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Effects of NPY and the specific Y1 receptor agonist [D-His²⁶]-NPY on the deficit in brain reward function and somatic signs associated with nicotine withdrawal in rats

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Abstract

Tobacco addiction is a chronic disorder that is characterized by dysphoria upon smoking cessation and relapse after periods of abstinence. Previous research suggests that Neuropeptide Y (NPY) and Y1 receptor agonists attenuate negative affective states and somatic withdrawal signs. The aim of the present experiments was to investigate the effects of NPY and the specific Y1 receptor agonist [D-His²⁶]-NPY on the deficit in brain reward function and somatic signs associated with nicotine withdrawal in rats. The intracranial self-stimulation procedure was used to assess the effects of nicotine withdrawal on brain reward function as this procedure can provide a quantitative measure of emotional states in rodents. Elevations in brain reward thresholds are indicative of a deficit in brain reward function. In the first experiment, NPY did not prevent the elevations in brain reward thresholds associated with precipitated nicotine withdrawal and elevated the brain reward thresholds of the saline-treated control rats. Similar to NPY, [D-His²⁶]-NPY did not prevent the elevations in brain reward thresholds associated with precipitated nicotine withdrawal and elevated the brain reward thresholds of the saline-treated control rats. Neither NPY nor [D-His²⁶]-NPY affected the response latencies. In a separate experiment, it was demonstrated that the specific Y1 receptor antagonist BIBP-3226 prevented the NPY-induced elevations in brain reward thresholds. NPY attenuated the overall somatic signs associated with precipitated nicotine withdrawal. [D-His²⁶]-NPY did not affect the overall somatic signs associated with precipitated nicotine withdrawal, but decreased the number of abdominal constrictions. Both NPY and [D-His²⁶]-NPY attenuated the overall somatic signs associated with spontaneous nicotine withdrawal. These findings indicate that NPY and [D-His²⁶]-NPY attenuate somatic nicotine withdrawal signs, but do not prevent the deficit in brain reward function associated with precipitated nicotine withdrawal. In addition, NPY decreases the sensitivity to rewarding electrical stimuli via an Y1 dependent mechanism.

Keywords

Nicotine; withdrawal; rats; Neuropeptide Y; [D-His²⁶]-NPY; BIBP-3226

1. Introduction

Tobacco dependence is a chronic disorder that is characterized by a negative affective state upon smoking cessation, continuation of smoking behavior despite negative physical and social consequences, and relapse after periods of abstinence (American Psychiatric Association, 2000; Dackis and O'Brien, 2005). Nicotine, tobacco's main psychoactive component,

stimulates the brain reward system, which has been suggested to play an important role in the initiation of tobacco smoking behavior (Corrigall et al., 1994; Hyman and Malenka, 2001). Preclinical studies indicate that nicotine mediates its positive reinforcing effects at least partly via the activation of neuronal nicotinic acetylcholine receptors (nAChR) and dopamine D1 receptors (Corrigall and Coen, 1991; Picciotto et al., 1998). It has been hypothesized that the negative emotional state associated with the discontinuation of tobacco smoking is partially mediated by the activation of brain stress systems and provides a major motivational force in the continuation of smoking behavior (Bruijnzeel and Gold, 2005; Koob and Le Moal, 2005). Animal experiments have shown that antagonism of nAChR or discontinuation of nicotine administration induces an elevation in brain reward thresholds in the intracranial self-stimulation procedure (Bruijnzeel et al., 2007; Epping-Jordan et al., 1998). Elevations in brain reward thresholds are mediated by a decrease in the positive reinforcing properties of pleasurable electrical stimuli and have been suggested to reflect an anhedonic-state (Markou et al., 1998). Elevations in brain reward thresholds have also been observed after the discontinuation of the administration of drugs of abuse such as amphetamine, fentanyl, and alcohol (Bruijnzeel et al., 2006; Schulteis et al., 1995; Wise and Munn, 1995). Antidepressant treatments, such as co-administration of the selective serotonin reuptake inhibitor fluoxetine and the serotonin-1A receptor antagonist p-MPPI ([4-(2'-methoxy-phenyl)-1-[2'-(n-(2''-pyridinyl)-p-iodobenzamido]-ethyl-piperazine)], partly prevent the elevations in brain reward thresholds associated with spontaneous nicotine withdrawal (Harrison et al., 2001). In addition, in a recent study we showed that antagonism of corticotropin releasing factor receptors attenuates the deficit in brain reward function associated with precipitated nicotine withdrawal (Bruijnzeel et al., 2007).

Several lines of experimental evidence suggest that the orexigenic neurotransmitter Neuropeptide Y (NPY) attenuates somatic drug withdrawal signs. Woldbye and colleagues showed that intraventricular (icv) administration of NPY dose-dependently attenuates somatic morphine withdrawal signs (Woldbye et al., 1998). In addition, the same research group reported that NPY reduces somatic ethanol withdrawal signs (Woldbye et al., 2002). Clinical and preclinical studies suggest that NPY counteracts the effects of stressors and low central NPY levels could play a role in depressive disorders (Redrobe et al., 2002b). NPY levels in the cerebrospinal fluid of depressed patients are lower than those in schizophrenics or healthy controls (Heilig et al., 2004; Widerlov et al., 1988). In addition, the central administration of NPY has antidepressant-like effects in animal models such as the rat forced swim test (Redrobe et al., 2002a; Stogner and Holmes, 2000) and clinically effective antidepressant treatments (e.g., repeated electroconvulsive shock stimulation and the selective serotonin reuptake inhibitor fluoxetine) elevate central NPY levels in rats (Baker et al., 1996; Heilig et al., 1988; Stenfors et al., 1989). Taken together, the above-discussed studies suggest that NPY attenuates somatic opioid and alcohol withdrawal signs and low levels of NPY may be implicated in the etiology of negative mood states. Although discontinuation of nicotine administration has been associated with negative affective and somatic withdrawal signs, the role of NPY in nicotine withdrawal has not been investigated. It is hypothesized here that stimulation of NPY receptors prevents the deficit in brain reward function and somatic signs associated with nicotine withdrawal in rats. The aim of the first two experiments (Experiment 1 and 2) was to investigate the effects of NPY and the selective Y1 receptor agonist [D-His²⁶]-NPY on the deficit in brain reward function associated with precipitated nicotine withdrawal. The state of the brain reward system was investigated using a discrete-trial intracranial self-stimulation procedure. This procedure was used as it provides a quantitative measure of the emotional aspects of drug withdrawal (Bruijnzeel et al., 2006; Schulteis et al., 1995; Wise and Munn, 1995). The effect of [D-His²⁶]-NPY on nicotine withdrawal was investigated as this compound, in contrast to NPY, has a low affinity for the Y5 receptor (NPY, Ki of 0.28 nM for Y1, Ki of 1.5 nM for Y5; [D-His²⁶]-NPY, Ki of 2.0 nM for Y1, Ki of 34.6 nM for Y5) (Mullins et al., 2001). Activation of the Y5 receptor has been suggested to mediate

