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Neuropeptide Y reduces expression of social fear via simultaneous activation of Y1 and Y2 receptors

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Abstract

Background: Neuropeptide Y (NPY) has anxiolytic effects and facilitates extinction of cued and contextual fear in rodents, thereby acting as a resilience factor against exaggerated fear responses after adverse events. We investigated whether NPY influences acquisition, expression and extinction of social fear in a mouse model of social fear conditioning (SFC).

Methods: NPY was administered intracerebroventricularly before SFC or before social fear extinction with or without prior administration of Y1 and/or Y2 receptor antagonists.

Results: We show that NPY affects SFC-induced social fear in a time point–dependent manner. When administered before SFC, NPY did not affect acquisition, expression and extinction of social fear. However, when administered before social fear extinction, NPY reduced expression of social fear via simultaneous activation of Y1 and Y2 receptors. As such, neither the Y1 receptor antagonist BIBO3304 trifluoroacetate nor the Y2 receptor antagonist BIIE0246 was able to block the effects of NPY completely. However, when administered in combination, they completely blocked the effects of NPY on social fear expression.

Conclusions: These findings have important clinical implications, as they suggest that although medication strategies aimed at increasing brain NPY activity are unlikely to prevent the formation of aversive memories after a traumatic social experience, they might improve the recovery from a traumatic social experience by reducing the expression of social fear.

Keywords

Social fear, extinction, social investigation, neuropeptide Y, fear learning, fear expression

Introduction

The appropriate display of social behaviours is essential for the well-being and survival of social species, and disorders associated with social deficits, such as social anxiety disorder (SAD), are highly debilitating (Morrison and Heimberg, 2013). Given that social fear and avoidance of social situations are the main behavioural symptoms of SAD, the best treatment outcomes are obtained with cognitive–behavioural therapy (Fedoroff and Taylor, 2001), which leads to gradual fear extinction, that is, a decline in the fear response as a result of repeated exposure to the feared situation. The pharmacotherapy for SAD is limited to medication originally designed for depression or generalised anxiety, such as antidepressants and benzodiazepines (Blanco et al., 2013; Fedoroff and Taylor, 2001; Gould et al., 1997). However, many SAD patients achieve only partial remission of symptoms or show a high rate of relapse after treatment discontinuation (Blanco et al., 2002), highlighting the necessity for more specific treatment options.

Recently, neuropeptides have emerged as viable research candidates due to their role in stress-related and social behaviours. Neuropeptide Y (NPY), a peptide that is 36 amino acids long, is the most abundant and widely distributed neuropeptide in the mammalian brain. It is expressed in brain regions involved in social behaviour and the fear circuitry, such as the amygdala, hippocampus, septum, periaqueductal grey, locus coeruleus, cerebral cortex, basal ganglia, hypothalamus and thalamus (Chang et al., 1985; De Quindt and Emson, 1986; Lynch et al., 1989). NPY exerts its biological effects through five subtypes of G_i-protein-coupled receptors termed Y1, Y2, Y4, Y5 and Y6 (Blomqvist and Herzog, 1997). Y1, Y2 and Y5 receptors are the most prominent in the brain and are expressed in limbic brain areas, such as the hippocampus, amygdala, cingulate cortex, thalamus, hypothalamus and cerebral cortex (Dumont et al., 1993, 1996; Parker and Herzog, 1999). NPY and its receptors regulate important biological and pathophysiological functions, such as blood pressure, neuroendocrine secretions, seizures, neuronal excitability and neuroplasticity (Stanley and Leibowitz, 1984; Colmers and Bleakman, 1994; Vezzani et al., 1999; Michalkiewicz et al., 2001; Magni, 2003; Hökfelt et al., 2008). NPY has also been shown to cause a variety of behavioural effects, such as stimulating food intake (Stanley and Leibowitz, 1984), promoting social interaction by acting on Y1 and possibly on Y2 receptors (Sajdyk et al., 1999; Sajdyk et al., 2002) and exerting anxiolytic and antidepressant-like effects by acting mainly on Y1

