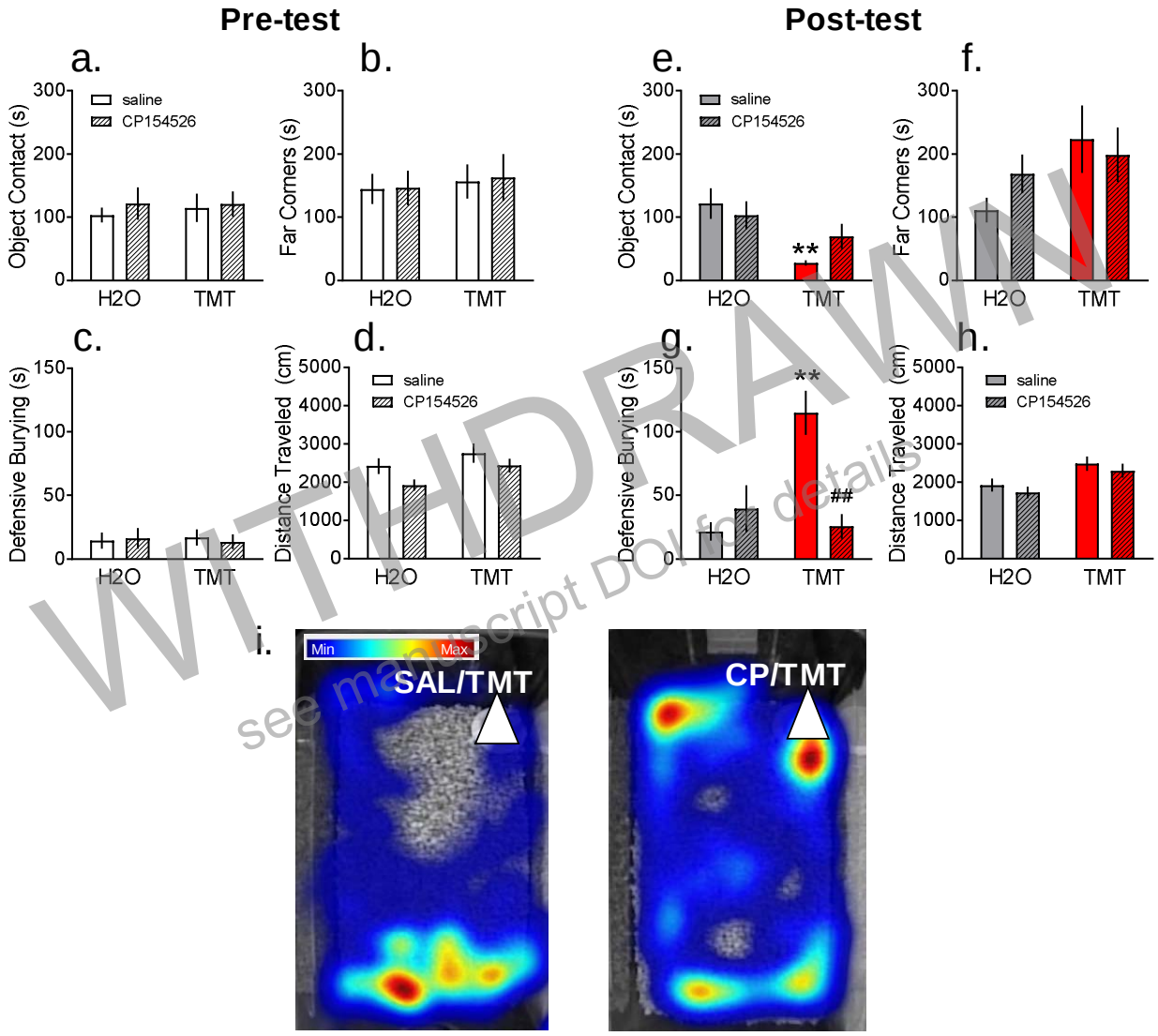

Figure 5.