NPY's sedative effects while NPY's anxiolytic and antidepressant-like effects have been suggested to be mediated via the activation of the Y1 receptor (Ishida et al., 2007; Redrobe et al., 2002a; Sorensen et al., 2004). Experiment 1 and 2 indicated that NPY and [D-His²⁶]-NPY elevated the brain reward thresholds of the saline-treated control rats. Therefore, a separate experiment (Experiment 3) was conducted to investigate the effect of the selective Y1 receptor antagonist BIBP-3226 on NPY-induced elevations in brain reward thresholds (Doods et al., 1995). The last three experiments (Experiment 4 – 6) investigated the effects of NPY and [D-His²⁶]-NPY on somatic withdrawal signs associated with precipitated and spontaneous nicotine withdrawal.

2. Materials and Methods

2.1 Subjects

Male Wistar rats (Charles River, Raleigh, NC) weighing 250-300 g at the beginning of the experiments were used. Animals were group-housed (two per cage) in a temperature- and humidity-controlled vivarium and maintained on a 12 hr light-dark cycle (lights off at 6 p.m.). All testing occurred at the beginning of the light cycle. Food and water were available *ad libitum* in the home cages. All subjects were treated in accordance with the National Institutes of Health guidelines regarding the principles of animal care. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) and approved by the University of Florida Institutional Animal Care and Use Committee.

2.2 Surgical Procedures

Electrode and cannula implantation—For experiments 1 – 3, rats were prepared with an electrode in the lateral hypothalamic (LH) medial forebrain bundle and a cannula above the lateral ventricle. For experiments 4 - 6, the rats were prepared with a cannula above the lateral ventricle. At the beginning of all the intracranial surgeries, the rats were anesthetized with an isoflurane/oxygen vapor mixture (1-3% isoflurane) and placed in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the incisor bar set 3.3 mm below the interaural line (flat skull). The rats were prepared with a stainless steel 23 gauge cannula 11 mm in length located immediately above the lateral ventricle using the following flat skull coordinates: anterior posterior (AP) -0.9 mm, medial lateral (ML) ± 1.4 mm, dorsal ventral (DV) -3.0 mm from skull (Paxinos and Watson, 1998). At the end of the surgery, removable 30 gauge wire stylets 11 mm in length were inserted in the cannulae so that the cannula would maintain patency. For electrode implantations, the incisor bar was set 5 mm above the interaural line. The rats were prepared with stainless steel bipolar electrodes (model MS303/2 Plastics One, Roanoke, VA) 11 mm in length in the medial forebrain bundle at the level of the posterior LH (AP -0.5 mm; ML ± 1.7 mm; DV -8.3 mm from dura). The electrodes and cannulae were permanently secured to the skull using dental cement anchored with four skull screws.

Osmotic minipump implantation—Minipumps (Alzet model 2ML4 28 day pumps, Durect Corporation, Cupertino, CA) filled with either saline or nicotine hydrogen tartrate dissolved in saline, were implanted subcutaneously under isoflurane/oxygen (1-3% isoflurane) anesthesia. The nicotine concentration was adjusted to compensate for differences in body weight to deliver a dose of 9 mg/kg/day of nicotine salt (3.16 mg/kg/day nicotine base).

2.3 Apparatus

The experimental apparatus consisted of twelve Plexiglas chambers (30.5 $\times$ 30 $\times$ 17 cm; Med Associates, Georgia, VT), each housed in a sound-attenuating melamine chambers (Med Associates, Georgia, VT). The operant chamber consisted of a metal grid floor and a metal wheel (5 cm wide) centered on a sidewall. A photobeam detector was attached next to the

response wheel and recorded every 90 degrees of rotation. Brain stimulation was delivered using constant current stimulators (Model 1200C, Stimtek, Acton, MA). Subjects were connected to the stimulation circuit through bipolar leads (Plastics One, Roanoke, VA) attached to gold-contact swivel commutators (model SL2C Plastics One, Roanoke, VA). A computer controlled the stimulation parameters, data collection, and all test session functions.

2.4 Intracranial self-stimulation procedure

The subjects were initially trained to turn the wheel on a fixed ratio 1 (FR1) schedule of reinforcement. Each quarter turn of the wheel resulted in the delivery of a 0.5 sec train of 0.1 msec cathodal square-wave pulses at a frequency of 100 Hz. After the successful acquisition of responding for stimulation on this FR1 schedule, defined as 100 reinforcements within 10 minutes, the rats were trained gradually on a discrete-trial current-threshold procedure. The discrete-trial current-threshold procedure used was a modification of a task developed by Kornetsky and Esposito (Kornetsky and Esposito, 1979), and previously described in detail by Bruijnzeel and Markou (Bruijnzeel and Markou, 2004; Bruijnzeel and Markou, 2005). Each trial began with the delivery of a non-contingent electrical stimulus, followed by a 7.5 sec response window within which the animal can respond to receive a second contingent stimulus identical in all parameters to the initial non-contingent stimulus. A response during this 7.5 sec response window was labeled a positive response, while the lack of a response was labeled a negative response. During a 2 sec period immediately after a positive response, additional responses had no consequence. The inter-trial interval (ITI) that followed either a positive response or the end of the response window (in the case of a negative response), had an average duration of 10 sec (ranging from 7.5 sec to 12.5 sec). Responses that occurred during the ITI resulted in a further 12.5 sec delay of the onset of the next trial. During training on the discrete-trial procedure, the duration of the ITI and delay periods induced by time-out responses were gradually increased until animals performed consistently at standard test parameters. The subjects subsequently were tested on the current-threshold procedure in which stimulation intensities varied according to the classical psychophysical method of limits. A test session consisted of four alternating series of descending and ascending current intensities starting with a descending series. Blocks of three trials were presented to the subject at a given stimulation intensity, and the intensity was altered systematically between blocks of trials by 5 μ A steps. The initial stimulus intensity was set 40 μ A above the baseline current-threshold for each animal. Each test session typically lasted 30-40 minutes and provided two dependent variables for behavioral assessment: brain reward thresholds and response latencies. *Thresholds*: The current threshold for a descending series was defined as the midpoint between stimulation intensities that supported responding (i.e., positive responses on at least two of the three trials), and current intensities that failed to support responding (i.e., positive responses on fewer than two of the three trials for two consecutive blocks of trials). The threshold for an ascending series was defined as the midpoint between stimulation intensities that did not support responding and current intensities that supported responding for two consecutive blocks of trials. Thus, four threshold estimates were recorded, and the mean of these values was taken as the threshold for each subject on each test session. *Response Latencies*: The time interval between the beginning of the non-contingent stimulus and a positive response was recorded as the response latency. The response latency for each test session was defined as the mean response latency on all trials during which a positive response occurred.