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and Y2 receptors (Enman et al., 2015; Redrobe et al., 2002). These anxiolytic and prosocial effects of NPY suggest its potential benefit in disorders associated with social anxiety and fear. This might also be suggested by the fact that NPY affects different aspects of fear-related behaviour. As such, NPY was shown to impair acquisition and consolidation in cued and contextual fear conditioning by acting on Y1 receptors (Broqua et al., 1995; Karlsson et al., 2005; Lach and De Lima, 2013) and to impair consolidation and retrieval of fear memories by acting on Y1 and Y2 receptors (Fendt et al., 2009; Gutman et al., 2008; Verma et al., 2015). Furthermore, NPY was shown to facilitate the extinction of cued and contextual fear possibly by simultaneously acting on Y1 and Y2 receptors (Gutman et al., 2008; Lach and De Lima, 2013; Verma et al., 2012, 2015), but also on Y4 receptors (Verma et al., 2016). NPY is thereby acting as a resilience factor against exaggerated fear responses after stress and adverse events. However, it is not clear whether NPY may also alter acquisition, expression and extinction of social fear.

Therefore, we studied the effects of NPY in an animal model of social fear, namely social fear conditioning (SFC), which was established to mimic the major behavioural symptoms of SAD, that is, reduced social investigation and avoidance of conspecifics as indicative of social fear (Toth et al., 2012, 2013). In this model, social fear is induced by administration of mild electric foot-shocks during the investigation of a conspecific. Repeated exposure of the socially fear-conditioned (SFC^+) mice to unknown conspecifics leads to a gradual decline in the fear response – a process termed 'social fear extinction'. Importantly, treatment of SFC^+ mice with medication used for SAD, such as diazepam and paroxetine, reversed social fear (Toth et al., 2012), providing predictive validity to the SFC model. In the present study, NPY was administered intracerebroventricularly (i.c.v.) before either SFC or social fear extinction in order to determine whether it affects acquisition, expression and/or extinction of social fear. As NPY reduced expression of SFC-induced social fear when administered before social fear extinction, next we determined whether these effects were mediated by Y1 and/or Y2 receptors.

Materials and methods

Animals

CD1 mice (Charles River, Sulzfeld, Germany; 10 weeks old) were individually housed for one week before the experiments started and remained single housed throughout the experiments. Mice were kept under standard laboratory conditions (12-hour/12-hour light/dark cycle, lights on at 06:00 hours, 22°C, 60% humidity, food and water ad libitum). Experiments were performed during the light phase, between 09:00 and 14:00 hours, in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Unterfranken and the guidelines of the NIH. All efforts were made to minimise animal suffering and to reduce the number of animals used.

Stereotaxic cannula implantation

Implantation of the guide cannula (21 G, 8 mm long; Injecta GmbH, Klingenthal, Germany) for i.c.v. infusions was performed under ketamine-xylazine anaesthesia (intraperitoneal injection of 120 mg/kg Ketavet® and 16 mg/kg Rompun®, respectively) as previously described (Kornhuber and Zoicas, 2017; Zoicas et al., 2014, 2016), 2 mm above the right lateral ventricle (from the bregma: + 0.2 mm; lateral: + 1.0 mm; depth: + 1.4 mm). After surgery, mice were handled for five days before experiments started.

Intracerebral infusions

Mice received i.c.v. infusions of either vehicle (Veh; distilled H_2O; 2 µL), porcine NPY (1 nmol/2 µL; PeptaNova, Sandhausen, Germany), selective Y1 receptor antagonist BIBO3304 trifluoroacetate (BIBO; 2 nmol/2 µL; Tocris Bioscience, Bristol, UK), Y2 receptor antagonist BIIE0246 (BIIE; 2 nmol/2 µL; Tocris Bioscience) or a combination of Y1+Y2 receptor antagonists (1 nmol/1 µL BIBO + 1 nmol/1 µL BIIE) via an infusion cannula (23 G, 10 mm long) inserted into the guide cannula and connected via polyethylene tubing to a Hamilton syringe. The infusion system was left in place for 30 seconds following the infusion to allow diffusion of the solution.

