

Neuropeptide Y (NPY) suppresses yohimbine-induced reinstatement of alcohol seeking

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Abstract

Introduction Reinstatement of responding to a previously alcohol-associated lever following extinction is an established model of relapse-like behavior and can be triggered by stress exposure. Here, we examined whether neuropeptide Y (NPY), an endogenous anti-stress mediator, blocks reinstatement of alcohol-seeking induced by the pharmacological stressor yohimbine.

Materials and methods NPY [5.0 or 10.0 µg/rat, intracerebroventricularly (ICV)] dose-dependently blocked the reinstatement of alcohol seeking induced by yohimbine (1.25 mg/kg, i.p.) but failed to significantly suppress the maintenance of alcohol self-administration. We then used *c-fos* expression mapping to examine neuronal activation following treatment with yohimbine or NPY alone or yohimbine following NPY pre-treatment.

Results and discussion The analysis was focused on a network of structures previously implicated in yohimbine-induced reinstatement, comprised of central (CeA) and basolateral (BLA) amygdala and the shell of the nucleus accumbens (Nc AccS). Within this network, both yohimbine and NPY potently induced neuronal activation, and their

effects were additive, presumably indicating activation of excitatory and inhibitory neuronal populations, respectively. **Conclusion** These results suggest that NPY selectively suppresses relapse to alcohol seeking induced by stressful events and support the NPY system as an attractive target for the treatment of alcohol addiction.

Keywords Yohimbine · Reinstatement · Alcohol seeking · *c-fos* · NPY · Relapse

Introduction

Neuropeptide Y (NPY) is a 36-amino-acid peptide with potent anxiolytic properties (Heilig et al. 1989, 1993). It is thought to counteract behavioral stress responses mediated by corticotropin-releasing hormone (CRH), and an interaction between CRH and NPY has been proposed to regulate emotional behavior (Heilig et al. 1994; Sajdyk et al. 2004). In addition to a broad range of physiological functions, NPY is involved in control of alcohol intake, withdrawal, and dependence [for reviews, see Carvajal et al. (2006), Thiele et al. (2004), and Thorsell (2007)]. For instance, NPY selectively suppresses alcohol drinking in rats selectively bred for high alcohol preference or with a prior history of alcohol dependence (Badia-Elder et al. 2001, 2003; Gilpin et al. 2008; Thorsell et al. 2005a,b). Viral-mediated overexpression of NPY within the amygdala suppresses alcohol drinking in rats with a history of dependence (Thorsell et al. 2007), while a recent study showed that NPY abolished dependence-induced escalation of alcohol drinking when injected in the central nucleus of the amygdala (Gilpin et al. 2008).

Together, these findings suggest an important role for NPY to counteract escalation of alcohol consumption

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following transition to dependence. Specifically, it has been hypothesized that NPY acts by counteracting negative reinforcement that drives escalation of alcohol drinking and maintains alcohol seeking following development of dependence (Heilig and Koob 2007; Valdez and Koob 2004). The effects of NPY on alcohol consumption appear to be reciprocally modulated by NPY-Y1 and NPY-Y2 receptors, since, similar to genetic disruption of NPY itself (Thiele et al. 1998), deletion of Y1 receptors results in increased alcohol intake (Thiele et al. 2002), while potentiation of NPY signaling through antagonism at NPY-Y2 auto receptors with BIIE0246 decreased operant responding for alcohol, with an increased potency in rats with a history of dependence (Rimondini et al. 2005; Thorsell et al. 2002).

Alcoholism is as a chronic relapsing disorder, and relapse prevention is one of the key objectives in its treatment. Reinstatement of responding on a previously alcohol-associated lever following extinction is an established animal model of relapse-like behavior (Shaham et al. 2003). Three broad categories of stimuli, priming doses of the drug, drug-associated cues, or stress, can trigger relapse to alcohol seeking (Le et al. 1998; Liu and Weiss 2002). Stress and cues are additive in inducing relapse, and their role is mediated by distinct neuropharmacological substrates. Specifically, stress-induced reinstatement is blocked by CRH-receptor antagonists (Le et al. 2000; Liu and Weiss 2002), indicating that it is driven by an activation of the CRH system.