2.5 Drug administration procedure

For intracerebroventricular injections, 30 gauge stainless steel injectors extending 2.5 mm beyond the guide cannula were used. All icv drug injections were made by gravity induced by raising the Hamilton syringe above the animal's head. Five μ l of solution was administered over a 30-60 second period, and the injector was left in place for another 30 seconds to allow

diffusion from the injector tip. The dummy stylets were inserted immediately after drug-administration.

2.6 Somatic withdrawal signs

Rats were observed for 10 minutes in a Plexiglas observation chamber (10" × 10" × 18"; L × W × H). The rats were habituated to the observation chamber for 5 minutes per day on 3 consecutive days prior to testing. The following somatic signs were recorded based on checklist of nicotine abstinence signs: body shakes, cheek tremors, escape attempts, eye blinks, gasps, genital licks, head shakes, ptosis, teeth chattering, writhes and yawns (Cryan et al., 2003; Malin et al., 1992). Ptosis was counted once per minute if present continuously. The total number of somatic signs was defined as the sum of the individual occurrences. For the final statistical analyses the signs were divided into the following categories: abdominal constrictions, gasps and writhes; facial fasciculation, cheek tremors and teeth chattering; eye blinks; ptosis; and other recorded signs.

2.7 Experimental Design

2.7.1 Experiment 1, Effect of NPY on the elevations in brain reward thresholds associated with precipitated nicotine withdrawal—The aim of this experiment was to investigate the effect of NPY on the elevations in brain reward thresholds associated with precipitated nicotine withdrawal. After recovery from the electrode and cannula implantations, the rats were trained on the ICSS procedure. When stable baseline brain reward thresholds were achieved, defined as less than 10% variation within a 5 day period, the rats were prepared with 28-day osmotic minipumps containing either saline (n = 8) or nicotine (9 mg/kg/day of nicotine salt, n = 8) dissolved in saline. Brain reward thresholds and response latencies were assessed daily throughout the experiment between 9:00 am and 12:00 noon. The noncompetitive and nonspecific nAChR antagonist mecamylamine was used to precipitate withdrawal. Mecamylamine (2 mg/kg, sc) injections started at least 6 days after the implantation of the minipumps to allow the development of nicotine dependence. NPY (1 – 16 µg [0.23 – 3.75 nmol], icv) was administered according to a Latin-square design 30 minutes prior to treatment with mecamylamine. The pretreatment interval was based on previous studies that investigated the role of NPY in opioid withdrawal (Clausen et al., 2001; Woldbye et al., 1998). The rats were placed in the ICSS test chambers 5 minutes after mecamylamine administration. It was ensured that the minimum time-interval between the mecamylamine injections was at least 72 hours to reestablish / maintain nicotine dependence. The serum elimination half-life of mecamylamine is approximately 1 hour (Debruyne et al., 2003). At the end of all the experiments the rats were euthanized using an overdose of pentobarbital (150 mg/kg, intraperitoneally). Cannulae placements were verified by administering 5 µl of a 0.5% aqueous methyl blue solution at the injections site. After the administration of the methyl blue, the brain was removed and the injection site was determined.

2.7.2 Experiment 2, Effect of [D-His²⁶]-NPY on the elevations in brain reward thresholds associated with precipitated nicotine withdrawal—The aim of this experiment was to investigate the effect of [D-His²⁶]-NPY on the elevations in brain reward thresholds associated with precipitated nicotine withdrawal. The design of this study is the same as that of Experiment 1, with the exception that [D-His²⁶]-NPY (1 – 16 µg [0.23 – 3.75 nmol], icv) was administered to rats chronically treated with saline (n = 9) or nicotine (9 mg/kg/day of nicotine salt, n = 11). [D-His²⁶]-NPY is considered a selective Y1 receptor agonist as its affinity for the Y1 receptor is 14, 10, and 17-fold greater than for the Y2, Y4, and Y5 receptor subtypes, respectively (Mullins et al., 2001).

2.7.3 Experiment 3, Effect of BIBP-3226 on NPY-induced elevations in brain reward thresholds—In experiment 1 and 2, the administration of NPY was followed by the

administration of mecamylamine, which may have contributed to the NPY-induced elevations in brain reward thresholds. Therefore, an experiment was conducted to determine if NPY by itself elevates brain reward thresholds. Rats ($n = 6$) were prepared with an electrode in the LH and a cannula above the lateral ventricle. After stable brain reward thresholds were achieved (defined as less than 10% variation within a 5 day period) the rats were treated with vehicle (icv) or NPY (16 μg [3.75 nmol], icv). One half of the rats received NPY first and the other half of the rats received vehicle first. NPY was administered 30 minutes prior to testing. The aim of the second part was to investigate the role of Y1 receptors in the NPY-induced elevations in brain reward thresholds. Rats ($n = 14$) were prepared with an electrode in the LH and a cannula above the lateral ventricle. After recovery from the surgeries, the rats were trained and stabilized (defined as less than 10% variation within a 5 day period) on the ICSS procedure. In order to investigate the role of Y1 receptors in NPY-induced elevations in brain reward thresholds, the selective Y1 receptor antagonist BIBP-3226 (0, 16, 32 μg [0, 33.79, 67.57 nmol], icv) was administered according to a Latin-square designs 30 minutes prior to the administration of NPY (16 μg [3.75 nmol], icv). BIBP-3226 was dissolved in distilled water containing 3% dimethyl sulfoxide (DMSO) and a 3% DMSO solution served as vehicle. The rats were placed in the ICSS test chambers 30 minutes after the administration of NPY. There were at least two days between subsequent injections.