The correct infusion site was histologically verified. Accordingly, all guide cannulas were implanted correctly. NPY, BIBO and BIIE doses and timing of administration were selected based on previous studies (Karlsson et al., 2005; Kornhuber and Zoicas, 2017; Redrobe et al., 2002; Zoicas et al., 2014).

SFC paradigm

To induce social fear, mice were conditioned during SFC, and social investigation was assessed during social fear extinction as a read-out of social fear.

SFC. SFC was performed with a computerised fear conditioning system (TSE System GmbH, Bad Homburg, Germany) as previously described (Kornhuber et al., 2019; Toth et al., 2012; Zoicas et al., 2014, 2016; see Toth and Neumann, 2013 for a schematic representation of the SFC paradigm). Mice were placed in the conditioning chamber (45 cm×22 cm×40 cm), and after a 30-second habituation period, an empty wire mesh cage (7 cm×7 cm×6 cm) was placed as a non-social stimulus near one of the short walls. After three minutes, the non-social stimulus was replaced by an identical cage containing an unfamiliar mouse. Unconditioned mice (SFC^-) were allowed to investigate this social stimulus for three minutes, whereas conditioned mice (SFC^+) were given a one-second mild electric foot-shock (0.7 mA) each time they investigated (i.e. made direct contact with) the social stimulus. Mice received between one and four foot-shocks, with a variable inter-shock interval, depending on when direct social contact was made. The number of foot-shocks was assessed as a measure of distress. Mice were returned to their home cage when no further social contact was made for two minutes (average duration of SFC ~10 minutes). All SFC^+ mice investigated the social stimulus and could be conditioned. The time mice spent investigating the non-social stimulus, as a pre-conditioning measure of non-social anxiety, was analysed by an observer blind to the treatment.

Social fear extinction. One day after SFC, mice were exposed in their home cage to three non-social stimuli (i.e. empty cages

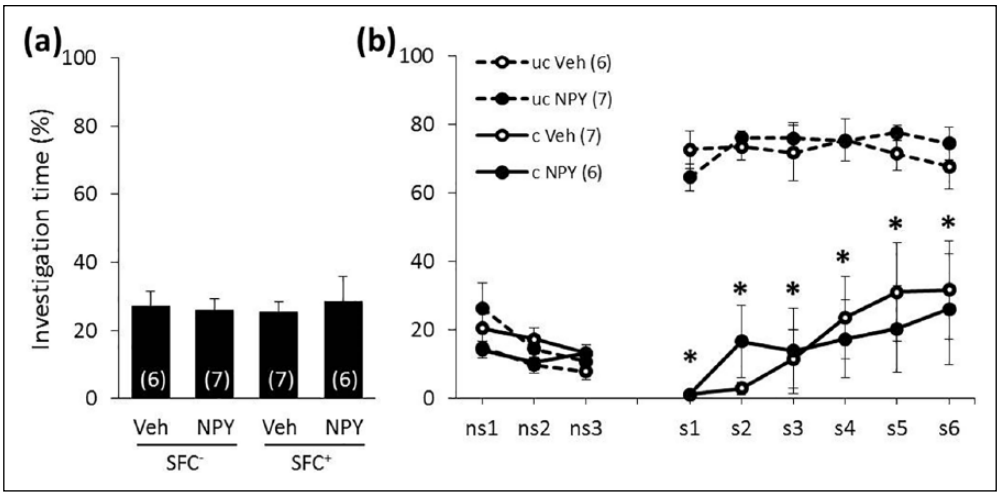

Figure 1. Neuropeptide Y (NPY) does not affect acquisition, expression or extinction of social fear when administered before social fear conditioning. (a) Preconditioning investigation of the non-social stimulus (empty cage) during social fear conditioning (SFC). (b) Investigation of the non-social (ns1–ns3) and social (cages with mice; s1–s6) stimuli during social fear extinction. Unconditioned (SFC^-) and conditioned (SFC^+) mice were infused intracerebroventricularly (i.c.v.) with either vehicle (Veh; 2 µL) or NPY (1 nmol/2 µL) 10 minutes before SFC on day 1. Data represent means±standard error of the mean (SEM), and numbers in parentheses indicate group size. *$p<0.05$ versus respective SFC^- controls.

identical to the cage used on day 1) to assess non-social investigation as a parameter of non-social fear. Mice were then exposed to six unfamiliar social stimuli (i.e. mice enclosed in wire mesh cages) to assess social investigation as a parameter of social fear. Each stimulus was placed near a short wall of the home cage and presented for three minutes, with a three-minute inter-exposure interval. The test was recorded and analysed using JWatcher v1.0 (Macquarie University and UCLA). Non-social investigation was defined as direct sniffing of the empty cage, whereas social investigation was defined as direct sniffing of the cage and/or of the social stimulus inside the cage.

