Published in final edited form as:

Eur Neuropsychopharmacol. 2016 September ; 26(9): 1378–1389. doi:10.1016/j.euroneuro.2016.07.007.

Overexpression of CRF in the BNST diminishes dysphoria but not anxiety-like behavior in nicotine withdrawing rats

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Abstract

Smoking cessation leads to dysphoria and anxiety, which both increase the risk for relapse. This negative affective state is partly mediated by an increase in activity in brain stress systems. Recent studies indicate that prolonged viral vector-mediated overexpression of stress peptides diminishes stress sensitivity. Here we investigated whether the overexpression of corticotropin-releasing factor (CRF) in the bed nucleus of the stria terminalis (BNST) diminishes nicotine withdrawal symptoms in rats. The effect of nicotine withdrawal on brain reward function was investigated with an intracranial self-stimulation (ICSS) procedure. Anxiety-like behavior was investigated in the elevated plus maze test and a large open field. An adeno-associated virus (AAV) pseudotype 2/5 vector was used to overexpress CRF in the lateral BNST and nicotine dependence was induced using minipumps. Administration of the nicotinic receptor antagonist mecamylamine and cessation of nicotine administration led to a dysphoria-like state, which was prevented by the overexpression of CRF in the BNST. Nicotine withdrawal also increased anxiety-like behavior in the elevated plus maze test and large open field test and slightly decreased locomotor activity in the open field. The overexpression of CRF in the BNST did not prevent the increase in anxiety-like behavior or decrease in locomotor activity. The overexpression of CRF increased CRF₁ and CRF₂ receptor gene expression and increased the CRF₂/CRF₁ receptor ratio. In conclusion, the overexpression of CRF in the BNST prevents the dysphoria-like state associated with nicotine withdrawal and increases the CRF₂/CRF₁ receptor ratio, which may diminish the negative effects of CRF on mood.

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Conflict of interest

There are no conflicts of interest.

Contributors

XQ and AB designed the studies, conducted the statistical analysis, and wrote the manuscript. XQ, LG, MF and AB conducted the behavioral experiments. XQ, ZY, ZS, KW did the Western blotting and PCR, and XQ and ZS created and injected the viral vectors.

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Keywords

Nicotine; dependence; dysphoria; CRF; overexpression; AAV vectors

1. Introduction

The rewarding and cognitive enhancing effects of nicotine play an important role in the initiation of smoking (Bruijnzeel, 2012; Rezvani and Levin, 2001). After the development of nicotine addiction, people mainly continue to smoke to prevent the dysphoria and anxiety associated with smoking cessation (Koob and Volkow, 2010). This is supported by the observation that most smokers relapse during the first week of abstinence when affective withdrawal signs are most severe and relapse rates are higher in people with depression (Hughes et al., 2004; Jarvis, 2004). Furthermore, smokers are more likely to have an anxiety disorder than non-smokers and smokers with an anxiety disorder are less motivated to quit (Johnson et al., 2000; Zvolensky et al., 2007).

Corticotropin-releasing (CRF) factor is a peptide that plays a critical role in depression and anxiety disorders and there is extensive interest in the role of this peptide in drug addiction (de Kloet et al., 2005; Koob and Volkow, 2010). CRF mediates its effects via CRF₁ and CRF₂ receptors (Dautzenberg and Hauger, 2002). Stimulation of CRF₁ receptors induces a negative mood state and anxiety-like behavior (Overstreet and Griebel, 2004; Zorrilla et al., 2002). The role of the CRF₂ receptor in the regulation of mood states is not as clear yet. However, growing evidence suggests that CRF₂ receptor activation opposes the effect of CRF₁ receptor activation and has anxiolytic and antidepressant-like effects (Bale and Chen, 2012; Bale and Vale, 2004).

Animal studies have provided critical insight into the effects of nicotine withdrawal on mood and anxiety-like behavior (Bruijnzeel, 2012). The intracranial self-stimulation (ICSS) procedure can provide insight into the effects of drugs of abuse on brain reward function (Der-Avakian and Markou, 2012). Acute nicotine administration lowers brain reward thresholds in the ICSS procedure, which is indicative of a potentiation of brain reward function (Harrison et al., 2002; Igari et al., 2013). In contrast, withdrawal from nicotine leads to elevations in brain reward thresholds, which is indicative of dysphoria-like state (Barr et al., 2002; Epping-Jordan et al., 1998). Nicotine also affects anxiety-like behavior with acute nicotine administration decreasing and cessation of chronic nicotine administration increasing anxiety-like behavior (George et al., 2007; Irvine et al., 2001). Blockade of CRF₁ receptors prevents dysphoria- and anxiety-like behavior associated with nicotine withdrawal (Bruijnzeel et al., 2009; Cohen et al., 2015; George et al., 2007). Both CRF₁ and CRF₂ receptors have been detected in the bed nucleus of the stria terminalis (BNST) and preclinical studies have provide evidence for a role of this brain site in regulating mood and anxiety-like behavior (Chalmers et al., 1995; Holmes et al., 2003; Shimada et al., 1989; Swanson et al., 1983). There is extensive evidence for a role of CRF in the BNST in stress-induced consummatory behavior and anxiety-like behavior (Elharrar et al., 2013; Micioni Di Bonaventura et al., 2014). Furthermore, the BNST plays a role in fear

responses (Walker et al., 2009). At this point little is known about the role of CRF in the BNST in the negative affective state associated with nicotine withdrawal.