2.7.4 Experiment 4, Effects of NPY and [D-His²⁶]-NPY on somatic signs associated with precipitated nicotine withdrawal—The aim of this experiment was to investigate the effects of NPY and [D-His²⁶]-NPY on the somatic signs associated with precipitated nicotine withdrawal. Rats were prepared with 28-day osmotic minipumps containing either saline ($n = 10$) or nicotine (9 mg/kg/day of nicotine salt, $n = 20$) dissolved in saline. The nAChR antagonist mecamylamine was used to precipitate nicotine withdrawal. Mecamylamine (2 mg/kg, sc) injections started at least 6 days after the implantation of the minipumps. The saline-treated rats received a single injection with vehicle (icv) followed by mecamylamine. This allowed us to investigate if precipitated withdrawal was associated with an increase in somatic signs (vehicle [icv]-mecamylamine-chronic saline group vs. vehicle [icv]-mecamylamine-chronic nicotine group). The nicotine-treated rats received NPY (1 – 16 μg [0.23 – 3.75 nmol], icv) or [D-His²⁶]-NPY (1 – 16 μg [0.23 – 3.75 nmol], icv) followed by mecamylamine. NPY or [D-His²⁶]-NPY was administered according to a Latin-square design 30 minutes prior to the administration of mecamylamine. Five minutes after the administration of mecamylamine, the rats were placed in Plexiglas observation cages and somatic withdrawal signs were recorded for 10 minutes by an experienced observer who was blind to the treatment conditions. The time-interval between the mecamylamine injections was at least 72 hours.

2.7.5 Experiment 5, Effect of NPY on somatic signs associated with spontaneous nicotine withdrawal—The aim of this experiment was to investigate the effect of NPY on the somatic signs associated with spontaneous nicotine withdrawal. At the end of experiment 4 (Day 28), the minipumps (saline, $n = 9$, nicotine $n = 18$) were removed in order to investigate the effect of NPY on spontaneous somatic withdrawal signs. Somatic withdrawal signs were recorded 12 hours and 24 hours after minipump explantation. At the 12-hour time point, somatic withdrawal signs were recorded in saline- and nicotine-treated rats. This allowed us to investigate if the discontinuations of nicotine administration lead to an increase in somatic withdrawal signs. Thirty minutes prior to the 24-hour time point, half of the nicotine-treated rats received NPY (16 μg [3.75 nmol], icv, $n = 9$) and the other half of the nicotine-treated rats ($n = 9$) received vehicle. Rats were randomly attributed to the NPY or vehicle group. Three of the rats (1 saline and 2 nicotine rats) were excluded because of a blocked cannula. At the end of the experiment the rats were euthanized and cannulae placement were verified.

2.7.6 Experiment 6, Effect of [D-His²⁶]-NPY on somatic signs associated with spontaneous nicotine withdrawal—The aim of this experiment was to investigate the effect of [D-His²⁶]-NPY on the somatic signs associated with spontaneous nicotine withdrawal. Rats were prepared with 28-day osmotic minipumps that contained nicotine (9 mg/kg/day of nicotine salt, n = 20) dissolved in saline. The minipumps were removed at Day 28 and somatic withdrawal signs were recorded 12 hours and 24 hours after minipump explantation. Thirty minutes prior to the 24-hour time point, half the rats were treated with [D-His²⁶]-NPY (16 µg [3.75 nmol], icv, n = 10) and the other half of the rats (n = 10) were treated with vehicle. Rats were randomly assigned to the [D-His²⁶]-NPY or vehicle group. At the end of the experiment the rats were euthanized and cannulae placement were verified.

2.8 Drugs

Nicotine, mecamylamine, and BIBP-3226 were obtained from Sigma-Aldrich (St. Louis, MO, USA). NPY (human, rat) was obtained from (GenScript Corp, Piscataway, NJ, USA) and [D-His²⁶]-NPY was kindly provided by Dr. Jean Rivier (The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, San Diego, CA). Nicotine and mecamylamine were dissolved in saline. NPY and [D-His²⁶]-NPY were dissolved in distilled water. NPY and [D-His²⁶]-NPY were dissolved immediately prior to the experiment and kept on ice until administration. BIBP-3226 was dissolved in distilled water that contained 3% DMSO.

2.9 Statistics

For experiment 1 and 2, ICSS parameters (brain reward thresholds and response latencies) were expressed as a percentage of the pre-test day values. Percent changes in ICSS parameters were analyzed using two-way repeated-measures analyses of variance (ANOVA), with the dose of NPY or [D-His²⁶]-NPY as the within-subject factor and pump content (saline or nicotine) as the between-subject factor. For experiment 3, ICSS parameters were also expressed as a percentage of the pre-test day values. The effect of BIBP-3226 was analyzed using one-way repeated-measures ANOVA, with the dose of BIBP-3226 as the within-subject factor. For experiments 1 - 3, statistically significant results in the ANOVA were followed by the Newman-Keuls post-hoc test. The Student's t-test was used to compare the mean absolute brain reward thresholds and response latencies of the saline- and nicotine-treated rats before minipump implantation. For experiment 4 - 6, the effects of NPY or [D-His²⁶]-NPY on overall somatic signs and individual somatic signs were analyzed using the non parametric Mann-Whitney U Test (2 groups) or the Kruskal-Wallis Test (3 or more groups). For all experiments, the criterion for significance was set at 0.05. The statistical analyses were performed using SPSS for Windows software, version 9.0.

3. Results

3.1 Experiment 1, Effect of NPY on the elevations in brain reward thresholds associated with precipitated nicotine withdrawal

Mean (±S.E.M.) absolute brain reward thresholds before pump-implantation for saline- and nicotine-treated rats were 89.03 ± 3.13 and 100.68 ± 3.28 µA [$t(14)=1.25$, n.s.], respectively. Mean (±S.E.M.) absolute response latencies for saline- and nicotine-treated rats were 3.13 ± 0.07 and 3.28 ± 0.11 s [$t(14)=1.17$, n.s.], respectively. Figure 1A indicates that mecamylamine elevated the brain reward thresholds of the nicotine-treated rats, but not of the saline-treated control rats (Treatment $F_{1,14}=10.93$, $P < 0.005$). Pretreatment with NPY did not affect the brain reward thresholds of the nicotine-treated rats. In contrast, NPY elevated the brain reward thresholds of the saline-treated rats (Dose × Treatment interaction: $F_{3,42}=2.98$, $P<0.042$). Newman-Keuls post-hoc comparisons indicated that the brain reward thresholds of the rats chronically treated with saline and acutely treated with 4 or 16 µg of NPY were elevated

compared to those of the rats chronically treated with saline and acutely treated with vehicle (0 µg of NPY). Mecamylamine increased the response latencies of the nicotine-treated rats compared to those of the saline-treated rats (Figure 1B, Treatment $F_{1,14}=10.71$, $P < 0.006$). NPY did not affect the response latencies of the saline- or nicotine-treated rats (Dose: $F_{3,42}=0.24$, n.s.; Dose $\times$ Treatment interaction: $F_{3,42}=0.49$, n.s.).