Statistical analysis

For statistical analysis, IBM SPSS Statistics for windows v21 (IBM Corp., Armonk, NY) was used. Data were analysed by one-, two- or three-way analyses of variance for repeated measures, followed by Bonferroni's post hoc analysis whenever appropriate. Statistical significance was set at $p<0.05$.

Results

NPY does not affect social fear learning when administered before SFC

To investigate whether NPY alters the acquisition of social fear (i.e. social fear learning), SFC^+ and SFC^- mice were infused i.c.v. with either Veh or NPY 10 minutes before SFC on day 1.

All mice showed similar investigation of the non-social stimulus during SFC (Figure 1(a); $F(3, 22)=0.08$; $p=0.97$), reflecting similar preconditioning non-social anxiety. All SFC^+ mice received a similar number of foot-shocks during SFC ($T(11)=0.07$; $p=0.95$), reflecting similar distress. During social fear extinction on day 2, mice showed similar non-social investigation, reflecting similar non-social fear after SFC. SFC^+ mice showed reduced social investigation compared to SFC^- mice, independent of treatment, reflecting social fear (Figure 1(b); conditioning effect $F(1, 22)=59.29$; $p<0.001$; conditioning×treatment effect $F(1, 22)=0.33$; $p=0.58$; stimulus×conditioning×treatment effect $F(1, 22)=2.81$; $p=0.96$).

As 1 nmol/2 µL NPY did not alter the acquisition of social fear, other doses of NPY were also tested to verify whether higher or lower NPY concentrations might be needed to modulate social fear learning. Higher doses of NPY (1.5 and 2 nmol/2µL) induced compulsive burying (1.5 nmol/2 µL: observed in 4/5 mice; 2 nmol/2 µL: observed in 5/5 mice) and short-term seizures (1.5 nmol/2 µL: observed in 1/5 mice; 2 nmol/2 µL: observed in 2.5 mice). Lower doses of NPY (0.1 and 0.5 nmol/2 µL) did not affect social fear learning (n=4–5 mice/NPY dose; $F(2, 10)=0.51$; $p=0.61$; data not shown).

NPY reduces expression of social fear when administered before social fear extinction

To investigate whether NPY alters expression and/or extinction of social fear, SFC^+ and SFC^- mice were infused i.c.v. with either Veh or NPY 10 minutes before social fear extinction on day 2.

All mice showed similar investigation of the non-social stimulus during SFC on day 1 (Figure 2(a); $F(3, 32)=0.08$; $p=0.97$), reflecting similar preconditioning non-social anxiety. All SFC^+ mice received a similar number of foot-shocks during SFC ($T(16)=0.00$; $p=1.0$), reflecting similar distress. During social fear extinction on day 2, mice showed similar non-social investigation, reflecting similar non-social fear after SFC. While Veh-treated SFC^+ mice showed reduced social investigation compared with all other groups, reflecting social fear (Figure 2(b); conditioning×treatment effect $F(1, 32)=26.41$; $p<0.001$; stimulus×conditioning×treatment effect $F(8, 256)=5.44$; $p<0.001$), NPY increased social investigation starting from the first social stimulus, reflecting reduced expression of social fear.