Acute administration of CRF decreases the sensitivity to rewarding electrical stimuli and increases anxiety-like behavior (Adamec et al., 1991; Macey et al., 2000; Takahashi et al., 1989). However, the overexpression of CRF in the CeA attenuates the elevations in brain reward thresholds associated with nicotine withdrawal (Qi et al., 2014). This suggests that the effects of prolonged CRF overexpression resemble those of CRF₁ receptor blockade or CRF₂ receptor activation. The goal of the present study was to evaluate the effect of AAV pseudotype 2/5-mediated overexpression of CRF in the BNST on dysphoria- and anxiety-like behavior associated with nicotine withdrawal. It was hypothesized that the overexpression of CRF in the BNST would diminish nicotine withdrawal. The BNST expresses a relatively high level of CRF peptide and CRF₁ receptors and a low level of CRF₂ receptors (Chalmers et al., 1995; Swanson et al., 1983). An AAV vector was used to overexpress CRF because these vectors mediate long-term transgene expression with minimal off-target effects (Samulski and Muzyczka, 2014). Before the CRF overexpression studies, we determined the time course of transgene expression and tropism of this vector. This was done with a vector that expresses green fluorescent protein (GFP), which can be used as a marker for gene expression (Chalfie et al., 1994; Tsien, 1998). The effect CRF overexpression on baseline brain reward function and precipitated and spontaneous nicotine withdrawal was investigated with an ICSS procedure. Both CRF₁ and CRF₂ receptor gene expression was determined to explore the neuronal mechanisms by which CRF overexpression might affect nicotine withdrawal. Nicotine withdrawal increases anxiety-like behavior and decreases locomotor activity (George et al., 2007; Irvine et al., 2001; Malin et al., 1992). In the present study, the effect of CRF overexpression on withdrawal-induced anxiety-like behavior was investigated in the elevated plus maze test and a large open field. Overall, these studies will provide insight into the effect of CRF overexpression in the BNST on CRF receptor gene expression and the dysphoria- and anxiety-like behavior associated with nicotine withdrawal.

2. Experimental procedures

2.1. Animals

Male Wistar rats (200–225 g, Charles River, Raleigh, NC) were socially housed (2 per cage) in a climate-controlled vivarium on a reversed 12 h light-dark cycle. Food and water were available ad libitum. The experimental protocols were approved by the University of Florida (UF) Institutional Animal Care and Use Committee.

2.2. Electrode and cannula implantations

Rats were anesthetized with an isoflurane and oxygen vapor mixture (2% isoflurane) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with the incisor bar set 3.3 mm below the interaural line (flat skull). The coordinates for the electrodes and cannulas were based on previous studies (Bruijnzeel and Markou, 2004; Marcinkiewicz et al., 2009). Stainless steel cannulas (11 mm in length, 23-gauge) were implanted bilaterally 2.0 mm above the lateral BNST using flat skull coordinates: anterior-posterior (AP) –0.6,

medial-lateral (ML) ± 3.7 mm and 15° vertical tilt, dorsal-ventral (DV) -4.6 from dura. The electrodes (11 mm in length, Plastics One, Roanoke, VA, USA) were implanted in the medial forebrain bundle with the incisor bar 5 mm above the interaural line (AP -0.5 mm, ML ± 1.7 mm, DV -8.3 mm from dura). After the intracranial surgeries, the rats were allowed to recover for at least one week.

2.3. ICSS procedure

Rats were trained on a modified discrete-trial ICSS procedure as described previously (Bruijnzeel et al., 2009; Markou and Koob, 1992). The operant conditioning chambers were housed in sound-attenuating chambers (Med Associates, Georgia, VT, USA). The operant conditioning chambers had a 5 cm wide metal response wheel that was centered on a sidewall and a photobeam detector recorded every 90 degrees of rotation. Brain stimulation was delivered by constant current stimulators (Model 1200C, Stimtek, Acton, MA, USA). Each test session provided a brain reward threshold and response latency. The reward threshold was defined as the midpoint between stimulation intensities that supported responding and current intensities that failed to support responding. The response latency was defined as the time interval between the beginning of the non-contingent stimulus and a positive response. Elevations in brain reward thresholds reflect dysphoria (Barr et al., 2002). Drugs that have sedative effects or induce motor impairments increase the response latency and stimulants decrease the response latency (Igari et al., 2013; Liebman, 1985).