3.2 Experiment 2, Effect of [D-His²⁶]-NPY on the elevations in brain reward thresholds associated with precipitated nicotine withdrawal

Mean ($\pm$ S.E.M.) absolute brain reward thresholds before pump-implantation for saline- and nicotine-treated rats were 101.02 ± 14.38 and $101.70 \pm 8.77 \mu A$ [$t(18)=0.042$, n.s.], respectively. Mean ($\pm$ S.E.M.) absolute response latencies for saline- and nicotine-treated rats were 3.52 ± 0.42 and 3.61 ± 0.25 s [$t(18)=0.57$, n.s.], respectively. Mecamylamine elevated the brain-reward thresholds of the nicotine-treated rats and did not affect the brain reward thresholds of the saline-treated control rats (Figure 2A, Treatment $F_{1,19}=7.18$, $P < 0.015$). [D-His²⁶]-NPY did not have an effect on brain reward thresholds of the nicotine-withdrawing rats, but [D-His²⁶]-NPY increased the brain reward thresholds of the saline treated rats (Dose $\times$ Treatment interaction: $F_{3,57} = 3.05$, $p < 0.036$). Newman-Keuls post-hoc comparisons revealed that brain reward thresholds of animals chronically treated with saline and acutely treated with 16 µg [D-His²⁶]-NPY were elevated compared to those of rats chronically treated with saline and acutely treated with vehicle (0 µg of [D-His²⁶]-NPY) or 1 µg of [D-His²⁶]-NPY. Brain reward thresholds of rats chronically treated with nicotine and acutely treated with either 0 or 1 µg [D-His²⁶]-NPY were elevated compared to those of their saline-treated control group. The administration of [D-His²⁶]-NPY or mecamylamine did not have an effect on the response latencies (Figure 2B, Dose: $F_{3,57}=1.88$, n.s.; Treatment: $F_{1,19}=0.40$, n.s.; Dose $\times$ Treatment interaction: $F_{3,57}=0.56$, n.s.).

3.3 Experiment 3, Effect of BIBP-3226 on NPY-induced elevations in brain reward thresholds

The aim of the first part of this experiment was to investigate the effect of NPY on brain reward thresholds. Statistical analyses indicated that 16 µg of NPY elevated brain reward thresholds (Dose: $F_{1,5}=7.63$, $p < 0.04$) and did not affect response latencies (Dose: $F_{1,5}=1.78$, n.s.). The brain reward thresholds after the administration of vehicle or NPY were 103.85 ± 1.64 and 123.14 ± 6.29 percent of baseline, respectively. The response latencies after the administration of vehicle or NPY were 98.26 ± 3.38 and 104.79 ± 4.14 percent of baseline, respectively. The brain reward thresholds and response latencies on the pre-test day served as baseline. The second part of this experiment investigated the effect of the Y1 receptor antagonist BIBP-3226 on NPY-induced elevations in brain reward thresholds. The brain reward thresholds of the rats treated with vehicle (3% DMSO) and 16 µg NPY were elevated compared to those of the rats treated with vehicle (3% DMSO) and vehicle (distilled water) (Figure 3A, Dose: $F_{1,12}=4.87$, $p < 0.048$). There were no differences in the response latencies of the vehicle (3% DMSO) - 16µg NPY group and the vehicle (3% DMSO) - vehicle (distilled water) group (Figure 3B, $F_{1,12}=0.64$, n.s.). Pretreatment with BIBP-3226 prevented the NPY-induced elevations in brain reward thresholds (Dose: $F_{2,26}=3.38$, $P < 0.0497$). Posthoc analyses indicated that pretreatment with 32 µg of BIBP-3226 prevented the NPY-induced elevations in brain reward thresholds ($P < 0.05$). This suggests that the NPY-induced elevations in brain reward thresholds are at least partly mediated via the activation of central Y1 receptors.

3.4 Experiment 4, Effects of NPY and [D-His²⁶]-NPY on somatic signs associated with precipitated nicotine withdrawal

The nAChR antagonist mecamylamine induced more somatic signs in rats chronically-treated with nicotine than in the saline-treated control rats (nicotine-treated rats, 67.15 ± 2.70 vs. saline-treated rats, 7.20 ± 1.39 , $P < 0.001$). The highest dose of NPY (16 µg) decreased the overall

number of somatic signs associated with precipitated nicotine withdrawal (Figure 4A, $P < 0.032$). In contrast, the selective Y1 receptor antagonist [D-His²⁶]-NPY did not have an effect on the overall number of somatic signs associated with precipitated nicotine withdrawal (Figure 4B). A detailed analysis indicated that NPY lowered the number of abdominal constrictions (Table 1, 16 μg of NPY, $P < 0.021$) and eye blinks (4 μg of NPY, $P < 0.021$) associated with precipitated nicotine withdrawal, but increased the number of facial fasciculations (4 μg of NPY, $P < 0.011$; 16 μg of NPY, $P < 0.017$) and other signs (4 μg of NPY, $P < 0.033$). Similar to NPY, [D-His²⁶]-NPY decreased the number of abdominal constrictions (Table 2, 16 μg of NPY, $P < 0.036$). [D-His²⁶]-NPY increased the number of facial fasciculations (16 μg of [D-His²⁶]-NPY, $P < 0.017$) and other signs (1 μg of [D-His²⁶]-NPY, $P < 0.031$; 4 μg of [D-His²⁶]-NPY, $P < 0.0496$) associated with precipitated nicotine withdrawal.