Among several stressors examined, only footshock and systemic injection of the α_2 -adrenoceptor antagonist yohimbine robustly reinstate alcohol seeking (Cippitelli et al. 2008; Le et al. 2005; Marinelli et al. 2007). Yohimbine disinhibits central noradrenergic signaling (Aghajanian and Vandermaelen 1982), produces peripheral sympathomimetic effects, activates the hypothalamic–pituitary–adrenal (HPA) axis, and induces feelings of anxiety and panic attacks in humans (Charney et al. 1983, 1989). Similar to other anxiogenic stimuli, yohimbine increases *c-fos* expression in specific areas of fear circuitry (Singewald et al. 2003; Tsujino et al. 1992). This activation pattern overlaps with that induced by footshock stress. Specifically, among numerous brain structures where either footshock or yohimbine induces *c-fos* expression, a limited network of interconnected structures comprised of the BLA and CeA, and Nc AccS is activated by both these stimuli (Funk et al. 2006). Given that footshock and yohimbine appear to be unique among stressors to induced relapse-like behavior, structures within which they both induce neuronal activation are plausible candidates for constituting neural substrates of stress-induced relapse to alcohol seeking.

Here, we examined whether NPY, given its established anti-stress profile, would suppress relapse to alcohol-seeking induced by yohimbine. We then analyzed modula-

tion of neuronal activation in the network of brain structures associated with stress- and yohimbine-induced relapse-like behavior, as measured by *c-fos* mRNA expression, following treatment with yohimbine or NPY alone or yohimbine following pretreatment with NPY. Finally, we examined the potential involvement of the HPA axis in mediation of behavioral NPY effects in the relapse model.

Material and methods

Animals

Male Wistar rats (Charles River, Wilmington, MA, USA) weighing 200–240 g at the beginning of the experiments were pair-housed with water and food available ad libitum. Animals were maintained in a temperature and humidity-controlled vivarium on a 12-h light/dark cycle (lights on at 6:00 A.M.–6:00 P.M.), and all the experiments were carried out during the light phase of the cycle. All animal use was according to NIH guidelines and approved by the NIAAA Animal Care and Use Committee. Each experiment was conducted with an independent group of rats.

Drugs

Human/rat NPY was purchased from American Peptide Company (Sunnyvale, CA, USA), dissolved in sterile saline (0.9%) and infused via ICV administration at 5 and 10 $\mu\text{g}/\text{rat}$ based on prior work (Badia-Elder et al. 2001). NPY solutions were delivered via a 10- μl Hamilton syringe in a volume of 2 $\mu\text{l}/\text{rat}$. Yohimbine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Yohimbine (1.25 mg/kg) was dissolved in distilled water in a dose chosen on the basis of previous studies (Le et al. 2005; Shepard et al. 2004) and injected intraperitoneally (i.p.) in a volume of 1 ml/kg.

Stereotactic surgeries

ICV cannulation was performed as described previously (e.g., Thorsell et al. 2002), except that rats were anesthetized via inhalation of isoflurane. Unilateral guide cannulas (Plastics One Inc., Roanoke, VA, USA) aimed 1.0 mm dorsal to the final injection site were implanted and cemented to the skull using stainless steel screws and kept patent using dummy cannulas. Flat skull position was used, and final injection coordinates were as follows: AP, –1 mm; ML, ± 1.8 mm; and DV, –4.3 mm rel. bregma (Paxinos and Watson 1986). Recovery was monitored for 7 days, after which cannula placement was confirmed by the observation of sustained bouts of water drinking subsequent to infusions of angiotensin II (0.1 $\mu\text{g}/\mu\text{l}$).

Operant training

Training and testing were conducted in operant chambers housed in sound-attenuating cubicles (Med Associates Inc., Georgia, VT, USA). Each chamber was equipped with two retractable levers positioned laterally to a drinking reservoir, which was located 4 cm above the grid floor in the center of the front panel. A microcomputer controlled the delivery of the fluids, presentation of visual stimuli, and recording of the behavioral data. Rats were trained to self-administer 10% alcohol (v/v) in 30-min daily sessions on a fixed ratio 1 (FR-1) schedule of reinforcement, in which each response resulted in delivery of 0.1 ml of fluid. For the first 3 days, rats were allowed to lever press for a 0.2% (w/v) saccharin solution and then trained to self-administer 10% alcohol by fading the saccharin (Weiss et al. 1993).