2.4. Plasmid construction and production of the AAV2/5 vectors

The viral vectors were produced as described previously (Qi et al., 2014). The AAV2/5-CRF vector was obtained from Vector BioLabs (Philadelphia, PA, USA) and the AAV2/5-GFP vector from the UF Powell Gene Therapy Center (Gainesville, FL, USA). Rat CRF cDNA or humanized GFP cDNA was inserted between AAV type 2 terminal repeats and the vectors had AAV type 5 capsids. The transduction efficiency of the vectors was evaluated by assessing transgene expression in primary neuronal cultures and the brain (see supplementary experimental procedures for details).

2.5. Intracerebral microinjection

In the first experiment the rats were anesthetized with an isoflurane and oxygen vapor mixture, placed in a stereotaxic frame, and unilaterally injected with AAV2/5-GFP (9.6×10^9 genome copies) in the lateral BNST. In the second experiment the rats were anesthetized and bilaterally injected with AAV2/5-CRF (5.6×10^{10} genome copies) or AAV2/5-GFP (1.5×10^{10} genome copies) with a stainless steel injector that extended 2.0 mm beyond the previously implanted guide cannula. In the third experiment the rats were anesthetized, placed in a stereotaxic frame, and then AAV2/5-CRF or AAV2/5-GFP was bilaterally infused into the lateral BNST (same doses as experiment 2). In all experiments, the injection volume was 0.8 μ l per side and this was infused over a 3-min period. The infusion speed was controlled by a Harvard Apparatus syringe pump (model 975) that was equipped with 10 μ l syringes (Hamilton, Reno, NE, USA). The injectors were left in place for 5 min to allow diffusion from the injector tip.

2.6. Osmotic minipump implantation

Rats were anesthetized with isoflurane and received minipumps (28 day pumps; Durect Corporation, Cupertino, CA, USA) filled with either saline or nicotine dissolved in saline (Sigma-Aldrich, St Louis, MO, USA). The nicotine concentration was adjusted to compensate for differences in body weight and to deliver a dose of 3.16 mg/kg/day nicotine base.

2.7. Small open field test

The small open field test was conducted to assess locomotor activity (Febo et al., 2003). The total distance traveled and vertical beam breaks (i.e., rearing) were measured with an automated animal activity cage system in a dark room (AccuScan Instruments, Columbus, OH, USA). The system consisted of four animal activity cages made of clear acrylic (40 cm × 40 cm × 30 cm; length [L] × width [W] × height [H]), with 16 equally spaced (2.5 cm) infrared beams across the length and width of the cage. One set of infrared beams was positioned 2 cm above the cage floor (horizontal activity beams) and another set of beams was positioned 14 cm above the cage floor (vertical activity beams). All beams were connected to a VersaMax analyzer which sent information to a computer that displayed beam data through VersaDat software. The test setup was cleaned with a Nolvasan solution between animals.

2.8. Large open field test

The large open field test was conducted in a dimly lit room (75 lux). The open field consisted of a large square arena measuring 120 × 120 × 60 cm (L × W × H). The arena was made of black high-density polyethylene panels that were placed on a plastic bottom plate (Faulkner Plastics, Miami, FL). The behavior of the animals was recorded with a camera that was mounted above the open field and the videos were analyzed with EthoVision XT 8.5 software (Noldus Information Technology, Leesburg, VA). The open field was divided into a border zone (20 cm wide) and a center zone (80 × 80 cm; L × W). The following behaviors were analyzed: total distance traveled, distance traveled in the border and center zone, and number of entries into the center zone.

2.9. Elevated plus maze test

The elevated plus maze test was conducted as described previously (Rylkova et al., 2009). The test apparatus consisted of four black polypropylene arms (Coulbourn Instruments, Whitehall, PA, USA). The two open arms had 0.5 cm ledges and the two closed arms had 30 cm tall walls. The open arms were placed opposite of each other. The arms were 10 cm wide, 50 cm long, and mounted on 55 cm tall acrylic legs. Testing was conducted in a dimly lit (75 lux) room and each test session lasted 5 min. At the beginning of each test the animals were placed on an open arm facing the center of the apparatus. The behavior of the animals was recorded with a camera and analyzed with EthoVision XT 8.5 software (Noldus Information Technology, Leesburg, VA, USA). The elevated plus maze was divided into 5 zones (two open arms, two closed arms, and center) and from each rat the center of the body and the nose were tracked. The following behaviors were analyzed: time on open arms (% of total; total is time in open and closed arms), distance traveled on open arms (% of total), and

entries into the open arms (% of total), total number of head dips into the open arms, and total number of closed arm entries. It was considered an open arm entry when the center of the rat was in one of the open arms. Head dips into the open arms were calculated by subtracting the total number of whole-body entries from the total number of nose-point (head) entries into the open arms (i.e., head dips that led to whole body entries were not included). Head dips into the open arms have been suggested to reflect risk assessment behavior (Griebel et al., 1997).

2.10. Immunohistochemistry, RT-PCR, and Western blotting

Immunohistochemistry, quantitative RT-PCR, and Western blotting was conducted as described previously with some minor modifications (Qi et al., 2014; Yang et al., 2014). For immunohistochemistry, the animals were perfused and 40 μ m coronal brain sections were cut with a cryostat. The brain sections containing the BNST were incubated with antibodies, mounted on slides, and examined with a confocal microscope. To determine CRF and CRF receptor mRNA levels, mRNA was isolated from brain tissue and analyzed using quantitative RT-PCR. Corticotropin releasing factor protein levels were determined by Western blotting. The BNST was punched out and homogenized and proteins were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were incubated with CRF antibodies and visualized on the membrane (see also supplementary experimental procedures).

