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The Role of the Medial Prefrontal Cortex in Regulating Social Familiarity-Induced Anxiolysis

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Overcoming specific fears and subsequent anxiety can be greatly enhanced by the presence of familiar social partners, but the neural circuitry that controls this phenomenon remains unclear. To overcome this, the social interaction (SI) habituation test was developed in this lab to systematically investigate the effects of social familiarity on anxiety-like behavior in rats. Here, we show that social familiarity selectively reduced anxiety-like behaviors induced by an ethological anxiogenic stimulus. The anxiolytic effect of social familiarity could be elicited over multiple training sessions and was specific to both the presence of the anxiogenic stimulus and the familiar social partner. In addition, socially familiar conspecifics served as a safety signal, as anxiety-like responses returned in the absence of the familiar partner. The expression of the social familiarity-induced anxiolysis (SFiA) appears dependent on the prefrontal cortex (PFC), an area associated with cortical regulation of fear and anxiety behaviors. Inhibition of the PFC, with bilateral injections of the $GABA_A$ agonist muscimol, selectively blocked the expression of SFiA while having no effect on SI with a novel partner. Finally, the effect of D-cycloserine, a cognitive enhancer that clinically enhances behavioral treatments for anxiety, was investigated with SFiA. D-cycloserine, when paired with familiarity training sessions, selectively enhanced the rate at which SFiA was acquired. Collectively, these outcomes suggest that the PFC has a pivotal role in SFiA, a complex behavior involving the integration of social cues of familiarity with contextual and emotional information to regulate anxiety-like behavior.

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INTRODUCTION

To ensure individual and species survival, complex social behaviors have developed over time (Amodio and Frith, 2006; Cohen, 2004; Strodl and Schausberger, 2012), and in multiple mammalian species, the presence of a conspecific reduces behavioral and autonomic responses to a threat (Davitz and Mason, 1955; DeVries *et al*, 2003; Hennessy *et al*, 2000; Hennessy *et al*, 2002; Kiyokawa *et al*, 2007, 2009, 2012; Nakayasu and Kato, 2011; Terranova *et al*, 1999). Such reductions in threat responses can be even greater when the conspecific is familiar. In addition, perception of pain and emotional distress to the threat of a painful stimulus is reduced when the subject is in contact with or viewing a picture of a familiar person compared with an unfamiliar person (Coan *et al*, 2006; Eisenberger *et al*, 2011). This effect of social familiarity is also at the core of many behavioral and cognitive therapies for anxiety where the subject's perceived alliance with the therapist is integral to the success of the treatment (Martin *et al*, 2000). Although alleviation of anxiety through social familiarity is widely accepted, very few studies have systematically investigated this effect, and thus little is known about the mechanisms and neural circuits that regulate it.

Preclinical modeling of social familiarity-induced inhibition of fear and/or anxiety is difficult, as many validated preclinical tests such as fear conditioning, elevated plus maze, or open field are confounded by the addition of a conspecific. However, the presence of a conspecific is at the core of the social interaction (SI) test (File and Hyde, 1978). Previously, we developed a modified version of the SI test, termed as the social interaction–habituation test (SI-hab), which permits using the same conspecific partner repeatedly to investigate the regulation of anxiety-like behavior, by social familiarity (Truitt *et al*, 2007). Social familiarity, as

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investigated by SI-hab in rats, reduces chronic anxiety-like behavior induced by pharmacological activation of the basolateral amygdala (BLA) (Truitt *et al*, 2007). In addition, this ability of social familiarity to inhibit anxiety-like behavior was dependent on a subpopulation of inhibitory BLA interneurons that may be activated by inputs from the medial prefrontal cortex (mPFC).

The mPFC has been implicated in the regulation of both social behavior (Adolphs, 2010; Fossati, 2012; Meyer-Lindenberg and Tost, 2012) and top-down regulation of anxiety (Hartley and Phelps, 2010; Kim *et al*, 2011b), making it a likely neural substrate for regulating social familiarity-induced reductions in anxiety. Damage within the mPFC is linked specifically to social deficits occurring after traumatic brain injury (Spikman *et al*, 2012) and sub-structures within the mPFC, including the infralimbic cortex (IL) of the rat, have recently been demonstrated to be critical for expression of key social behaviors during development (van Kerkhof *et al*, 2013a, 2013b). The IL and the similar human structure, the subgenual ventral mPFC (vmPFC), are well known sites for cortically driven reductions in anxiety through safety learning. The IL/vmPFC is also activated in response to stimuli that signal safety (Gupta *et al*, 2013; Herry and Mons, 2004; Knapska and Maren, 2009; Phelps *et al*, 2004; Schiller *et al*, 2008). Activating the IL enhances and emulates extinction of fear conditioning (Milad and Quirk, 2002; Thompson *et al*, 2010), likely via connections with the amygdala (Knapska *et al*, 2012).

The vmPFC and its connectivity to the amygdala are also tightly linked to emotion regulation including anxiety, and the strength of this connection can predict positive outcome for cognitive behavioral therapies (Bishop *et al*, 2004; Kim *et al*, 2011a, 2011b; Pezawas *et al*, 2005). Cognitive behavioral therapies, such as exposure therapy, are a form of safety learning that can be enhanced (reduction in the number of pairings) in numerous animal and human studies by D-cycloserine, an allosteric NMDA receptor partial agonist (Davis *et al*, 2006; Ganasen *et al*, 2010; Gupta *et al*, 2013; Hofmann *et al*, 2006a, 2006b; Myers and Carlezon, 2012; Walker *et al*, 2002; Watson *et al*, 1990). In addition, D-cycloserine effects on safety learning appear to occur by augmenting the IL–amygdala circuitry (Chang and Maren, 2011; Gupta *et al*, 2013; Ledgerwood *et al*, 2003; Walker *et al*, 2002). In the current study, we investigated the ability of social familiarity to selectively reduce anxiety-like responses, determined the role of the PFC in this process, and then examined the enhancement of this process with D-cycloserine.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (Harlan Laboratories, Indianapolis, IN) between 300 and 350 g were used for all behavioral experiments. Upon arrival at the facility, rats were individually housed in a temperature-controlled room (22 °C) and kept on a 12-h light–dark cycle (lights on at 0700 hours) with free access to food and water for at least 1 week before behavioral testing (Truitt *et al*, 2007). Rats were handled daily for a minimum of 3 days before any behavior testing. Cages were changed weekly. All cage changes occurred after behavior testing and a minimum of 20 h before the next day's behavior testing. All experiments were conducted in accordance to the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication no. 80-23, revised in 1996) and according to the guidelines of the Indiana University Purdue University at Indianapolis Institutional Animal Care and Use Committee.