Alcohol self-administration

Following completion of the saccharin-fading procedure, Wistar rats ($N=10$) were trained to self-administer 10% (v/v) alcohol during 30-min sessions under an FR-1 schedule. Concurrently with the lever pressing, a 5-s time-out period was in effect, during which the house light was on and responses were recorded but not followed by activation of the pump. After three consecutive days of alcohol self-administration sessions, the rats were subjected to stereotactic surgeries (see above). After 1 week, animals were returned to the self-administration boxes, and sessions continued until a stable baseline of responses was reached. We studied the effect of NPY (0.0, 5.0, 10.0 $\mu\text{g}/\text{rat}$) given 5 min prior to a self-administration session. Experiments were conducted every fourth day using a Latin square counterbalanced design. After each round of the Latin square, animals were given one day off. This was followed by two consecutive days of alcohol self-administration to allow for a return to baseline. Responding on the inactive lever was recorded throughout the experiment to monitor nonspecific behavioral effects.

Yohimbine-induced reinstatement of alcohol-seeking behavior

This experiment was performed as described in Cippitelli et al. (2008). Briefly, after completion of the saccharin-fading procedure, rats ($N=27$) were allowed daily 30-min self-administration sessions under an FR-1 schedule of reinforcement for 15 days in order to reach a robust and stable level of alcohol self-administration. Sessions were performed 5 days a week. Once self-administration was established as described, rats were subjected to stereotactic surgeries. After recovery, alcohol-reinforced responding was re-established and then extinguished over 15 consecutive daily sessions. Extinction sessions were identical to self-administration

sessions, except that alcohol was no longer available. After the last extinction session, animals were pre-treated with NPY (0, 5, or 10 $\mu\text{g}/\text{rat}$, ICV) 5-min prior to administration of yohimbine (1.25 mg/kg, i.p.). Thirty minutes following yohimbine treatment, the reinstatement test was started under the same conditions as extinction sessions. House light was still contingently presented during both extinction and reinstatement phases. Responding on the inactive lever was recorded throughout all the experiment to monitor possible nonspecific behavioral effects.

In a separate control experiment, the possible effects of NPY on response rates following extinction were examined. Procedures for this experiment were identical to those described above, with the exception that a within subjects design was used.

In situ hybridization and corticosterone assay

Rats ($N=25$) were ICV cannulated as described above and divided into four groups ($N=6-7$ per group). Following recovery, NPY (10 $\mu\text{g}/\text{rat}$) or vehicle were infused ICV 5 min prior to i.p. administration of either 1.25 mg/kg of yohimbine or vehicle. Animals were decapitated 40 min later; brains were rapidly removed, frozen in isopentane (-40°C), and stored at -80°C . In situ hybridization was carried out as previously described (Hansson et al. 2008). Briefly, 10- μm coronal sections at bregma levels +2.20 (Nc AccS) and -2.80 (BLA and CeA) according to (Paxinos and Watson 1998) were hybridized with a rat-specific, [^{35}S]-UTP labeled riboprobe for *c-fos* (NM_022197.1, bp 306–864). Sections were analyzed using a phosphorimager (BAS-5000, Fujifilm Corp., Japan) and MCID Image Analysis Software (Imaging Research Inc., UK). ^{14}C standards were used to obtain absolute measures of radioactivity (nCi/g). For detailed visualization, slides were exposed for 1 month to Kodak BioMax MR film (Eastman Kodak Company, UK). A few sections of each animal were counterstained with cresyl violet for control of cannula placement. For corticosterone analysis, trunk blood from the same animals was collected in 300 μl vials containing EDTA dipotassium salt (Sarstedt, Nümbrecht, Germany) and stored at -80°C until used. Samples were assayed for corticosterone with a coat-a-count RIA kit from Siemens Medical Solutions Diagnostic (Los Angeles, CA, USA). The radioimmunoassay was performed with rat [^{125}I]CORT and had a detection limit of about 57 ng/mL.

Statistics

Data were assessed for homogeneity of variance and analyzed with ANOVA followed by post hoc (Newman–Keuls) tests when appropriate. To establish that reinstatement was successfully induced, responding during the last extinction

session and the respective reinstatement session were separately compared in the vehicle-treated group by one-way within-subject ANOVA. NPY effect on yohimbine-induced reinstatement was determined using one-way ANOVA with treatment (drug dose) as a between-subject factor, followed by separate linear regression analysis. The effect of NPY on alcohol self-administration was analyzed using one-way repeated measures ANOVA with treatment (drug dose) as a within-subject factor. Corticosterone data were analyzed by two-way ANOVA with i.p. yohimbine vs vehicle as one factor and ICV NPY vs vehicle as the other, followed by Newman Keuls post hoc testing. *c-fos* mRNA data were analyzed using two-way ANOVA with the same factors. To preserve power, analysis was restricted to the three brain regions previously described as areas of overlap for yohimbine- and footshock-induced reinstatement, i.e., BLA, CeA, and Nc AccS. All *P* values were corrected for multiple tests using the Bonferroni method.