2.11. Experimental design

2.11.1. Time-course of AAV2/5-mediated GFP expression and tropism in BNST

—To investigate the time course of AAV2/5-mediated GFP expression and whether the AAV2/5 vector transduces neurons, the viral vector (AAV2/5-GFP) was infused into the BNST of 18 rats. Two ($n = 6$), four ($n = 6$), and eight ($n = 6$) weeks later the rats were perfused and the brains were removed to determine GFP expression and tropism in the BNST (Figure 1).

2.11.2. Overexpression of CRF in the BNST and elevations in brain reward thresholds in nicotine withdrawing rats

—The rats were trained on the ICSS procedure and when the brain reward thresholds were stable, AAV2/5-GFP ($n = 23$) or AAV2/5-CRF ($n = 25$) was infused bilaterally into the BNST. Brain reward thresholds and response latencies were assessed the following day and continued to be assessed daily throughout the experiment. Twenty-eight days after the viral vector infusions, the rats were prepared with 28-day minipumps (sc) that delivered saline (Saline-GFP, $n = 11$; Saline-CRF, $n = 12$) or nicotine (Nicotine-GFP, $n = 12$; Nicotine-CRF, $n = 13$; 3.16 mg/kg/day of nicotine base). The electrodes of two rats (one saline-CRF and one nicotine-CRF rat) broke after the precipitated withdrawal phase and these rats were excluded from the rest of the experiment. The non-selective nAChR antagonist mecamylamine (0.33–3.0 mg/kg, sc) was used to precipitate nicotine withdrawal. Mecamylamine was administered according to a Latin square design 10-min before ICSS testing. The rats received the first injection 7 days after the minipump implantations and there were at least 3 days between subsequent mecamylamine injections. The minipumps were removed on day 28 to study spontaneous withdrawal (up to 96 h after pump removal). One day after the last ICSS session, the brains

of the rats were removed to assess CRF and CRF receptor levels. About half the animals from each group were used for Western blotting (GFP, n = 12; CRF, n = 12) and the other half for RT-PCR (GFP, n = 13; CRF, n = 11).

2.11.3. Overexpression of CRF in the BNST and anxiety-like behavior and locomotor activity in nicotine withdrawing rats

A new group of rats was bilaterally infused with AAV2/5-GFP (n = 24) or AAV2/5-CRF (n = 24) into the BNST. Twenty-eight days later the rats were prepared with 28-day minipumps that delivered saline (Saline-GFP, n = 12; Saline-CRF, n = 12) or nicotine (Nicotine-GFP, n = 12; Nicotine-CRF, n = 12; 3.16 mg/kg/day of nicotine base). Mecamylamine (1.5 mg/kg, sc) injections started at least 7 days after the pump implantations to allow for the development of dependence. There were at least 3 days between subsequent mecamylamine injections. The rats were tested first in a small open field, then in a large open field, and last in an elevated plus maze. After these studies, the minipumps were removed and three weeks later the effect of mecamylamine (mecamylamine vs. saline) on locomotor activity and rearing in the small open field was investigated. This was done to rule out the possibility that mecamylamine affects locomotor activity in control rats that were prepared with saline pumps. Only rats that had received saline pumps were used because nicotine induces a long-term increase in sensitivity to mecamylamine (Paterson and Markou, 2004). Half of the rats received a mecamylamine injection (GFP/mecamylamine, n = 6; CRF/mecamylamine, n = 6, sc) and the other half of the rats received a saline injection (GFP/saline, n=6; CRF/saline, n = 6; 1.5 mg/kg, sc). One day after the last small open field test, the brains of half the animals of each group were collected to assess the effect of the AAV-CRF vector on CRF protein levels in the BNST (GFP, n=12; CRF, n = 12). The brains of the rats that were not used for Western blotting were removed to validate the correct placement of the cannulas.

2.12. Statistics

The ICSS parameters were expressed as a percentage of the pre-test day values obtained on the day before the viral vector injections, mecamylamine injections, or minipump removal. The effect of the viral vectors on ICSS parameters (28-day period) was analyzed with a two-way repeated-measures ANOVA with time as within-subjects factor and vector (GFP vs CRF) as between-subjects factor. The effect of precipitated nicotine withdrawal on ICSS parameters was analyzed using a three-way repeated-measures ANOVA with mecamylamine dose as within-subjects factor and the vector and pump content (nicotine vs. saline) as between-subjects factors. The spontaneous nicotine withdrawal data were analyzed with a three-way repeated-measures ANOVA with time as within-subjects factor and vector and pump as between-subjects factors. The data from the small open field, large open field, and elevated plus maze test were analyzed with two-way ANOVAs with vector and pump as between-subjects factors. Western blot and PCR data were analyzed with Student's t-tests. Statistically significant results in the ANOVAs were followed by Bonferroni post hoc comparisons. The data were analyzed with GraphPad Prism version 6 and IBM SPSS Statistics version 22.