Bright Light Challenge

Anxiety-like behavior during SI testing was induced using a bright light challenge (File and Hyde, 1978). The bright light challenge consisted of an abrupt transition from dim red light (40-watt red light, 1 lux) to bright white fluorescent lighting (488 lux at the approximate eye level of the rats 8.5 feet from the light source) during the 5-min SI testing session.

Behavioral Testing

Habituation and staging. Before behavioral testing or habituation to the SI testing arena, rats were moved in their home cages from the animal housing facility to a staging room in the behavioral testing suite. The staging room was constantly maintained under dim red light conditions and is located adjacent to the room with the SI testing arena. Rats habituated to the dim lighting in the staging room for a minimum of 30 min. Twenty-four hours before the first SI test, rats were habituated to the SI apparatus by placing the rat into the chamber alone for 5 min under dim red lighting conditions.

SI Test. The SI testing arena consists of a plexiglas open top box with dimensions 91.44 cm length × 91.44 cm width × 30.48 cm height. Just before SI testing, the experimental rat and the partner rat were both carried into the testing room within their home cages. Only one test was performed within the testing room at a time and the behavior box was thoroughly wiped down with a disinfectant cleaner between testing sessions. The protocol used for the SI test has been described previously (Sanders and Shekhar, 1995b; Shekhar and Katner, 1995). In brief, SI testing consists of placing the experimental rat into the SI box simultaneously with an age-, weight-, and sex-matched conspecific partner for a 5-min test session. Each session is video recorded from above and subsequently scored using ODlog for Mac OS X version 2.6.1 by Macropod Software by a treatment blind observer. SI time is measured as the amount of time, in seconds, that the experimental rat spends engaging in non-aggressive physical investigation of the partner rat; defined by the experimental rat sniffing the partner rat (none of the experimental rats displayed aggressive behavior in these studies). Partner initiated contact or investigation was independent of SI time, thus SI times are independent of the partner's behavior (none of the partner rats used in these studies displayed avoidant or aggressive behavior). Partner rats were used for a maximum of two sessions in a single day and these sessions were separated by at least 30 min. This SI procedure has been used for nearly two decades and has been validated as an anxiety measure (Rainnie *et al*, 2004; Sajdyk and Gehlert,

2000; Sanders and Shekhar, 1995a; Shekhar, 1994, 1996, 2002; Shekhar and Katner, 1995; Truitt *et al*, 2007). All SI testing occurred between 0900 and 1300 hours (during the rat's light period).

SI-hab testing paradigm. The SI-hab test, described previously (Truitt *et al*, 2007), consists of daily repeated SI test sessions for a minimum of 5 consecutive days. Variables that were manipulated during SI-hab testing include lighting condition (bright light challenge or dim light control), familiarity of partner rat (novel or familiar partner (FP) rat for each day of SI-hab testing) and pharmacological interventions.

Surgical Techniques

Rats were anesthetized by placing them in a Plexiglas box connected to an IsoFlurane system (MGX Research Machine, Vetamac, Rossville, IN). The animals were then placed on a stereotaxic instrument (Kopf Instruments, Tujunga, CA) with the incisor bar set at -4.5 mm and kept under a constant flow of isoflurane through a Plexiglas nose cone. Rats were implanted bilaterally with 26-gauge microinjection guide cannula (Plastics One, Roanoke, VA) directed toward the IL PFC (AP $+3.2$ mm, ML ± 0.7, DV -5 mm) according to Paxinos and Watson (2005) atlas of rat brain. All rats were given a minimum of 4 days recovery before any behavioral testing. During recovery, rats were gently handled each day for a minimum of 2 min.

Histology

Rats with guide cannula were killed following the conclusion of experiments, and brains were removed, flash frozen, and stored at $-80\,^{\circ}$C until processed. Frozen brains were sliced coronally at 30 μm and every third section (separated by 90 μm) was placed on a microscope slide. The sections were counterstained on the slides with cresyl violet. The location of bilateral injection sites was determined by damage left by cannula and injectors from these 30-μm coronal Nissl-stained sections through the frontal cortex at $\times 5$ magnification, and confirmation at $\times 40$ (when needed), using rat brain atlas for guidance (Paxinos and Watson, 2005). In all cases both injection sites were located within the area designated IL cortex within the PFC (Paxinos and Watson, 2005).

Statistics

All data were analyzed using Prism 6.0 Software (La Jolla, CA) and all data are presented as mean ± SEM. The dependent variable for SI testing was duration of SI (seconds). Comparisons of these data between two groups with only one time point were made using Student's *t*-test. Comparisons from a single treatment group over multiple days were made using a repeated measures one-way ANOVA, whereas comparisons between two groups over multiple days were made using a repeated measures two-way ANOVA. In the presence of significant main effects of day or day-by-treatment interaction, *post hoc* pairwise comparisons were conducted. Dunnett's test was used for pairwise comparisons with the control day (first day of SI-hab testing) within a treatment group; Tukey's HSD test was used for pairwise comparisons of a challenge day with other days within treatment groups (or across days regardless of group when main effect of day was observed in the absence of an interaction); comparisons between treatment groups for a given day were made using Bonferroni's test or Fisher's LSD (where noted). The confidence level for significance in all tests was set at $p<0.05$.

Specific Experimental Protocols

Experiment 1. Rats were first given a baseline SI test in dim red light with a novel partner (NP) rat. Forty-eight hours later, the SI-hab testing paradigm was initiated. Rats were divided into two groups based on lighting conditions during SI-hab testing: dim light ($n=8$) and bright light challenge ($n=7$). On the first SI-hab day, rats were paired with a NP for the SI test. On SI-hab days 2–5, rats were then re-exposed to the same partner (familiar) used in SI-hab day 1.