Results

NPY does not affect alcohol self-administration in non-dependent rats

As shown in Fig. 1a, alcohol self-administration was not significantly altered by NPY treatment [main treatment effect, $F_{(2,18)}=1.8$, $P=0.2$]. Total responses on the inactive lever were also unaffected [$F_{(2,18)}=0.7$, $P=0.5$].

NPY prevents yohimbine-induced reinstatement of alcohol seeking

A stable baseline of responding for 10% (v/v) alcohol was established over the course of 15 days. Responses on the alcohol lever on the last alcohol session were 56.9 ± 6.4 (mean \pm SEM). During extinction, responding progressively decreased from 39.3 ± 6.9 on the first day to 13.7 ± 4.0 on the last extinction day. Treatment with yohimbine (1.25 mg/kg, i.p.) induced a robust increase in the number of responses on the alcohol-associated lever compared to the last extinction day [$F_{(1,8)}=31.0$, $P<0.01$]. Inactive lever responding was unaffected [$F_{(1,8)}=3.0$, NS]. Pre-treatment with NPY (5, 10 μ g/rat) dose-dependently reversed yohimbine-induced responding on the alcohol-associated lever [$F_{(2,24)}=4.5$, $P<0.05$]. Post hoc analysis showed a statistically significant effect of NPY at the higher dose ($P=0.02$). Linear regression analysis confirmed the dose–response effect [$R^2=0.25$, $P<0.01$]. Responding on the inactive lever was not modified by NPY [$F_{(2,24)}=1.3$, NS; Fig. 1b]. NPY alone (5 or 10 μ g/rat) did not affect response rates on the alcohol associated lever (Table 1).

Additive neuronal activation in CeA and Nc AccS by yohimbine and NPY

Analysis was focused on the network of structures previously reported as associated with stress- as well as yohimbine-induced reinstatement, i.e., BLA, CeA, and Nc AccS (Funk et al. 2006). Among these structures, we found a robust activation in response to yohimbine in CeA (main yohimbine effect, $F_{(1,18)}=98.5$, corrected $P<0.001$; Fig. 2) and the Nc AccS (main yohimbine effect, $F_{(1,20)}=98.6$, corrected $P<0.001$; Fig. 3). In contrast, activation in the BLA was marginal (control, 61.8 ± 4.2 ; yohimbine alone, 71.2 ± 8.4 , nCi/g, mean \pm SEM; main yohimbine effect, $F_{(1,20)}=4.4$, nominal $P=0.05$, corrected $P=0.15$, NS). Within these structures, NPY also induced neuronal activation, at a level similar to that observed with yohimbine, both in CeA (main NPY effect, $F_{(1,18)}=67.0$, corrected $P<0.001$, Fig. 2) and Nc AccS (main NPY effect, $F_{(1,20)}=87.3$, corrected $P<0.001$; Fig. 3). There was also a robust NPY-induced activation in the BLA (control, 61.8 ± 4.2 ; NPY alone, 116.3 ± 3.7 ; main NPY effect, $F_{(1,20)}=30.2$, corrected $P<0.001$). The effects of yohimbine and NPY in CeA and Nc AccS were additive; post hoc analysis showed in each case that the NPY-yohimbine group was significantly higher than either the group treated with yohimbine alone (CeA, corrected $P<0.01$; Nc AccS, corrected $P<0.001$) or that administered NPY alone (CeA, corrected $P<0.001$; Nc AccS, corrected $P<0.001$) group.

Activation of HPA axis following yohimbine, NPY, and the combination

A robust activation of the HPA axis occurred both in response to NPY, yohimbine alone, and yohimbine following NPY. ANOVA showed a highly significant main effect of the ICV NPY pre-treatment [$F_{(1,21)}=16.4$, $P<0.001$] as well as the i.p. yohimbine treatment [$F_{(1,21)}=13.3$, $P<0.01$] to increase CORT response. Post hoc analysis showed that the combination of both agents did not result in an additive effect on serum corticosterone levels, potentially indicating a ceiling effect (veh–veh, 220.4 ± 54.5 ; veh–YOH, 521.5 ± 41.1 ; NPY–veh: 540.0 ± 18.4 ; and NPY–YOH, 566.0 ± 52.1 ng/ml; mean \pm SEM, $N=6-7$; $P<0.001$ vs. veh–veh).