3. Results

3.1. Time-course of AAV2/5-mediated GFP expression and tropism in BNST

Time course of GFP expression in BNST—The immunostainings showed that GFP was expressed in the BNST after the AAV2/5-GFP infusions (Figure 2). Green fluorescent protein expression in the brain increased over time and its levels were higher at 4 and 8 weeks than at 2 weeks after infusion of the viral vectors (Time: $F_{2,15}=7.17$, $p<0.05$, Figure S1a).

Tropism of AAV2/5 vector in the BNST—The colocalization of GFP and the neuronal marker NeuN was examined to determine the AAV2/5-mediated transduction efficiency of neurons in the BNST. Almost all GFP-positive cells were NeuN-positive and this was the same for all time points (98%, Figure S1b). This indicates that almost all the cells that were transduced were neurons. To investigate if AAV2/5 transduces CRF neurons in the BNST, the colocalization of GFP, NeuN, and CRF was examined. The percentage of CRF-positive cells that was also GFP and NeuN-positive slightly increased over time (79–96%; Time: $F_{2,11}=24.67$, $p<0.0001$, Figure S1c,d).

3.2. Overexpression of CRF in the BNST and elevations in brain reward thresholds in nicotine withdrawing rats

Precipitated nicotine withdrawal and brain reward function—Prior to the administration of the viral vectors there were no differences in the brain reward thresholds or response latencies between the experimental groups (Table S1). The administration of the viral vectors led to a brief increase in brain reward thresholds (Time: $F_{27,1242}=14.95$, $p<0.0001$; Figure S2a) and response latencies (Time: $F_{27,1242}=3.96$, $p<0.0001$; Figure S2b) in the GFP and CRF group. However, there was no significant difference in brain reward thresholds or response latencies between the GFP and CRF group. On the test-day before the onset of the mecamylamine injections there was no difference in brain reward thresholds or response latencies between the groups (Saline-GFP, Saline-CRF, Nicotine-GFP, Nicotine-CRF; Table S1). The nAChR antagonist mecamylamine induced a dose-dependent devaluation of reward (elevation in brain reward thresholds) in the nicotine-treated rats but did not affect the brain reward thresholds of the saline-treated control rats (Dose: $F_{3,132}=58.55$, $P<0.0001$; Pump: $F_{1,44}=181.96$, $P<0.0001$; Dose x Pump: $F_{3,132}=44.87$, $P<0.0001$, Figure 3a). The overexpression of CRF in the BNST diminished the elevations in brain reward thresholds in the nicotine-treated rats but did not affect the brain reward thresholds of the saline-treated rats (Vector: $F_{1,44}=30.43$, $P<0.0001$; Vector x Pump: $F_{1,44}=16.93$, $P<0.0001$). Furthermore, the overexpression of CRF had the greatest effect in the nicotine-treated rats that received the highest dose of mecamylamine (Dose x Vector: $F_{3,132}=10.81$, $P<0.0001$; Dose x Vector x Pump: $F_{3,132}=11.82$, $P<0.0001$). The administration of mecamylamine increased the response latencies in the nicotine-treated rats but did not affect the response latencies of the saline-treated control rats (Dose: $F_{3,132}=6.49$, $P<0.0001$; Dose x Pump: $F_{3,132}=3.62$, $P<0.05$, Figure 3b). The overexpression of CRF did not affect the response latencies of the nicotine or saline-treated rats.

Spontaneous nicotine withdrawal and brain reward function—On the test-day before the removal of the minipumps there were no differences in baseline brain reward thresholds or response latencies between the groups (saline-GFP, Saline-CRF, Nicotine-GFP, Nicotine-CRF; Table S1). Removal of the minipumps led to an elevation in the brain reward thresholds of the nicotine-treated rats but did not affect the brain reward thresholds of the saline-treated control rats (Time: $F_{6,252}=19.78$, $P<0.0001$; Pump: $F_{1,42}=15.62$, $P<0.0001$; Time x Pump: $F_{6,252}=18.47$, $P<0.0001$; Figure 3c). Overexpression of CRF in the BNST prevented the elevations in brain reward thresholds in the nicotine-dependent rats but did not affect the brain reward thresholds of the saline-treated control rats (Vector: $F_{1,42}=7.93$, $P<0.01$; Time x Vector: $F_{6,252}=4.79$, $P<0.0001$; Time x Vector x Pump: $F_{6,252}=3.37$, $P<0.01$). The post hoc analyses showed that the brain reward thresholds of the nicotine-GFP rats were increased compared to those of the saline-GFP rats during the first 24 h after pump removal. Furthermore, during the first 24 h post pump removal the brain reward thresholds of the nicotine-CRF rats were lower than those of the nicotine-GFP rats, which indicates that the overexpression of CRF diminishes the negative mood state associated with nicotine withdrawal. Removal of the minipumps also increased the response latencies of the nicotine-treated rats and this effect was attenuated by the overexpression of CRF in the BNST (Time: $F_{6,252}=9.34$, $P<0.0001$; Time x Pump: $F_{6,252}=2.25$, $P<0.05$; Time x Vector: $F_{6,252}=2.24$, $P<0.05$; Figure 3d).