3.5 Experiment 5, Effect of NPY on somatic signs associated with spontaneous nicotine withdrawal

Twelve hours after the explantation of the minipumps, the total number of somatic withdrawal signs was higher in the nicotine-treated rats than in the saline-treated control rats ($P < 0.0001$). The total number of somatic signs in the saline-treated group was 5.78 ± 1.50 and the total number of somatic signs in the nicotine-treated group was 22.33 ± 2.19 . This indicates that discontinuation of nicotine administration lead to a somatic nicotine withdrawal syndrome. Administration of NPY prior to the 24-hour time point decreased the overall number of somatic withdrawal signs (Figure 5, 16 μg of NPY, $P < 0.021$). A detailed analysis indicated that NPY decreased the number of abdominal constrictions (Table 3, 16 μg of NPY, $P < 0.021$) and eye blinks (16 μg of NPY, $P < 0.021$).

3.6 Experiment 6, Effect of [D-His²⁶]-NPY on somatic signs associated with spontaneous nicotine withdrawal

The administration of [D-His²⁶]-NPY prior to the 24-hour time point decreased the total number of somatic withdrawal signs (Figure 6, 16 μg of NPY, $P < 0.00031$). Additional statistical analyses indicated that [D-His²⁶]-NPY decreased the number of abdominal constrictions (Table 4, 16 μg of [D-His²⁶]-NPY, $P < 0.00067$), eye blinks (16 μg of [D-His²⁶]-NPY, $P < 0.0035$), and ptosis (16 μg of [D-His²⁶]-NPY, $P < 0.0069$).

Discussion

The results presented here indicate that NPY and the selective Y1 receptor agonist [D-His²⁶]-NPY do not prevent the elevations in brain reward thresholds associated with precipitated nicotine withdrawal in rats. In addition, it was shown that NPY and [D-His²⁶]-NPY elevated the brain reward thresholds of the saline-treated control rats. The selective Y1 receptor antagonist BIBP-3226 attenuated the NPY-induced elevations in brain reward thresholds, which suggests that NPY elevated brain reward thresholds at least partly by stimulating Y1 receptors. NPY and [D-His²⁶]-NPY did not increase the response latencies of the saline- and nicotine-treated rats. This indicates that NPY or [D-His²⁶]-NPY did not induce sedative- or locomotor-suppressant effects, which might have affected the outcome of the ICSS test sessions.

In a separate series of experiments, NPY attenuated precipitated somatic nicotine withdrawal signs and NPY and [D-His²⁶]-NPY attenuated spontaneous somatic withdrawal signs. Detailed analyses of the effects of NPY and [D-His²⁶]-NPY on somatic withdrawal signs indicated that these compounds have similar effects on specific somatic signs. The highest dose of NPY (16 μg) and [D-His²⁶]-NPY (16 μg) decreased the number of precipitated abdominal constrictions and increased precipitated facial fasciculations. The effect of [D-His²⁶]-NPY on facial fasciculations was so pronounced (1.45 ± 0.51 vehicle vs. 8.42 ± 1.69 [D-His²⁶]-NPY) that an

effect of [D-His²⁶]-NPY on overall somatic signs could not be detected. NPY decreased the number of abdominal constrictions and eye blinks associated with spontaneous nicotine withdrawal. A similar pattern was observed after the administration of the Y1 receptor agonist. [D-His²⁶]-NPY decreased the number of abdominal constrictions, eye blinks, and ptosis. An effect of NPY on ptosis was not detected. This was probably due to the fact that the number of occurrences was relatively low in the vehicle / chronic nicotine control group. It is interesting to note that NPY and [D-His²⁶]-NPY increased the number of facial fasciculations associated with precipitated nicotine withdrawal and did not have an effect on the number of facial fasciculations associated with spontaneous nicotine withdrawal. The precipitated and spontaneous nicotine withdrawal experiments were conducted under the same conditions and the animals were from the same strain and provided by the same vendor. Therefore, it is most likely that the NPY and [D-His²⁶]-NPY-induced increase in facial fasciculations in the precipitated withdrawal experiment was due to a complex interaction between the nAChR antagonist and the NPY receptor agonists. The observation that NPY and [D-His²⁶]-NPY decreased somatic nicotine withdrawal signs is in line with previous studies which reported that NPY attenuates somatic opioid and alcohol withdrawal signs (Woldbye et al., 1998; Woldbye et al., 2002).

The finding that the nAChR antagonist mecamylamine elevated the brain reward thresholds of the nicotine-treated rats, but not of the saline-treated rats, is in line with previous studies that investigated the effects of antagonism of nAChRs in nicotine dependent rats. Previous research has shown that systemic administration of the nAChR antagonists mecamylamine and dihydro- β -erythroidine (DH β E) or intra-ventral tegmental area administration of DH β E elevates the brain reward thresholds of nicotine-treated rats and does not affect the brain reward thresholds of saline-treated control rats (Bruijnzeel and Markou, 2004; Epping-Jordan et al., 1998; Harrison et al., 2001). It has been suggested that elevations in brain reward thresholds reflect a negative emotional state that resembles the dysphoria experienced by drug dependent patients after the discontinuation of drug use. Previous research indicates that inhibition of serotonin reuptake or antagonism of CRF receptors prevents the negative emotional state of nicotine withdrawal (Bruijnzeel et al., 2007; Harrison et al., 2001). It has been suggested that NPY counteracts the effects of stressors and possesses antidepressant-like properties (Heilig, 2004). Therefore, we hypothesized that NPY would prevent the negative affective state associated with precipitated nicotine withdrawal. A possible explanation for the fact that the administration of NPY and [D-His²⁶]-NPY did not affect nicotine withdrawal induced elevations in brain reward thresholds, elevated brain reward thresholds in the saline-treated rats, and decreased the overall number of somatic withdrawal signs is that NPY induces a powerful decrease in neuronal excitability. NPY has been reported to decrease neuronal excitability by decreasing excitatory synaptic transmission and increasing inhibitory synaptic transmission (Bacci et al., 2002). In addition, NPY has been shown to inhibit the release of a wide range of neurotransmitters including glutamate and GABA (Chen and van den Pol, 1996; Rhim et al., 1997). It has been suggested that the somatic signs of drug withdrawal are at least partly mediated by an increased release of glutamate in brainstem areas such as the locus coeruleus (Aghajanian et al., 1994; Van Bockstaele et al., 2001). Therefore, NPY-induced inhibition of glutamate release could possibly explain the decreased number of somatic signs in rats treated with NPY or [D-His²⁶]-NPY.