Discussion

We report that reinstatement of alcohol seeking induced by the pharmacological stressor yohimbine is dose-dependently blocked by NPY. Similar to what has been reported previously in non-dependent rats [for review, see Thorsell (2007)], NPY did not suppress alcohol self-administration, making it unlikely that its blockade of

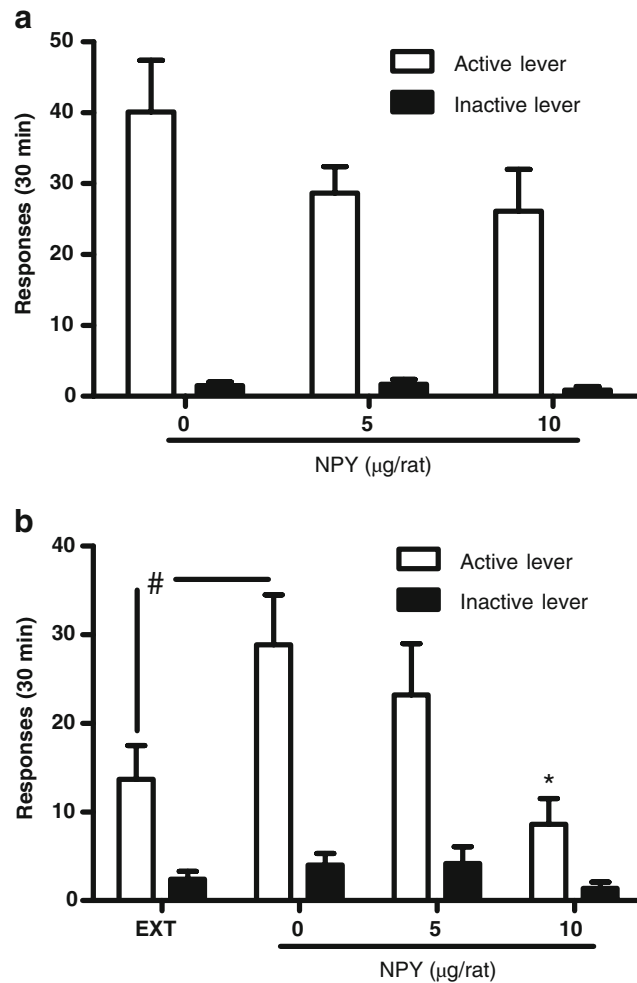


Fig. 1 a NPY (5.0 and 10.0 µg/rat, ICV) did not suppress alcohol self-administration under an FR-1 schedule of reinforcement: *white bars* active lever, *black bars* inactive lever. Values are mean (±SEM) number of reinforced responses on the active lever or non-reinforced responses at the inactive lever ($N=10$). There was no main effect of treatment. For detailed statistics, see **Results**. **b** ICV injection of NPY (5.0 and 10.0 µg/rat) decreases yohimbine (1.25 mg/kg, i.p.)-induced reinstatement of alcohol-seeking ($N=27$): *white bars* active lever, *black bars* inactive lever. Values represent the mean (±SEM) number

of total responses on the active and inactive levers, respectively. There was significant reinstatement induction by yohimbine, as indicated by responses in rats ($N=9$) exposed to yohimbine (in the absence of reward delivery) compared with extinction responding by the same **Animals**; $^{\#}P<0.01$ vs. last day of extinction (EXT). There was a significant main effect of NPY treatment, a significant dose-dependence relationship, and a significant suppression of responding in the 10.0 µg group on post hoc analysis. $*P=0.02$ vs. vehicle-treated controls. For detailed statistics, see **Results**

yohimbine-induced reinstatement results from non-specific behavioral impairment, such as sedation or ataxia. Among structures previously reported to be associated with stress- and yohimbine-induced reinstatement, yohim-

bine administered alone induced a marked neuronal activation in the CeA and Nc AccS but not in the BLA. An NPY dose that blocked yohimbine-induced reinstatement, when given alone, resulted in a potent *c-fos* induction within the same structures where yohimbine-induced activity was found. When both NPY and yohimbine were administered, the activation observed was additive.