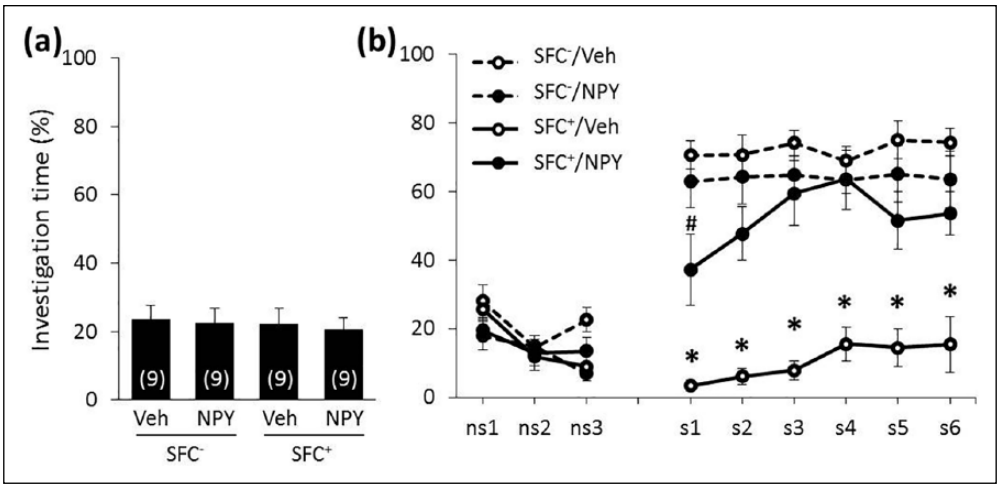

Figure 2. NPY reduces expression of social fear when administered before social fear extinction. (a) Preconditioning investigation of the non-social stimulus (empty cage) during social fear conditioning. (b) Investigation of the non-social (ns1–ns3) and social (cages with mice; s1–s6) stimuli during social fear extinction. Unconditioned (SFC^-) and conditioned (SFC^+) mice were infused i.c.v. with either vehicle (2 µL) or NPY (1 nmol/2 µL) 10 minutes before social fear extinction on day 2. Data represent means±SEM, and numbers in parentheses indicate group size. *$p<0.05$ versus all groups; #$p<0.05$ versus SFC^-/NPY and SFC^+/Veh groups.

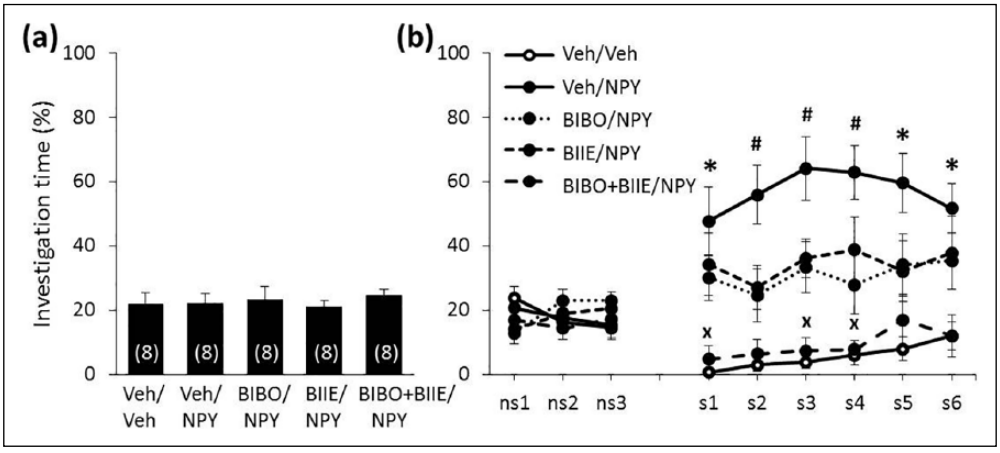

Figure 3. NPY reduces expression of social fear via simultaneous activation of Y1 and Y2 receptors. (a) Preconditioning investigation of the non-social stimulus (empty cage) during social fear conditioning. (b) Investigation of the non-social (ns1–ns3) and social (cages with mice; s1–s6) stimuli during social fear extinction. Conditioned mice (SFC^+) were infused i.c.v. with either vehicle (2 µL), BIBO3304 trifluoroacetate (BIBO; Y1 receptor antagonist; 2 nmol/2 µL), BIIE0246 (BIIE; Y2 receptor antagonist; 2 nmol/2 µL) or a combination of BIBO and BIIE (1 nmol/1 µL BIBO + 1 nmol/1 µL BIIE) 20 minutes before social fear extinction on day 2. After 10 minutes, mice were infused again with Veh (2 µL) or NPY (1 nmol/2 µL). Data represent means±SEM, and numbers in parentheses indicate group size. *$p<0.05$ versus Veh/Veh and BIBO+BIIE/NPY; #$p<0.05$ versus all groups; x$p<0.05$ versus BIBO/NPY and BIIE/NPY.