It is of interest to note that previous studies reported that the administration of NPY into the nucleus accumbens induces conditioned place preference (CPP) via a dopamine dependent mechanism (Brown et al., 2000; Josselyn and Beninger, 1993). The outcome of these CPP experiments does not necessarily contradict the outcome of the present studies in which the administration of NPY or [D-His²⁶]-NPY into the lateral ventricles elevated brain reward thresholds. A major difference between the place preference studies and the present studies is that in the place preference studies NPY was administered into a specific brain site, nucleus

accumbens, while in the present studies NPY was administered into the lateral ventricles and therefore mediated its effect in the whole brain. NPY might have brain site specific effects on brain reward function and it cannot be ruled out that the administration of NPY in the nucleus accumbens might have lowered brain reward thresholds, which would be in line with the observation that the administration of NPY into the nucleus accumbens induces place preference. However, caution is warranted when comparing the outcome of CPP and ICSS experiments. A major difference between the ICSS procedure and the CPP test is that in the ICSS test procedure the acute effects of a drug is assessed (animal is under the influence of the drug) while in the CPP test the rats are tested in a drug-free state and the outcome of the test is dependent on a previously formed association between the subjective effects of the drug and the environment (Carlezon, Jr., 2003).

To our knowledge, this is the first study to report that NPY or a specific Y1 receptor agonist reduces the reinforcing effects of LH self-stimulation in rats. The outcome of this experiment is not in line with two other studies that concluded that NPY does not affect the reinforcing effects of LH self-stimulation (Cabeza de Vaca et al., 1998; Fulton et al., 2002). It is unlikely that this discrepancy is due to the doses of NPY used. In the present study, 4 and 16 µg of NPY induced an elevation in brain reward thresholds. The highest dose of NPY in the study conducted by Fulton and colleagues, 4 µg of NPY, and the highest dose in the study conducted by Cabeza de Vaca and colleagues, 12.5 µg of NPY, did not affect LH self-stimulation (Cabeza de Vaca et al., 1998; Fulton et al., 2002). These doses are the same or higher than the doses that elevated brain reward thresholds in the present study. The drug infusion protocols were slightly different in these three NPY studies. In the present study, NPY (5 µl) or [D-His²⁶]-NPY (5 µl) was administered by gravity over a 30-60 second period. Fulton and colleagues administered NPY in a volume of 4 µl over a 4-minute period (Fulton et al., 2002). Cabeza de Vaca and colleagues administered NPY in a volume of 5 µl over a 95-second period using a syringe pump (Cabeza de Vaca et al., 1998). We are not aware of any studies that reported that minor differences in injection volume or injection speed could lead to different outcomes. However, it cannot be ruled out that differences in the injection procedure might account for some of the observed differences between the present study and the studies conducted by Cabeza de Vaca and Fulton and colleagues. Another possible explanation for the discrepancy in the effects of NPY on LH self-stimulation might be that a different ICSS method was used in the present study than in the studies that reported that NPY does not affect the reinforcing effects of LH self-stimulation (Cabeza de Vaca et al., 1998; Fulton et al., 2002). In the present study a rate-independent discrete-trial method was used to determine the effects of NPY on LH self-stimulation (Esposito and Kornetsky, 1977). In the studies conducted by Fulton, Cabeza de Vaca and colleagues a rate-dependent ICSS method was used to investigate the effects of NPY on LH self-stimulation (Cabeza de Vaca et al., 1998; Fulton et al., 2002). A major difference between these two methods is that in the rate-independent method reward thresholds are determined by systematically changing the stimulus intensities whereas in the rate-dependent method the stimulus intensity is fixed and a change in response rate is interpreted as a change in the reinforcing value of LH self-stimulation. Previous research suggest that drugs might have a different effect on LH self-stimulation depending on the self-stimulation methodology used (Esposito and Kornetsky, 1978; Liebman, 1983). For example, low doses of the typical antipsychotic pimozide elevate brain reward thresholds in a rate-independent ICSS procedure, but do not affect response-rates in a rate-dependent ICSS procedure (Zarevics and Setler, 1979). Therefore, it may be possible that rate-independent procedures are more sensitive at detecting the effects of drugs on the reinforcing effects of LH self-stimulation. Finally, it may be possible that the discrepancy in the effect of NPY on LH self-stimulation is due to differences in pre-treatment intervals. In the present study, NPY was administered 30 or 35 minutes prior to testing. In the study conducted by Cabeza de Vaca and colleagues, NPY was administered 5 minutes prior to testing and in the study conducted by Fulton and colleagues NPY was administered 15 minutes prior to testing (Cabeza de Vaca et

al., 1998; Fulton et al., 2002). Therefore, it cannot be ruled out that Cabeza de Vaca and Fulton and colleagues might have detected an effect on LH self-stimulation if the pre-treatment intervals had been longer.

In conclusion, the present studies suggest that stimulation of Y1 receptors attenuates the somatic signs associated with nicotine withdrawal but does not reverse the elevations in brain reward thresholds associated with precipitated nicotine withdrawal. This suggests that NPY or Y1 receptor agonists may be an effective treatment for the physical discomfort associated with the discontinuation of tobacco smoking. In addition, it was shown that NPY elevates brain reward thresholds via an Y1 dependent mechanism. Future studies may investigate if the administration of NPY or selective Y1 receptor agonists affects the deficit in brain reward function associated with spontaneous nicotine withdrawal or nicotine withdrawal-induced anxiety-like behavior. Experiments that investigate the effects of novel treatments on the somatic and affective aspects of nicotine withdrawal are warranted as treatments that counteract acute and protracted nicotine-withdrawal symptoms may improve relapse rates.

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Figure 1.

Effects of NPY (saline, $n = 8$; nicotine, $n = 8$) on brain reward thresholds (A) and response latencies (B) during nicotine withdrawal. Brain reward thresholds are expressed as a percentage of the pre-test day values. Asterisks (** $P < 0.01$) indicate elevations in brain reward thresholds compared to those of the corresponding saline-treated control group. Crosses (+ $P < 0.05$, ++ $P < 0.01$) indicate elevations in brain reward thresholds compared to those of rats chronically treated with saline and acutely treated with vehicle (0 μg of NPY). Data are expressed as means $\pm$ SEM.

Figure 2.

Effects of [D-His²⁶]-NPY (saline, n = 9; nicotine, n = 11) on brain reward thresholds (A) and response latencies (B) during nicotine withdrawal. Brain reward thresholds are expressed as a percentage of the pre-test day values. Asterisks (** P<0.01) indicate elevations in brain reward thresholds compared to those of the corresponding saline-treated control group. Crosses (++ P<0.01) indicate elevations in brain reward thresholds compared to those of rats chronically treated with saline and acutely treated with vehicle (0 µg of [D-His²⁶]-NPY). Data are expressed as means ± SEM.