Table 1 Lack of influence of intracerebroventricular NPY on response rates on the alcohol-associated lever following extinction

	NPY (µg/rat)		
	0	5	10
Active lever	11.3±2.9	10.6±2.8	13.1±3.6
Inactive lever	1.9±0.6	1.8±0.7	1.9±0.9

The finding that NPY suppressed yohimbine-induced reinstatement is consistent with the notion, proposed more than 15 years ago, that NPY is an endogenous anti-stress mediator that can buffer behavioral effects of stress mediated by CRH (Heilig et al. 1994; Heilig and Koob 2007; Sajdyk et al. 2004; Valdez and Koob 2004). Similar

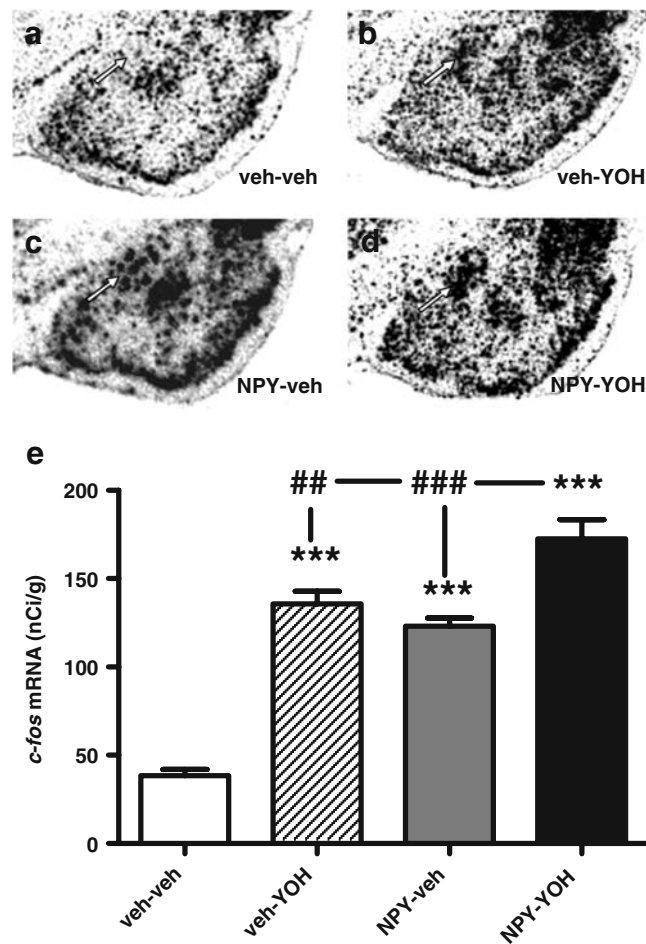


Fig. 2 Induction of *c-fos* expression within central amygdala (CeA) by yohimbine (1.25 mg/kg, i.p.), NPY (10 μ g/rat ICV), and the combination of the two treatments. Subpanels a–d show representative sections generated using film autoradiography, visualizing *c-fos* mRNA expression in the CeA following ICV pre-treatment with vehicle followed by i.p. vehicle injection (veh–veh, a), yohimbine alone (veh–YOH, b), NPY alone (NPY–veh, c), or pre-treatment with NPY followed by treatment with yohimbine (NPY–YOH, d). Panel e

shows quantitative phosphorimaging results from the respective group. Both ICV NPY treatment alone and i.p. yohimbine treatment alone resulted in an approximately fourfold increase in *c-fos* expression. When both drugs were administered, the results were additive. *** $P < 0.001$ vs. veh–veh. ## $P < 0.01$, ### $P < 0.001$ vs. NPY–YOH. Data are reported as nCi/g (mean \pm SEM; $n = 5–7$). For detailed statistics, see Results

to footshock-stress-induced reinstatement of alcohol seeking (Gehlert et al. 2007; Hansson et al. 2006; Le et al. 2000; Liu and Weiss 2002), yohimbine-induced reinstatement is mediated through a CRH-dependent mechanism, since it is blocked by systemic administration of the CRH1 receptor antagonist antalarmin (Marinelli et al. 2007). Our present finding that NPY can block yohimbine-induced reinstatement thus provides additional support for the notion that this peptide can act as a functional CRH-receptor antagonist. The ability of NPY to prevent stress-induced relapse offers a potentially attractive mechanism for treatment development.