Experiment 2. The SI-hab paradigm was performed for 6 consecutive days. Rats were divided into two groups based on partner condition. Rats in the novel partner group ($n=8$) were paired with a novel (unfamiliar) partner rat each SI-hab day. Rats in the FP group ($n=8$) were paired with the same partner rat in each SI test. All SI testing sessions were performed under the bright light challenge conditions.

Experiment 3. The SI-hab paradigm was performed in bright light challenge conditions with the same partner (familiar) for SI-hab days 1–5. On SI-hab day 6, the SI testing was done with a NP rat in bright light challenge conditions. The rats were then tested on day 13 with their original FP under bright light challenge conditions, but this time in a novel environment (different SI box in same testing room).

Experiment 4. SI-hab paradigm was performed for 6 consecutive days. Days 1–5 were under dim light conditions and with the same partner rat. On day 6, rats were divided into two groups based on partner condition. Here, rats were paired with either a NP ($n=5$) or the same partner they had been paired with for the first 5 days. The SI session on day 6 was performed under bright light challenge conditions.

Experiment 5. All SI tests were performed under bright light challenge conditions. SI-hab testing was performed for 8 consecutive days with the same partner rat. On SI-hab days 1–5 and again on day 8, rats were given a sham intracranial (i.c.) injection 10 min before SI testing (consisting of a mock i.c. injection). Ten minutes before SI testing on days 6 and 7, rats were given bilateral i.c. injections into the IL of the mPFC of either 90 pmol muscimol (Musc; Sigma-Aldrich, St Louis, MO) dissolved in 0.9% saline or 0.9% saline vehicle (Veh) at an injection volume of 100 μl. The experiment was done in a counter-balanced, cross-over design; an injection of one treatment (either Veh or Musc) on day 6 and the opposite treatment on day 7. This dose of Musc is similar to what has been used to suppress mPFC nuclei specifically in relation to social or fear/anxiety studies (Sierra-Mercado *et al*, 2011; van Kerkhof *et al*, 2013a). Infusions were done at a rate of

100 μl/min and injectors were allowed to remain in for an additional minute before removal. On days 11 and 12, rats once again were given bilateral i.c. injections into the IL of either 90 pmol Musc or Veh in another counterbalanced cross-over design, 10 min before SI testing with a NP rat under bright light challenge conditions.

Experiment 6. To habituate rats to being injected, rats were brought into the behavior staging room and were subcutaneously (s.c.) injected with 0.9% saline (1.0 ml/kg) once per day for the 2 days before the SI-hab paradigm. The SI-hab paradigm was performed using the following protocol; rats were injected 30 min before SI testing, each SI session was under bright light challenge conditions and with the same partner rat for SI-hab days 1–5. On SI-hab day 6, rats were challenged with a NP under bright light challenge conditions and 30 min following injection. All rats were injected with saline (1.0 ml/kg s.c.) on SI-hab day 1, the first exposure to the partner rat. Rats were divided into two groups based on the type of injection they received on SI-hab days 2–6. On these days, rats were either injected with saline (Veh group, $n=5$) or D-cycloserine (10 mg/kg in a volume of 1.0 ml/kg; DCS group, $n=5$). The dose of D-cycloserine was chosen because it was in the low-dose range that was still effective at enhancing safety learning (Ledgerwood *et al*, 2003; Walker *et al*, 2002).

RESULTS

Anxiety-Like Behavior is Reduced with Repeated Exposures to a Familiar Conspecific

To determine whether the bright light challenge reduced SI times, rats were divided into two groups based on lighting conditions during SI testing, dim red lights (control, $n=11$) or bright light challenge, transitioning from dim light to bright light immediately after being placed in the SI chamber (bright light challenge, $n=11$). Bright light challenge rats had significantly reduced SI time compared with control rats (SI time (mean ± SEM) control = 21.45 ± 1.10 and challenge = 12.84 ± 1.08; two-tailed unpaired *t*-test $t_{20}=5.57$, $p<0.0001$, data not shown). In experiment 1, the effects of the bright light challenge were investigated in the SI-hab paradigm. The basic experimental protocol for experiment 1 is illustrated in the top of Figure 1a. In brief, rats received a baseline SI testing session under dim light and NP conditions (baseline). After 48 h, the SI-hab protocol began and continued for 5 consecutive days (SI-hab days 1–5). On the first SI-hab day, rats were paired with a novel SI partner and then re-exposed to that same partner (familiar) for the remainder of the experiment (SI-hab days 2–5). Rats were divided into two groups based on lighting conditions during SI-hab testing: dim light ($n=8$) and bright light challenge ($n=7$). This paradigm produced a main effect of day and day × lighting condition interaction on SI times (two-way repeated measures ANOVA, day $F_{5,65}=7.56$, $p<0.0001$; day × lighting condition $F_{5,65}=3.95$, $p=0.0034$, Figure 1a). The bright light challenge significantly reduced SI times in the bright light group on the first 2 days of SI-hab testing compared with baseline (Tukey's $p=0.0043$ and $p=0.0088$, respectively) and control rats (Bonferroni's $p=0.013$ and $p=0.047$, respectively). In the bright light group, rats were repeatedly exposed to the same partner. SI times significantly increased on the fourth and fifth exposure to the same, familiar, partner rat compared with the SI times of the first exposure to the partner, SI-hab day 1 (Dunnett's $p=0.0028$ and $p<0.0001$, respectively). This increase in SI time observed over the SI-hab paradigm was not observed in