However, NPY did not completely reverse social fear in SFC^+ mice, as social investigation reached levels found in SFC^- mice starting from the second social stimulus.

NPY reduces expression of social fear via simultaneous activation of Y1 and Y2 receptors

To investigate whether the effects of NPY on social fear are mediated by the Y1 and/or Y2 receptors, SFC^+ mice were infused i.c.v. with either Veh, BIBO, BIIE or BIBO+BIIE 20 minutes before social fear extinction on day 2. After 10 minutes, mice were infused again with either Veh or NPY.

All mice showed similar investigation of the non-social stimulus during SFC on day 1 (Figure 3(a); $F(4, 35)=0.20$; $p=0.94$), reflecting similar preconditioning non-social anxiety. All mice received a similar number of foot-shocks during SFC ($F(4, 35)=0.21$; $p=0.93$), reflecting similar distress. During social fear extinction on day 2, mice showed similar non-social investigation, reflecting similar non-social fear after SFC. While Veh/NPY-treated mice showed increased social investigation compared to Veh/Veh-treated mice, reflecting reduced expression of social fear, BIBO and BIIE only partly blocked the effects of NPY (Figure 3(b); group effect $F(4, 35)=15.49$; $p<0.001$; group×stimulus effect $F(32, 280)=3.79$; $p<0.001$). As such,

BIBO/NPY- and BIIE/NPY-treated mice showed reduced social investigation compared to Veh/NPY-treated mice and increased social investigation compared to Veh/Veh-treated mice. However, a combination of BIBO and BIIE completely blocked NPY effects on social fear expression, as suggested by the similarly low social investigation between Veh/Veh- and BIBO+BIIE/NPY-treated mice (Figure 3(b); $p<0.05$).

Discussion

The present study demonstrates for the first time that i.c.v. administration of NPY affects SFC-induced social fear in a time point–dependent manner. In more detail, we show that when administered before SFC, NPY did not affect acquisition, expression and extinction of social fear. In contrast, when administered before social fear extinction, NPY reduced expression of social fear via simultaneous activation of Y1 and Y2 receptors. As such, neither the Y1 receptor antagonist BIBO nor the Y2 receptor antagonist BIIE was able to block the effects of NPY completely. However, when administered in combination, they completely blocked the effects of NPY on social fear expression. These results suggest that although NPY does not prevent the formation of aversive memories after a traumatic social experience, it can improve the recovery from a traumatic social experience by reducing the expression of social fear.

While NPY did not affect acquisition of social fear in the present study, it has previously been shown to impair acquisition of cued and contextual fear when administered i.c.v. or directly into the amygdala (Broqua et al., 1995; Gutman et al., 2008; Karlsson et al., 2005; Lach and De Lima, 2013). However, in operant conditioning tasks, such as passive and active avoidance tests, NPY did not affect acquisition (Bouchard et al., 1997; Ishida et al., 2007; Nakajima et al., 1994), supporting our results. Similar to passive and active avoidance tests, the SFC paradigm is based on operant conditioning, where animals learn to associate a voluntary behaviour with its consequences. When the consequence is favourable, the behaviour will occur more frequently, whereas when the consequence is unfavourable, the behaviour will occur less frequently (Thorndike, 1933; White, 1989). Therefore, differences in either the form of conditioning (e.g. classical versus operant) or the valence of the cue (non-social versus social) might contribute to the partially differential role of NPY on fear acquisition. Interestingly, Lacey et al. (2019) showed that NPY impaired the acquisition of conditioned defeat in Syrian hamsters, raising the question of whether the controllability of the aversive event might also play a role. As such, NPY might impair the acquisition in paradigms using uncontrollable aversive stimuli such as inescapable foot-shocks during cued and contextual fear conditioning and aggressive conspecifics during social defeat, but not in paradigms using controllable aversive stimuli such as escapable foot-shocks during SFC and passive and active avoidance tests.