Figure 3.

Effects of BIBP-3226 (16 or 32 μ g, $n = 14$) on NPY-induced (16 μ g, icv) elevations in brain reward thresholds (A) and response latencies (B). Brain reward thresholds and response latencies are expressed as a percentage of the pre-test day values. Asterisks (* $P < 0.05$) indicate a decrease in brain reward thresholds compared to the vehicle / NPY group. Crosses (+ $P < 0.05$) indicate an elevations in brain reward thresholds compared to the vehicle / vehicle group ($n = 13$). Data are expressed as means $\pm$ SEM. Abbreviations: B, BIBP-3226; veh, vehicle.

Figure 4. Effects of NPY (A, n = 10) and [D-His²⁶]-NPY (B, n = 10) on somatic signs associated with mecamylamine-precipitated nicotine withdrawal. Asterisks (* P<0.05) indicate a decrease in overall somatic signs compared to those of rats treated with vehicle (0 µg of NPY). Data are expressed as means ± SEM.

Figure 5.

Effects of NPY (16 μ g, icv, n = 9 per group) on somatic signs associated with spontaneous nicotine withdrawal. NPY or vehicle was administered 30 minutes prior to the 24-hour time point. Asterisks (* $P < 0.05$) indicate a decrease in overall somatic signs compared to those of rats treated with vehicle (0 μ g of NPY). Data are expressed as means $\pm$ SEM.

Figure 6.

Effects of [D-His²⁶]-NPY (16 µg, icv, n = 10 per group) on somatic signs associated with spontaneous nicotine withdrawal. [D-His²⁶]-NPY or vehicle was administered 30 minutes prior to the 24-hour time point. Asterisks (** P<0.01) indicate a decrease in overall somatic signs compared to those of rats treated with vehicle (0 µg of [D-His²⁶]-NPY). Data are expressed as means ± SEM.

Table 1

Effects of NPY on somatic signs associated with precipitated nicotine withdrawal.

	0	1	Dose of NPY (μ g, icv) 4	16
Abdominal const.	9.20 $\pm$ 2.80	5.10 $\pm$ 1.80	5.40 $\pm$ 1.87	2.00 $\pm$ 0.52*
Eye blinks	8.80 $\pm$ 1.87	6.00 $\pm$ 1.79	3.00 $\pm$ 1.06*	4.10 $\pm$ 1.66
Ptosis	5.10 $\pm$ 1.32	5.00 $\pm$ 0.99	4.80 $\pm$ 0.88	4.90 $\pm$ 0.98
Facial Fasc.	1.90 $\pm$ 0.55	2.00 $\pm$ 0.61	5.20 $\pm$ 0.88*	4.80 $\pm$ 1.14*
Other signs	2.40 $\pm$ 0.50	6.20 $\pm$ 2.03*	3.20 $\pm$ 0.83	2.20 $\pm$ 0.53

All rats were chronically treated with nicotine (9 mg/kg/day, sc, 28-days). NPY (n = 10) was administered 30 minutes prior to the administration of mecamylamine (2 mg/kg, sc). Asterisks (* P<0.05) indicate a difference in the number of somatic signs compared to rats treated with vehicle. Data are expressed as means $\pm$ SEM.

Table 2Effects of [D-His²⁶]-NPY on somatic signs associated with precipitated nicotine withdrawal.

	0	1	Dose of [D-His ²⁶]-NPY (μg, icv) 4	16
Abdominal const.	10.73 ± 3.86	6.27 ± 2.53	2.55 ± 0.81	4.59 ± 1.41*
Eye blinks	6.73 ± 2.01	6.36 ± 2.20	4.00 ± 1.00	6.30 ± 1.91
Ptosis	4.45 ± 1.07	4.73 ± 1.29	5.82 ± 1.28	6.00 ± 1.38
Facial Fasc.	1.45 ± 0.51	2.82 ± 0.71	4.45 ± 1.06	8.42 ± 1.69*
Other signs	2.00 ± 0.73	6.00 ± 1.58*	4.82 ± 1.26*	5.09 ± 1.32

All rats were chronically treated with nicotine (9 mg/kg/day, sc, 28-days). [D-His²⁶]-NPY (n = 10) was administered 30 minutes prior to the administration of mecamylamine (2 mg/kg, sc). Asterisks (* P<0.05) indicate a difference in the number of somatic signs compared to rats treated with vehicle. Data are expressed as means ± SEM.

Table 3

Effects of NPY on somatic signs associated with spontaneous nicotine withdrawal.

	0	Dose of NPY (µg, icv) 16
Abdominal const.	5.78 ± 1.74	1.00 ± 0.24*
Eye blinks	8.44 ± 1.48	3.11 ± 0.59**
Ptosis	2.44 ± 0.97	1.33 ± 0.37
Facial Fasc.	3.33 ± 0.99	2.89 ± 1.02
Other signs	1.67 ± 0.44	1.89 ± 1.30

All rats were chronically treated with nicotine (9 mg/kg/day, sc, 28-days). NPY (n = 9) or vehicle (n = 9) was administered 30 minutes prior to the 24-hour time point (post minipump explantation). Asterisks (* P<0.05, ** P<0.01) indicate a decrease in the number of somatic signs compared to rats treated with vehicle. Data are expressed as means ± SEM.

Table 4Effects of [D-His²⁶]-NPY on somatic signs associated with spontaneous nicotine withdrawal.

	0	Dose of [D-His ²⁶]-NPY (μg, icv) 16
Abdominal const.	13.40 ± 5.19	1.10 ± 0.38**
Eye blinks	13.30 ± 2.86	3.60 ± 0.97**
Ptosis	4.50 ± 0.89	1.20 ± 0.49**
Facial Fasc.	1.20 ± 0.63	2.90 ± 0.77
Other signs	0.80 ± 0.29	0.80 ± 0.29

All rats were chronically treated with nicotine (9 mg/kg/day, sc, 28-days). [D-His²⁶]-NPY (n = 10) or vehicle (n = 10) was administered 30 minutes prior to the 24-hour time point (post minipump explantation). Asterisks (** P<0.01) indicate a decrease in the number of somatic signs compared to rats treated with vehicle. Data are expressed as means ± SEM.