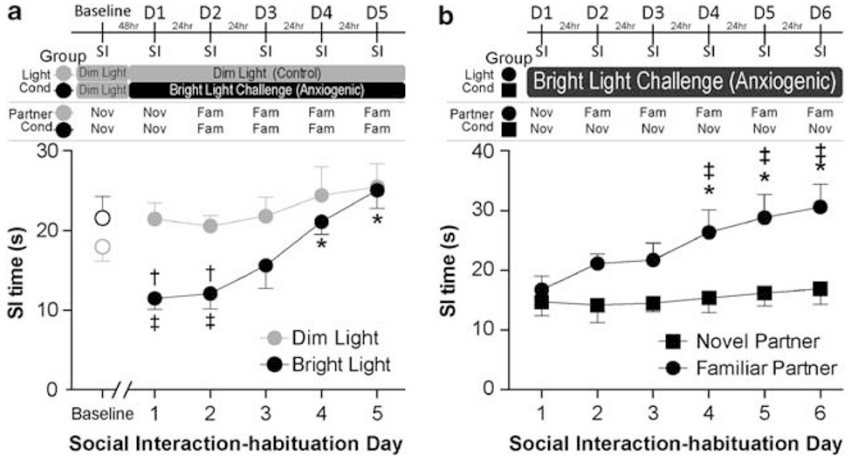

Figure 1 Anxiety-like behavior is reduced with repeated exposures to a familiar conspecific. (a) The effects of social familiarity on anxiety-like responses to bright light challenge was investigated using the procedure illustrated in (a, top). Presented here are the mean ± SEM of social interaction (SI) time in rats continually tested under dim light conditions (dim light, $n=8$) and rats tested under bright light challenge conditions (bright light, $n=7$) during social interaction–habituation test (SI-hab) days. Exposure to bright light challenge significantly reduced SI times in the bright light group compared with baseline and dim light group. Repeated exposure to the same partner rat (social interacton-habituation, days 1–5) increased SI times compared with SI-hab day 1 on the fourth and fifth exposure selectively in bright light rats, but not dim light rats. (b) Presence of a familiar partner (FP), but not a novel partner (NP), selectively increases SI time during bright light challenge. Presented in b (top) is the procedural schematic used for this experiment. Presented in b (bottom) are mean ± SEM SI times for six sessions of SI-hab testing with a NP rat (NP, $n=8$) or the same partner rat (FP, $n=8$) in each session. Repeated SI testing selectively increased SI times in the FP group but not the NP group. *Dunnett's $p<0.05$ different from D1; †Tukey's $p<0.05$ different from dim light condition within group (within group (a) and regardless of treatment group (b)); ‡Bonferroni $p<0.05$ different between treatment groups. Fam, familiar partner; Nov, novel partner.

the rats tested under dim light conditions (dim light group). Thus, the increase in SI time that is acquired with multiple exposures to a FP and bright light conditions can be interpreted as a reduction in anxiety-like behavior.

Experiment 2 was designed to investigate the role of social familiarity in the reduction in anxiety-like behavior observed in experiment 1. Here, rats were divided into two groups, familiar partner ($n=8$) or novel partner ($n=8$). Rats were exposed to the SI-hab protocol as described for experiment 1 with the following exceptions: all SI sessions were performed under bright light challenge conditions, and rats in the novel partner group were tested with a NP during each SI session, whereas rats in the FP group were tested with the same partner during each of the six SI-hab sessions (days 1–6, Figure 1b top). Here, main effects of day, partner condition, and a day × partner condition interaction were observed (two-way repeated measures ANOVA, day $F_{5,70}=7.53$, $p<0.0001$; partner condition $F_{1,14}=7.13$, $p=0.018$; day × partner condition $F_{5,70}=3.72$, $p=0.0048$ Figure 1b graph). SI times increased over the SI-hab days in the FP group but not in the NP group, with SI times in the fourth through sixth sessions being significantly increased compared with SI time of the first exposure to the partner (Dunnett's $p\leqslant 0.0003$) and with SI times of the NP group (Bonferroni's $p\leqslant 0.038$). Collectively, the results from experiments 1 and 2 suggest that social familiarity has a role in the anxiolytic-like response acquired over multiple exposures to the bright light challenge.

The Role of Contextual Cues During Acquisition and Expression of Anxiolytic-Like Responses to Social Familiarity

Experiment 3 was designed to determine the role of social and environmental context in the expression of anxiolytic-like behaviors observed following repeated pairing of a FP and bright light challenge. Rats were exposed to the SI-hab paradigm with the same partner rat under bright light challenge conditions on SI-hab days 1–6. As observed in previous experiments, repeated SI testing with the same partner under bright light challenge conditions leads to increases in SI times (one-way repeated measures ANOVA $F_{5,30}=4.88$, $p=0.015$). SI times on days 5 and 6 were significantly increased compared with the first exposure to the partner (Dunnett's $p\leqslant 0.014$; Figure 2a). Rats were exposed to a NP challenge on SI-hab day 7. Exposure to the NP, under bright light challenge conditions, resulted in SI times similar to day 1 and significantly reduced from day 6 (Tukey's $p=0.043$), suggesting that the presence of the FP is required for the expression of the anxiolytic-like behavior. Rats were then exposed to a novel environment challenge, where rats were once again paired with the FP (used on days 1–6) under bright light challenge conditions but tested in a different SI apparatus (black colored walls compared with the light blue colored walls of the SI apparatus used for all previous SI sessions). SI times in the novel environment were once again significantly greater than day 1 SI times (Dunnett's $p=0.025$) and SI times during the NP challenge (Tukey's $p=0.0012$).

Experiment 4 was designed to investigate whether rats will acquire anxiolytic-like responses to social familiarity when the repeated exposure to a FP is performed in the absence of an anxiogenic context. Here, rats were paired with the same partner rat (FP) for 5 consecutive SI-hab days under dim light conditions (FP:dim). The sixth SI-hab day was a challenge day where all SI testing was performed under bright light challenge conditions, and rats were either paired with a NP or with the same partner (FP) they had been paired with in the previous five SI-hab sessions that were all performed under dim light conditions. This