We also show that NPY reduced expression of social fear when administered before social fear extinction, an effect which was mediated via simultaneous activation of Y1 and Y2 receptors. This supports previous findings showing that NPY reduced expression and facilitated extinction of cued and contextual fear by acting on Y1 (Gutman et al., 2008; Lach and De Lima, 2013) and Y2 (Verma et al., 2015) receptors. Although previous studies suggested simultaneous involvement of multiple NPY receptors in the modulation of conditioned fear (Broqua et al., 1995; Gutman et al., 2008; Fendt et al., 2009; Lach and De Lima, 2013), a simultaneous pharmacological manipulation of multiple NPY receptors has not been used before. Consistent with our findings, Verma et al. (2012) provided evidence for a combined role of Y1 and Y2 receptors in extinction of cued fear by using knockout mice. In this study, the phenotype of impaired cued fear extinction observed in NPY knockout mice was replicated only in double Y1 and Y2 receptor knockout mice, whereas extinction of cued fear was only moderately impaired in Y1 receptor knockout mice and unaltered in Y2 receptor knockout mice (Verma et al., 2012). Nevertheless, our findings need to be interpreted with caution, as the effects of Y1 and Y2 antagonists alone in the SFC paradigm are yet unknown and need to be investigated in future studies. It also cannot be excluded that higher doses of each antagonist on its own might completely block the effects of NPY on social fear expression. However, this is unlikely, given that the antagonist doses used in our study and even lower doses of these antagonists were effective in blocking NPY effects in several behavioural paradigms (Gutman et al., 2008; Kornhuber and Zoicas, 2017; Lach and De Lima, 2013).

Although NPY was shown to increase social investigation in naïve rats and mice when infused directly into the basolateral amygdala (Sajdyk et al., 1999; Sajdyk et al., 2002), it did not alter social investigation when infused i.c.v. (Kornhuber and Zoicas, 2017) or into the central amygdala (Sajdyk et al., 1999), suggesting possible brain region–specific effects of NPY on social investigation. In our study, i.c.v. NPY increased social investigation in SFC^+ mice but not in SFC^- mice, suggesting that i.c.v. NPY increases social investigation only in individuals with low or impaired sociability. This is similar to the effects of other neuropeptides, such as oxytocin and neuropeptide S, which were also shown to reduce social fear in SFC^+ mice without further increasing social investigation in SFC^- mice when administered i.c.v. (Zoicas et al., 2014, 2016).

Although the mechanisms underlying the effects of NPY on social fear are yet unknown, they might include modulatory effects of NPY on corticosterone (CORT) secretion and on cardiovascular function. As such, i.c.v. NPY was shown to increase plasma CORT concentrations (Dimitrov et al., 2007; Sainsbury et al., 1996), and increasing CORT concentration by intraperitoneal or intra-basolateral amygdala administration of glucocorticoid receptor agonists was shown to facilitate fear extinction (Yang et al., 2006, 2007). On the other hand, i.c.v. NPY was shown to blunt elevations in blood pressure and heart rate following exposure to the resident-intruder paradigm, an established model of social stress (Klemfuss et al., 1998), and to blunt fear-induced tachycardia in a cued fear conditioning paradigm (Tovote, 2004). By decreasing cardiovascular function in response to stressful stimuli, NPY might enable SFC^+ mice to approach the social stimuli faster and thereby to express less social fear.

Taken together, we show that i.c.v. NPY, while not affecting baseline social investigation and acquisition of social fear, reduces expression of social fear via simultaneous activation of Y1 and Y2 receptors. These findings have important clinical implications, as they suggest that although medication strategies aimed at increasing brain NPY activity are unlikely to prevent the formation of traumatic social memories, they might improve the recovery from a traumatic social experience by reducing the expression of social fear.

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Declaration of Conflicting Interests

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