Gene transfer and CRF and CRF receptor gene expression in the BNST—At the end of the ICSS experiment, the brains of the rats were removed and CRF protein and mRNA levels were assessed. Western blotting revealed a band of about 21 kDa for the CRF precursor prepro-CRF³². The CRF level in the BNST of animals that had received AAV2/5-CRF was 41% higher than in the animals that had received AAV2/5-GFP ($t_{(22)} = 4.24$, $P<0.001$, Figures 4a, b). Furthermore, the CRF mRNA level was 81% higher in the AAV2/5-CRF rats than in rats that had received the control vector ($t_{(22)} = 2.13$, $P<0.05$, Figure 4a). The overexpression of CRF led to a 175% increase in CRF₁ receptor mRNA levels ($t_{(22)} = 2.54$, $P<0.05$, Figure 4c) and a 412% increase in CRF₂ receptor mRNA levels ($t_{(22)} = 2.41$, $P<0.05$, Figure 4c), and increased the CRF₂/CRF₁ receptor ratio ($t_{(22)} = 2.41$, $P<0.05$, Figure 4d). In the third experiment (section 3.3), the administration of the AAV-CRF vector in the BNST led to a 27% increase in CRF protein levels ($t_{(22)}=3.15$, $P<0.01$, Figure S3a,b). In summary, this experiment indicates that the overexpression of CRF in the BNST increases the CRF₂/CRF₁ receptor ratio and diminishes the elevations in brain reward thresholds associated with nicotine withdrawal.

3.3. Overexpression of CRF in the BNST and anxiety-like behavior and locomotor activity in nicotine withdrawing rats

Small open field test—Precipitated nicotine withdrawal led to a decrease in the distance traveled (Pump: $F_{1,44}=4.99$, $P<0.05$, Table 1) and a decrease in rearing (Pump: $F_{1,44}=24.04$, $P<0.0001$, Table 1) in the GFP and CRF group. The overexpression of CRF slightly potentiated the nicotine withdrawal-induced decrease in rearing (Pump x Vector: $F_{1,44}=5.74$, $P<0.05$, Table 1). At the end of the experiment, it was investigated if mecamylamine affects locomotor activity and rearing in rats that have not been exposed to nicotine. The administration of mecamylamine did not affect the distance traveled or rearing

in the small open field (Table S2). In addition, there was no effect of CRF-overexpression on the behavior of the rats. Thus, mecamylamine does not affect locomotor activity or rearing in rats not exposed to nicotine.

Large open field test—Precipitated nicotine withdrawal was associated with a decrease in the total distance traveled (Pump: $F_{1,44}=5.32$, $P<0.05$, Table 1). This was due to a decrease in the distance traveled in the border zone (Pump: $F_{1,44}=4.13$, $P<0.05$, Table 1) and a small, non-significant, decrease in the distance traveled in the center zone (Pump: $F_{1,44}=3.07$, $P=0.09$, Table 1) in the nicotine withdrawing rats. Nicotine withdrawal also decreased the number of entries into the center zone (Pump: $F_{1,44}=4.29$, $P<0.05$, Table 1). The overexpression of CRF in the BNST did not affect the behavior of the nicotine- or saline-treated rats.

Elevated plus maze test—Nicotine withdrawal decreased the percentage of time on the open arms (Pump $F_{1,44}=4.41$, $P<0.05$, center-point, Figure 5a) and decreased the number of head dips into the open arms (Pump $F_{1,44}=10.23$, $P<0.01$, nose-point, Figure 5b). Nicotine withdrawal did not affect the percentage of whole body (center-point) entries into the open arms (% of total; Table S3), which might have been due to the relatively low number of whole body entries into the open arms in all groups. There was a strong trend towards a nicotine withdrawal-induced decrease in the percentage of distance traveled in the open arms (% of total; Pump: $F_{1,44}=3.72$, $P=0.06$, center-point, Table S3). Nicotine withdrawal did not affect the total number of closed arm entries, which suggests that nicotine withdrawal did not affect locomotor activity (Table S3). The overexpression of CRF did not affect the percentage of time on the open arms (% of total), percentage of distance traveled on the open arms (% of total), the percentage of entries into the open arms (% of total), or the total number of closed arm entries. Taken together, this suggests that nicotine withdrawal increases anxiety-like behavior and this is not affected by the overexpression of CRF in the BNST. In summary, this experiment indicates that the overexpression of CRF does not diminish anxiety-like behavior associated with nicotine withdrawal.