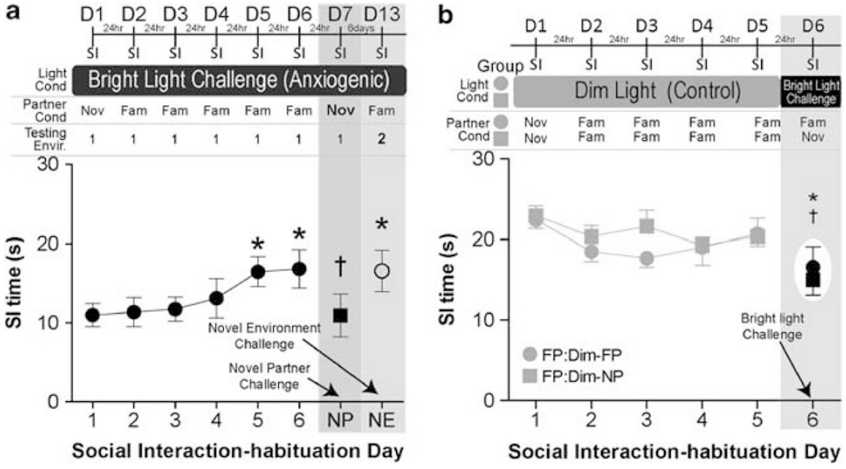

Figure 2 Effects of social, environmental and anxiogenic context on expression and acquisition of social familiarity-induced anxiolysis (SFiA). Presented in (a) are mean ± SEM social interaction (SI) times for rats tested under bright light challenge conditions with the same partner for six social interaction–habituation test (SI-hab) sessions, a novel partner challenge (NP) on the seventh (see a top for procedural schematic; NP challenge shaded region). (b) The effect of context during social familiarity trials on social familiarity-induced reductions in anxiety-like response was investigated using the procedure illustrated in b (top). All rats were paired with the same partner (familiar partner, FP) for SI testing under dim light conditions (FP:dim) for five SI-hab sessions. On the sixth SI-hab session, SI testing was done under bright light challenge conditions in rats paired with their FP (FP:dim-FP, $n=7$) or rats paired with a NP (FP:dim-NP, $n=7$). Bright light challenge reduced SI times regardless of testing with familiar or NP (NP challenge, shaded region). *Dunnett's $p<0.05$ different from Day 1; †Tukey's $p<0.05$ different from Day 5 FP session. Fam, familiar partner; NE, novel environment challenge day; Nov, novel partner; NP, novel partner challenge day.

produced two groups: rats trained with a FP in dim light conditions and exposed to a NP on the challenge day (FP:dim-NP, $n=7$), and rats trained with a FP in dim light conditions and exposed to the same partner on the challenge day ((FP:dim-FP, $n=7$), see procedural schematic Figure 2b top). This procedure resulted in a significant main effect of day/light condition (two-way repeated measures ANOVA, day $F_{5,55}=4.36$, $p=0.0021$), but neither partner condition main effect nor the interaction reached significance. SI times on day 6, the bright light challenge, were significantly lower than SI times on days 1 or 5, regardless of the presence of a familiar or NP rat (Tukey's $p=0.0005$ and $p=0.035$, respectively; Figure 2b). These results suggest that social familiarity-induced anxiolysis (SFiA) is not acquired when social familiarity pairings are done in the absence of the anxiogenic stimulus.

The role of the mPFC in Expression of SFiA

Experiment 5 was designed to investigate the role of the mPFC in the expression of the SFiA. Here, rats ($n=11$) were implanted with bilateral guide cannulae such that injections were into the IL of the mPFC (Figure 3a). These rats were tested in the SI-hab paradigm under bright light challenge conditions with the same partner rat for 8 consecutive days (see top of Figure 3b procedural schematic). Before the SI session on days 1–5 and 8, rats were given a sham injection 10 min before SI testing. Ten min before the SI session on days 6 and 7, rats received i.c. injections of either Musc (90 pmol/100 nl) or saline Veh (100 nl) in a counterbalanced, cross-over design, where six rats received Veh injections on day 6 and Musc injections on day 7, and the other five rats received Musc injections on day 6 and Veh on day 7. Repeated exposure to the same partner rat led to significant increases in SI time (repeated measures ANOVA $F_{10,90}=4.60$, $p<0.0001$, Figure 3b), with SI times significantly increased compared with day 1 on days 4, 5, and 8, and following the IL Veh injection days (Dunnett's $p\leqslant 0.037$). Interestingly, Musc injections into the IL blocked this increase in SI time from day 1 (Dunnett's $p=0.77$) and significantly reduced SI times compared with Veh injections into the IL (Tukey's $p=0.042$). The reduction in SI time following Musc injections into the IL appears specific to social familiarity-induced increases in SI; Musc injections into the IL before SI testing with a NP under bright light conditions had no effect on SI time compared with Veh injections (Figure 3a, NP challenge, shaded area of graph). SI times with NP following Veh or Musc injections were significantly reduced compared with SI times following Veh injections with a FP (Tukey's $p\leqslant 0.026$) and were similar to day 1 SI times. Conversely, under dim light conditions and pairing with a novel rat, Musc injections into the IL resulted in a slight but significant increase in SI time compared with Veh injections into the IL (mean ± SEM SI time, Veh 21.45 ± 0.79, Musc 25.93 ± 0.65; paired two-tailed *t*-test $t_{10}=2.28$, $p=0.046$).

D-Cycloserine Injections before Social Familiarity Pairings Increased the Rate that SFiA was Acquired

Experiment 6 was designed to determine whether the acquisition of SFiA could be enhanced by pairing social familiarity exposure with systemic injections of the cognitive enhancer D-cycloserine. Here, we *a priori* defined SFiA acquisition as a significant increase in SI time compared with the first exposure to the partner (SI-hab