Based on a careful comparative analysis of behavioral and pharmacological stressors that do or do not induce reinstatement of alcohol seeking, a network consisting of the BLA, CeA, and Nc AccS has previously been outlined

as the likely neural substrate of yohimbine-induced reinstatement (Funk et al. 2006). In excellent agreement with this analysis, we found that yohimbine induced a marked, approximately fourfold increase in *c-fos* expression in CeA and Nc AccS. We did not, however, replicate a robust yohimbine response in the BLA, where the increase was less than 15%, and not statistically significant. The previous *c-fos* mapping experiment employed a yohimbine dose of 2.5 mg/kg, while we used 1.25 mg/kg. It is by now evident, both from our present data and a study by the Lê group (Marinelli et al. 2007), that the lower 1.25 mg/kg yohimbine dose is sufficient to induce reinstatement to alcohol seeking. We therefore conclude that neuronal activation, as measured by *c-fos* expression, is less robust within the BLA than within CeA and Nc AccS at a behaviorally active dose of yohimbine and that activation of

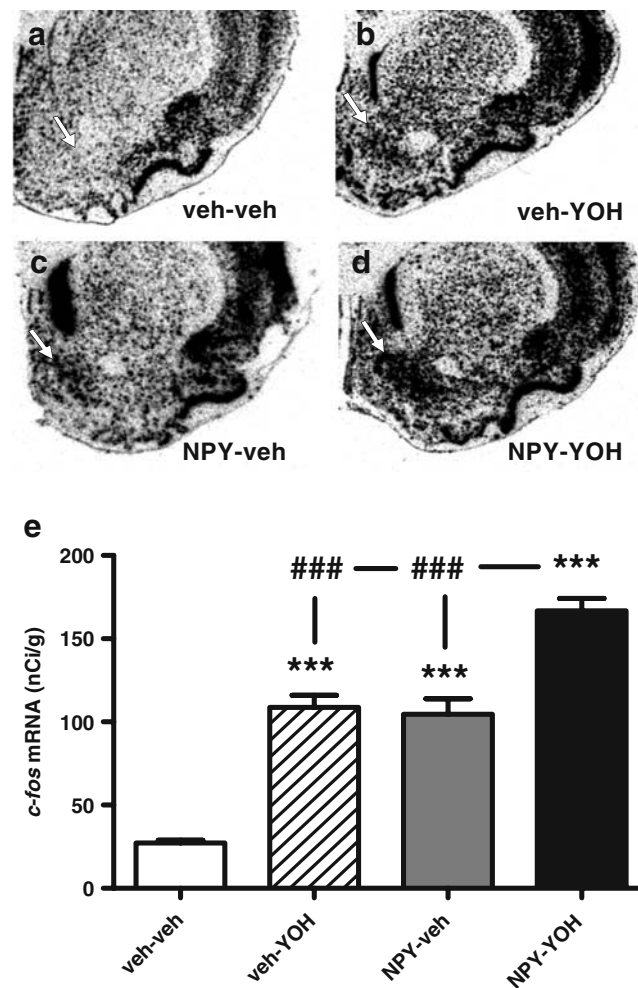


Fig. 3 Induction of *c-fos* expression within the nucleus accumbens shell (Nc AccS) by yohimbine (1.25 mg/kg i.p.), NPY (10 µg/rat ICV), and the combination of the two treatments. Subpanels **a–d** show representative sections generated using film autoradiography, visualizing *c-fos* mRNA expression in the Nc AccS following ICV pre-treatment with vehicle followed by i.p. vehicle injection (veh-veh, **a**), yohimbine alone (veh-YOH, **b**), NPY alone (NPY-veh, **c**), or pre-treatment with NPY followed by treatment with yohimbine

(NPY-YOH, **d**). Subpanel **e** shows quantitative phosphorimaging results from the respective group. In a pattern closely paralleling that observed in CeA, both NPY and i.p. yohimbine treatment resulted in an approximately fourfold increase in *c-fos* expression, and when both drugs were administered, the result was additive. *** $P < 0.001$ vs. veh-veh. ### $P < 0.001$ vs. NPY-YOH. Data are reported as nCi/g (mean \pm SEM; $n = 5-7$). For detailed statistics, see [Results](#)

the BLA may not be required for yohimbine-induced reinstatement of alcohol seeking. This leaves CeA and Nc AccS, both components of the extended amygdala (Heimer 2003; Heimer and Van Hoesen 2006), as the core network likely to subserve yohimbine-induced relapse-like behavior.