4. Discussion

The goal of these studies was to investigate the effect of prolonged overexpression of CRF in the BNST on the dysphoria- and anxiety-like behavior associated with nicotine withdrawal. Both precipitated and spontaneous nicotine withdrawal led to elevations in brain reward thresholds, which reflects a dysphoria-like state. The overexpression of CRF in the BNST prevented the elevations in brain reward thresholds associated with nicotine withdrawal and did not affect brain reward function in rats implanted with minipumps containing saline. The overexpression of CRF increased the $CRF_2:CRF_1$ receptor ratio and this might have diminished the elevations in brain reward thresholds in the nicotine withdrawing rats. Nicotine withdrawal also increased anxiety-like behavior in the elevated plus maze test and the large open field test. The overexpression of CRF in the BNST did not prevent this increase in anxiety-like behavior. Nicotine withdrawal (nicotine-GFP vs. saline-GFP) had a relatively small effect on locomotor activity in the border zone of the large open field (9% decrease) or in the small open field (12% decrease), but induced a large decrease in locomotor activity in the center of the large open field (45% decrease). Furthermore,

nicotine withdrawal did not affect the total number of closed arm entries in the elevated plus maze test. These findings suggest that it is unlikely that the withdrawal-induced increase in anxiety-like behavior was due to a decrease in locomotor activity.

Before the onset of the behavioral experiments the AAV2/5 vector was thoroughly characterized. We determined the time course of transgene expression, the transduction efficiency of the vector, and tropism in the BNST. High levels of GFP were detected, 2, 4, and 8 weeks after the administration of the viral vector in the BNST. The expression of GFP gradually increased over time, and GFP levels were higher at 4 and 8 weeks than at 2 weeks after the infusions. The gradual increase in GFP levels is in line with studies that investigated the time-course of AAV2/5 mediated transgene expression in other brain sites (Qi et al., 2014; Reimsnider et al., 2007). We also did a confocal analysis to investigate cell tropism of the viral vector and to determine if the viral vectors transduce CRF neurons in the BNST. At all three time points, 98% of the cells that were transduced were neurons. A very high percentage of the CRF neurons expressed GFP, namely 79% at the 2-week time point and 96% at the later time points.

One of the main goals of the present study was to investigate the effect of chronic overexpression of CRF in the BNST on the elevations in brain reward thresholds associated with nicotine withdrawal. Mecamylamine induced a dose-dependent increase in brain reward thresholds in the nicotine dependent rats that had received the AAV2/5-GFP vector. The overexpression of CRF diminished the elevations in brain reward thresholds associated with nicotine withdrawal. The overexpression of CRF dramatically diminished the elevations in brain reward thresholds induced by the higher doses of mecamylamine. Several days after the last mecamylamine injection the minipumps were removed to investigate spontaneous nicotine withdrawal. In the nicotine-GFP group there was a large increase in brain reward thresholds, which was detected at the 6, 12, and 24 h time points. At these time points, the brain reward thresholds of the nicotine-CRF rats were lower than those of the nicotine-GFP rats. These findings indicate that the overexpression of CRF prevents the elevations in brain reward thresholds associated with nicotine withdrawal.

There are several mechanisms by which the overexpression of CRF might have diminished the elevations in brain reward thresholds in the nicotine withdrawing rats. First of all, the overexpression of CRF might have led to the development of tolerance to the effects of CRF. Both in-vivo and in-vitro studies have shown that chronic exposure to peptides leads to the development of tolerance. Rats have been shown to develop tolerance to the behavioral effects of orphanin FQ and melanin-concentrating hormone (Devine et al., 1996; Rossi et al., 1997). Furthermore, in-vitro studies show that high levels of CRF desensitize CRF₁ receptors in pituitary corticotrophs and in recombinant cell lines (Hauger et al., 2009; Kageyama et al., 2006). In addition, stress-induced release of CRF in the CeA contributes to anxiety-like behavior, which is diminished by the overexpression of CRF (Regev et al., 2011). G-protein-coupled receptor kinases (GRK) play an important role in desensitizing receptors and GRK2, 3, and 6 have been shown to play a role in desensitizing the CRF₁ receptor (Hauger et al., 2009; Kageyama et al., 2006). Therefore, future studies might explore if the overexpression of CRF induces tolerance to the effects of CRF by increasing GRK2, 3, or 6 levels.

It might also be possible that the overexpression of CRF in the BNST diminished the elevations in brain reward thresholds associated with nicotine withdrawal by increasing the CRF₂:CRF₁ receptor ratio. The overexpression of CRF increased CRF₁ and CRF₂ mRNA levels but induced a greater increase in CRF₂ than CRF₁ receptor gene expression. There is accumulating evidence that a shift in the CRF₁:CRF₂ receptor ratio can increase or decrease the sensitivity to stressors. Stimulation of CRF₁ receptors increases stress responses and activation of CRF₂ receptors opposes the effects of CRF₁ receptor activation and dampens stress responses (Bale and Vale, 2004). This is supported by the observation that stimulation of CRF₁ receptors mediates depressive- and anxiety-like behavior, HPA-axis activation, and increases heart rate and blood pressure (Briscoe et al., 2000; Holsboer and Ising, 2010; Nijssen et al., 2000). Accumulating evidence suggests that CRF₂ receptor activation has antidepressant-like effects. Corticotropin-releasing factor type 2 receptor knock out mice display increased depressive-like behavior in the forced swim test and the tail suspension test and the CRF₂ agonists urocortin-2 and -3 decrease depressive-like behavior in the forced swim test (Bale and Vale, 2003; Tanaka and Telegdy, 2008; Todorovic et al., 2009). Interestingly, the CRF₂ receptor in the BNST also provides protection against the effects of stress. Rats that display strong fear responses in response to traumatic stress have low CRF₂ receptor levels in the BNST and overexpression of CRF₂ receptors in this brain site provides protection against the fear enhancing effects of traumatic stress (Elharrar et al., 2013). Therefore, the increase in the CRF₂:CRF₁ receptor ratio might have diminished the elevations in brain reward thresholds associated with nicotine withdrawal.