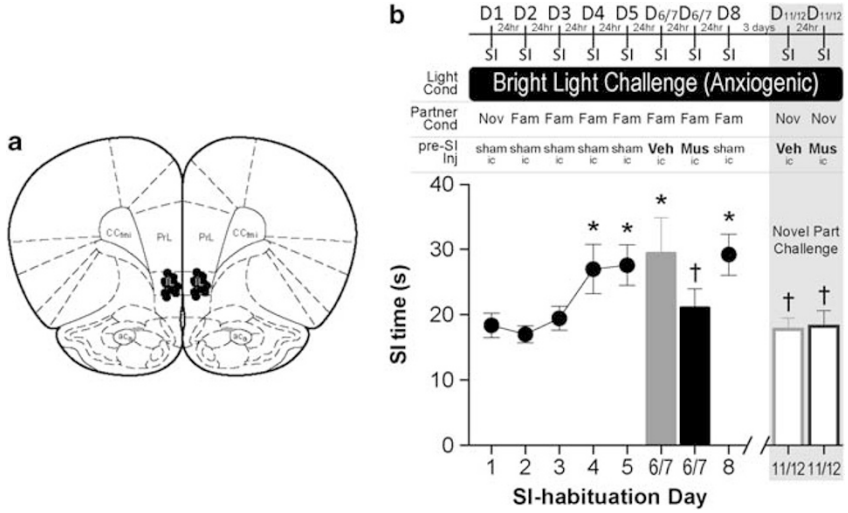

Figure 3 Inhibiting the mPFC selectively blocks expression of social familiarity-induced anxiolysis (SFiA). Presented in (a) is a schematic representation of the injection sites (modified from (Paxinos and Watson, 2005)). Pairs of black circles represent the bilateral injection site of each rat. Injections sites were tightly located in the IL from + 4.0 to + 3.5 mm bregma. ac_a, anterior commissure, anterior part; CC_{fmi}, corpus callosum forceps minor; IL, infralimbic cortex; PrL, prelimbic cortex. Presented in (b) are mean ± SEM social interaction (SI) times for rats ($n=11$) tested under bright light challenge conditions with the same partner for eight social interaction–habituation test (SI-hab) sessions (see top of figure for procedural schematic). Muscimol (Musc—solid black bar), but not vehicle (Veh—solid gray bar), injected into the IL 10 min before SI testing blocked the social familiarity-induced increase in SI times observed over the first five SI-hab sessions. The blockade of SFiA by Musc into the mPFC was temporary, as SI times were significantly increased compared with SI times following the Musc injections on the next SI-hab session. In the novel partner (NP) challenge (shaded region), SI times following both Veh (open gray bar) and Musc (open black bar) injections into the mPFC were reduced equally compared with those of Veh injections with familiar partner (FP, solid gray bar). *Dunnett's $p<0.05$ different from D1; †Tukey's $p<0.05$ different Veh injection with FP.

day 1), and the rate of acquisition as the number of SI-hab pairings required to achieve this significant increase in SI time. To habituate rats to being injected, rats were brought into the behavior staging room and were subcutaneously (s.c.) injected with 0.9% saline (1.0 ml/kg) once per day for two days before the SI-hab paradigm. The SI-hab paradigm was performed using the following protocol (Figure 4 top); rats were injected 30 min before SI testing, each SI session was under bright light challenge conditions and with the same partner rat for SI-hab days 1–5. On SI-hab day 6, 30 min following injection, rats were challenged with a NP under bright light challenge conditions. All rats were injected with saline (1.0 ml/kg s.c.) on SI-hab day 1, the first exposure to the partner rat. Rats were divided into two groups based on the type of injection they received on SI-hab days 2–6. On these days, rats were either injected with saline (Veh group, $n=5$) or D-cycloserine (10 mg/kg in a volume of 1.0 ml/kg; DCS group, $n=5$). As previously observed, social familiarity produced an increase in SI time across days (two-way repeated measures ANOVA main day effect $F_{5,\ 40}=13.16$, $p<0.0001$ Figure 4). However, D-cycloserine treatment affected the rate at which this increase in SI time occurred over the first 3 days of repeated exposure to the partner rat (day × treatment interaction $F_{2,16}=7.84$, $p=0.0042$). Rats treated with D-cycloserine had significantly increased SI times on the third SI session (SI-hab day 3) and lasting through session 5, compared with the first day of exposure to the partner (Dunnett's $p\leqslant 0.031$), whereas SI times of Veh-treated rats were not significantly increased, compared with day 1, until the fifth exposure to the partner rat (Dunnett's $p=0.002$). In addition, the SI times of the DSC group were significantly increased compared with Veh group SI times on SI-hab days 3 and 4 (Fisher's LSD $p\leqslant 0.027$). As D-cycloserine has previously been reported to have prosocial effects in mice (Jacome *et al*, 2011), both Veh- and D-cycloserine-injected rats were exposed to a NP challenge for the sixth SI session. If D-cycloserine injections were producing prosocial effects, then it would be expected that the SI times during the NP challenge would remain elevated and increased compared with the SI times of the first SI-hab session (day 1) and with SI times of the Veh group. SI pairing with a NP resulted in a significant reduction in SI time compared with SI times of the rat's previous SI session (day 5) for both D-cycloserine- and Veh-treated rats (Tukey's $p=0.018$ and $p=0.005$, respectively). Furthermore, the SI times for each group were not significantly different compared with day 1 SI times (Dunnett's (Veh) $p=0.999$ and (DCS) $p=0.073$) or between groups (day 6, Fisher's LSD $p=0.072$). However, comparing D-cycloserine effects on SI time only during the NP conditions (day 1 and day 6) resulted in a main effect of day (two-way repeated measures ANOVA main day effect $F_{1,\ 8}=5.89$, $p=0.041$) and a day × treatment interaction ($F_{1,\ 8}=6.40$ $p=0.035$). In this, less stringent analysis (as a result of reducing the multiple comparisons) SI times of DCS rats are significantly greater on day 6 compared with day 1 (within) and also compared with SI time of Veh rats on day 6 (Fisher's LSD $p=0.008$ and $p=0.024$, respectively). Thus, interpretations of these data are limited.