According to a simplistic hypothesis, NPY might be expected to inhibit neuronal activation within one or more reinstatement-associated structures activated by yohimbine. The results of our neuronal activation mapping study did not, however, produce results in agreement with this view. Instead, NPY gave rise to a similar level of *c-fos* expression in CeA and Nc AccS as did yohimbine and was additive with yohimbine when both drugs were administered. These findings are in agreement with prior observations that immediate-early gene expression is a non-specific

marker for both anxiolytic or anxiogenic drugs within several stress-related structures including CeA (Thompson and Rosen 2006). Specifically, yohimbine potentiates noradrenergic signaling in brain areas where norepinephrine stimulates CRH release, including CeA [for review, see Koob (2009)]. Neuronal activation in CeA as well as paraventricular nucleus (PVN) and other stress-related areas is, however, also produced by anti-anxiety agents that potentiate inhibitory GABA transmission, such as the prototypical benzodiazepine diazepam (Beck and Fibiger 1995; Salminen et al. 1996). Given the established anxiolytic role of NPY and its ability to potentiate GABA transmission, NPY-induced *c-fos* mRNA expression in stress-related structures is likely to reflect activation of inhibitory GABA synapses.

CeA is clearly a candidate site where these actions of NPY might mediate suppression of yohimbine-induced reinstatement. Prior studies show that NPY within this structure has anti-stress effects in other behavioral models (Heilig et al. 1993) and suppresses dependence-induced alcohol drinking (Gilpin et al. 2008; Thorsell et al. 2007). Thus, our data suggest that yohimbine may activate discrete output neurons of CeA that drive relapse-like behavior, while NPY would recruit a distinct population of neurons that may counteract this activation. These findings further underline the similarities between NPY and CRH1 antagonism, since CeA is also the site through which the latter mechanism inhibits behavioral stress responses and dependence-induced drinking (Funk et al. 2007; Rassnick et al. 1993).

To examine whether NPY suppression of yohimbine-induced reinstatement depends on the HPA axis, we evaluated corticosterone responses following treatment with yohimbine, NPY, or the combination. In agreement with prior studies (Banihashemi and Rinaman 2006; Marinelli et al. 2007), we found that yohimbine induced a robust increase in peripheral corticosterone levels. This effect is thought to depend on noradrenergic inputs to the PVN and/or BNST that activate CRH neurons in the PVN (Banihashemi and Rinaman 2006). Despite its ability to counteract behavioral consequences of yohimbine, NPY also activates the HPA axis in rats. Large numbers of nerve terminals of NPY-containing neurons, the cell bodies of which are located mainly in the hypothalamic arcuate nucleus and in part in the brainstem, are found in the hypothalamic PVN (Krysiak et al. 1999). *In vitro* (Tsagarakis et al. 1989) as well as *in vivo* (Suda et al. 1993) studies have shown that NPY stimulates CRH release and increases hypothalamic CRH gene expression. Furthermore, NPY directly injected into the PVN is more potent than other routes of administration to activate the HPA axis (Albers et al. 1990; Krysiak et al. 1999). Thus, centrally administered NPY increases corticosterone levels by triggering CRH release in the hypothalamus. In our study, a dose of NPY that effectively blocked yohimbine-induced reinstatement had no effect on yohimbine-induced corticosterone release. Thus, similar to the effects of CRH1 antagonists (Deak et al. 1999; Gehlert et al. 2007; Marinelli et al. 2007), suppression of yohimbine-induced reinstatement by NPY is independent of the HPA axis, and mediated through extra-hypothalamic regions.

A potential limitation in interpreting the present results is that the *in situ* and corticosterone data were obtained in animals without a history of alcohol self-administration. For two reasons, we believe that this is unlikely to be a major limitation. First, our data closely parallel a previous study (Funk et al. 2006), which showed that stressors capable of eliciting reinstatement,

such as footshock and yohimbine, are selective in their ability to induce an overlapping pattern of neuronal activation, also when assessed in alcohol naive animals. Secondly, our observation that NPY suppressed reinstatement induced by yohimbine without suppressing the corticosterone response to this drug closely parallels results previously obtained with CRH-receptor antagonism (Marinelli et al. 2007).

In conclusion, congruent with its anti-stress actions in other behavioral models, NPY inhibits reinstatement to alcohol seeking induced by the pharmacological stressor yohimbine. This finding is likely to be predictive of an ability to decrease relapse vulnerability in clinical alcoholism by NPY agonists. Together with prior findings on suppression of escalated, dependence-induced drinking by NPY (Gilpin et al. 2008; Thorsell et al. 2005a, 2007), our data point to the NPY system as an attractive target for the treatment of alcohol addiction.

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Disclosures/Conflict of interest The authors declare no conflicts of interest.

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