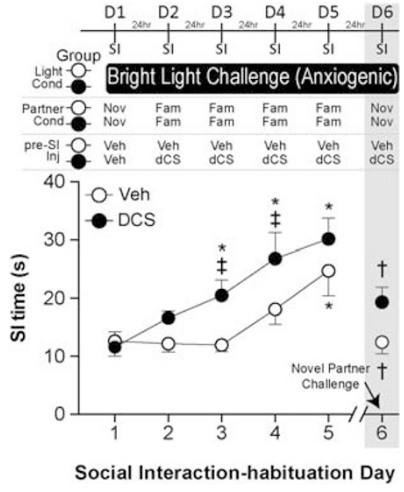

Figure 4 D-cylcoserine injections enhanced the rate at which SFiA is acquired. Presented in the top of the figure is a schematic representation of the protocol used for this experiment. Presented here are mean ± SEM social interaction (SI) times for rats injected with vehicle (Veh, $n=5$) or D-cycloserine (DCS, $n=5$). All SI testing was performed under bright light challenge conditions and all rats were initially injected with vehicle (0.9% saline) 30 min before SI testing on day 1. On subsequent days, the D-cycloserine (DCS 10 mg/kg) replaced vehicle injection in the DCS group. Both groups of rats expressed social familiarity-induced anxiolysis (SFiA). However, the DCS rats expressed it more rapidly than Veh rats (DSC rats day 3, Veh rats day 5). Novel partner (NP) challenge (day 6 shaded region) reduced SI times compared with day 5 in both DCS and Veh rats. *Dunnett's $p<0.05$ different from Day 1; †Tukey's $p<0.05$ different from Day 5 within group; ‡Fisher's LSD $p\leqslant 0.027$ different between treatment groups.

DISCUSSION

Social Familiarity-Induced Anxiolysis

The concept that overcoming fear and anxiety is easier in the presence of a familiar person (eg, friend or therapist) is commonly accepted and a critical component of cognitive behavioral therapy (Baldwin *et al*, 2007; Martin *et al*, 2000; McHugh *et al*, 2013; Roshanaei-Moghaddam *et al*, 2011). Yet, few studies have systematically investigated this effect and little is known of the neural mechanisms that regulate this SFiA. The current study is one of the first to systematically investigate SFiA in an animal model. Here, we present an animal model in which social familiarity selectively reduces anxiety-like responses to a naturally anxiogenic stimulus, bright light challenge, but does not alter baseline anxiety behaviors as measured by the SI test (Crawley and Goodwin, 1980; de Jongh *et al*, 2002; DeFries *et al*, 1966; Walker and Davis, 1997). Previous studies using the SI test reported that bright light only consistently reduced SI times when paired with a novel environment (File and Hyde, 1978). To enhance the anxiogenic effect of bright light in the SI test, a bright light challenge, consisting of quickly transitioning from dim light to bright light after the rats are in the SI chamber, was used in the current study. Here, the bright light challenge resulted in consistent and reliable reductions in SI times, and the rats did not habituate to this anxiogenic stimulus over 6 days. The SI times observed in these experiments are within the range of SI scores from similar experiments using the same SI scoring methodology (Sajdyk and Gehlert, 2000; Shekhar *et al*, 2002; Truitt *et al*, 2007).

The reduction in SI times induced by the bright light challenge was overridden by the fourth or fifth pairing with a familiar rat. These increases in SI time with repeated exposures to a familiar rat appear to be an anxiolytic-like response rather than a general increase in prosocial behavior. Social familiarity did not affect SI times in the absence of anxiogenic stimuli and anxiety-like responses to the bright light challenge returned when the FP rat was replaced with a NP. These results are similar to past findings in which social familiarity had no effect on control rats, but reduced anxiety-like behaviors in rats made persistently anxious by a pharmacological manipulation directly to the BLA (Truitt *et al*, 2007), a procedure that leads to lasting increases in anxiety-like behaviors and increased excitability of the BLA (Rainnie *et al*, 2004; Truitt *et al*, 2007). In addition, acquisition of this anxiolytic-like behavior appears to be specifically linked to the FP, as rats failed to acquire an anxiolytic-like response to the bright light challenge when a NP was used for each of the repeated SI sessions. Furthermore, the anxiolytic-like response in the presence of a socially FP remained even when the SI test was done in a different testing environment. Collectively, these results support the idea that the acquisition of anxiolytic-like behavior in this paradigm is dependent on social familiarity and unlikely to be a result of habituation to the testing environment or the bright light stimulus. In addition to social familiarity, acquisition of the anxiolytic-like behavior also appears to be linked to anxiety-like conditions during repeated pairing with the partner rat. Social familiarity had no effect on anxiety-like behavior when the repeated pairings with the same partner rat was done under dim light (low anxiety-like) conditions.

Social Familiarity and Safety Cues

SFiA appears to be acquired over multiple pairings of a FP rat and the anxiogenic stimulus. After SFiA is acquired, the presence of the familiar rat remains pivotal for the expression of the anxiolysis, as anxiety-like behavior returns when the familiar rat is replaced with a novel rat (current data and (Truitt *et al*, 2007)). Collectively, these data could be interpreted, as SFiA is a conditioned response and the familiar rat acts as a cue. However, typically when cues are repeatedly paired with unconditioned aversive stimuli, they are avoided or the cue itself starts to induce fear or anxiety responses (Maren and Quirk, 2004; Thielen and Shekhar, 2002). As the presence of the FP reduces anxiety-like behavior, the partner rat may be acting as a safety cue, in which case SFiA could be considered a form of safety learning (Christianson *et al*, 2012). In support of this concept, it was observed that the context during the repeated exposures to a conspecific determined the extent to which anxiolysis would result upon subsequent exposures. When the social familiarity training occurred under non-threatening conditions, social familiarity failed to induce anxiolysis in response to the bright light challenge. At this point, it is unclear whether the presence of the anxiogenic stimulus during the social familiarity training session enhances the social memory of the partner rat or is required to activate an anxiolytic pathway that is specific to the type of anxiogenic cue.

The mPFC and SFiA

The rodent and human mPFC are implicated in both social processing and cortical regulation of anxiety/fear, making it a compelling target as the cortical site for regulation of SFiA (Adolphs, 2010; Fossati, 2012; Hartley and Phelps, 2010; Meyer-Lindenberg and Tost, 2012; Milad and Quirk, 2002; van Kerkhof *et al*, 2013a, 2013b). In the current study, expression of SFiA appears to be dependent on an active mPFC. Temporary inhibition of the mPFC, by local injections of Musc into the IL (of the mPFC), blocked the social familiarity-induced reduction in anxiety-like responses to the bright light challenge. This effect of inhibiting the mPFC appeared selective to SFiA, as it did not alter anxiety-like responses to the bright light challenge in the presence of a novel rat. In this study, all of the injections were localized to the IL; however, it is possible that the effects of the Musc injections were as a result of diffusion beyond the IL, and thus our interpretation of these results are limited to the mPFC. Although inhibiting the mPFC appears to selectively suppress SFiA, it is unclear if this suppression is a result of disrupting top-down regulation of anxiety or a result of disrupting social cognition, such as the ability to recall the partner rat as familiar. In terms of social cognition, the mPFC is cited as a locus for integration of social stimuli and emotional responses (Adolphs, 2009; Amodio and Frith, 2006). Thickness of the (v)mPFC is associated with social functioning and ability to correctly interpret emotion from social cues (Holmes *et al*, 2012). In a recent animal study, the importance of the mPFC in developmentally relevant social behavior, social play, was demonstrated through inactivation of the mPFC (van Kerkhof *et al*, 2013a). The authors also reported that inactivation of the mPFC (in a non-threatening environment) increased social investigation, which is a similar measure to the increase in SI time reported under similar conditions in the current study. Thus under basal conditions, the mPFC appears to suppress SIs/investigations; however, under threatening conditions, the mPFC appears to be involved in the expression of anxiolytic social learning. This latter point is likely related to the mPFC's role in top-down regulation of anxiety. Suppression of anxiety or fear responses by different forms of cognitive behavioral therapies and extinction involves activation of the mPFC (Eisenberger *et al*, 2011; Hartley and Phelps, 2010; Kim *et al*, 2011a; Milad and Quirk, 2002; Phelps *et al*, 2004; Schiller *et al*, 2008; Sotres-Bayon and Quirk, 2010). The mPFC is a site where extinction of conditioned fear is consolidated in rodents (Laurent and Westbrook, 2009; Peters *et al*, 2010; Santini *et al*, 2012). Similarly, the mPFC responds greater to stimuli-signaling safety compared with fear predictive stimuli, in human imaging studies of fear-conditioning extinction (Kalisch *et al*, 2006; Milad *et al*, 2007; Phelps *et al*, 2004) and specifically safety cues in a fear-conditioning reversal paradigm (Schiller *et al*, 2008). In addition, activation of the mPFC during a threat was selectively increased while viewing images of a familiar person compared with an unfamiliar person, and the strength of the relationship with the familiar person correlated positively with threat-induced activation of the mPFC, suggesting that mPFC activation was related to the value of the person as a safety

signal (Eisenberger *et al*, 2011). The value of familiar rats as a safety signal in the current study was also dependent on the mPFC.

Enhancement of SFiA with D-Cycloserine

The current data support the idea that in the process of SFiA, the FP rat becomes a safety signal. This is based on the observations that acquisition of SFiA appears to require repeated pairings of the socially FP rat with the anxiogenic stimulus, the presence of the familiar conspecific is necessary for the expression of SFiA, and SFiA is dependent on an active mPFC that is a pivotal site for safety learning. Safety learning in humans and rodents can be enhanced by pairing the safety learning with D-cycloserine (Davis *et al*, 2006; Gupta *et al*, 2013; Hofmann *et al*, 2006b). D-cycloserine augmentation of cognitive behavioral therapy for social anxiety, in particular, was associated with a faster rate of improvement (Hofmann *et al*, 2013). Similarly, in the current study, pretreatment with D-cycloserine before pairings with the familiar conspecific reduced the number of SI training sessions required to reduce the anxiety-like response to the anxiogenic challenge. A potential caveat of this observation is that D-cycloserine treatment increased prosocial behavior in other rodent models (Myers and Carlezon, 2012) that could confound the current interpretations of the SI behavior. To determine the extent to which prosocial effects of D-cycloserine were contributing to the enhanced SFiA, rats were pretreated with D-cycloserine and tested with a NP after SFiA was established. Here, in the presence of a NP, SI times compared with the last SFiA session were significantly reduced compared with the last SFiA session and no longer significantly higher than day 1, regardless of receiving D-cycloserine or Veh injection. From these data, it can be interpreted that the enhanced acquisition of SFiA observed with D-cycloserine was at least in part due to enhanced social learning rather than enhanced prosocial behavior. Alternatively, the D-cycloserine-treated rats displayed a strong trend toward increased SI times during the NP challenge, which reached significance when not controlling for multiple comparisons, implying the possibility of a prosocial effect induced by D-cycloserine that appears to be additive with the SFiA response. Further studies are needed to fully resolve the mechanism by which D-cycloserine enhances the acquisition of SFiA.

Contrary to the concept that in SFiA, the FP becomes a safety signal is the idea of an innate social buffering response. Social buffering studies have demonstrated that the presence of a conspecific can reduce fear and stress responses in conditioned fear paradigms without any training (Davitz and Mason, 1955; Kiyokawa *et al*, 2009, 2012; Latane, 1969; Terranova *et al*, 1999), suggesting that a conspecific may serve as an external inhibitor of fear or anxiety rather than a safety signal (Christianson *et al*, 2012). Social buffering effects were not directly investigated in the current study of SFiA. However, SFiA differs from social buffering in several key areas. First, SFiA overrides an unconditioned anxiogenic stimulus, whereas most social buffering experiments use a conditioned fear as the stimulus. Next, SFiA was acquired only following 'training', requiring between 4–5 pairings of the familiar conspecific with the anxiogenic stimuli, suggesting that the social familiarity is acting more like a safety signal than external inhibitor. Finally, expression of SFiA appears to require an active mPFC, whereas social buffering effects in response to the presence of a conspecific at the time of testing appear to be independent of mPFC activation (Kiyokawa *et al*, 2007, 2009).

Concluding Remarks

The current study is one of the first to present a preclinical behavioral model by which the anxiolytic effects of social familiarity can be investigated. In this model, reductions in anxiety-like responses are selective to social familiarity and appear to require the presence of the FP. SFiA appears to be a learned response, where the context in which social familiarity is established determines the extent to which familiarity will induce anxiolysis. In addition, the mPFC is critical to expression of SFiA, similar to findings in other safety-learning paradigms. Finally, the cognitive enhancer D-cycloserine enhanced the acquisition of SFiA.

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