The present study also showed that nicotine withdrawal induced a small, but significant, decrease in locomotor activity in the open field tests. This is in line with other studies that reported that nicotine withdrawal as well as withdrawal from other stimulants such as amphetamine decreases locomotor activity (Che et al., 2013; Malin et al., 1992). Drug withdrawal-induced changes in locomotor activity are underinvestigated and are often considered confounding factors when assessing anxiety- and depressive-like behavior (Cryan et al., 2002; Dawson and Tricklebank, 1995). It has been suggested that exploration of a novel environment is rewarding and therefore a withdrawal-induced deficit in reward function might lead to a decrease in exploratory behavior (Alcaro and Panksepp, 2011). The withdrawal-induced decrease in locomotor activity might be mediated by changes in dopaminergic transmission. Drugs of abuse that increase dopamine transmission increase locomotor activity, and drugs that damage mesocorticolimbic dopaminergic terminals or block dopamine receptors decrease locomotor activity (Fink and Smith, 1980; Vezina and Stewart, 1989). Nicotine withdrawal decreases dopamine levels in the nucleus accumbens (Hildebrand et al., 1998). Considering the role of dopamine in locomotor activity, it might be possible that this contributes to the decrease in exploration in the nicotine withdrawing rats.

The rats displayed increased anxiety-like behavior in the elevated plus maze test during nicotine withdrawal. This is in line with other studies that reported that nicotine withdrawal increases anxiety-like behavior in the elevated plus maze test (Damaj et al., 2003; Irvine et al., 2001). Nicotine withdrawal also decreased the number of entries into the center of the large open field. A decrease in center entries is often interpreted as an increase in anxiety-like behavior. Previous studies have shown that experimental conditions that increase anxiety-like behavior (e.g., isolations rearing, chronic corticosterone treatment) decrease

center entries and drugs that decrease anxiety-like behavior increase center entries (David et al., 2009; Lukkes et al., 2009; Xu et al., 2004). Nicotine withdrawal also led to a small decrease in locomotor activity in the large open field test and this could have affected the number of center entries. However, the nicotine withdrawal-induced decrease in locomotor activity was relatively small (9% in border zone, saline-GFP vs. nicotine-GFP) and therefore it is unlikely that this caused the large decrease (50%) in center entries. Taken together, the overexpression of CRF in the BNST did not attenuate nicotine withdrawal-induced anxiety-like behavior in the elevated plus maze or large open field. Therefore, these studies suggest that the overexpression of CRF diminishes dysphoria-, but not anxiety-like, behavior associated with nicotine withdrawal.

In conclusion, the present studies show that the overexpression of CRF in the BNST prevents the dysphoria-like state associated with precipitated and spontaneous nicotine withdrawal. In contrast, the overexpression of CRF did not diminish anxiety-like behavior or the decrease in locomotor activity in the nicotine withdrawing rats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Role of funding source

This work was funded by a National Institute on Drug Abuse (NIDA) grant (DA023575) to A. Bruijnzeel; NIDA had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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• 2.11.1. Time-course of AAV2/5-mediated GFP expression

• 2.11.2. Overexpression of CRF and ICSS testing

• 2.11.3. Overexpression of CRF and open field tests and elevated plus maze test

Figure 1.
Schematic overview of experiments. Abbreviation: W, weeks.

Figure 2.

Viral vector mediated expression of GFP in the BNST. (a, b) The figures illustrate the localization of the BNST (anterior posterior = -0.3 mm from bregma)(Paxinos and Watson, 1998). (c) Immunofluorescent stainings revealed a high level of AAV2/5-mediated GFP protein expression in the BNST 4 weeks after the infusions. The scale bar corresponds to 200 μm. Abbreviations: 3v, 3rd ventricle; ac, anterior commissure; BNST, bed nucleus of the stria terminalis; CPu, Caudate putamen; LS, lateral septum; LV, lateral ventricle.

Figure 3. Overexpression of CRF in the BNST diminishes the dysphoria-like state associated with nicotine withdrawal. Administration of (a) mecamylamine (n = 11–13/group) and (c)

minipump removal (n = 11–12/group) elevated ICSS thresholds in the nicotine-treated rats. Withdrawal was diminished by the overexpression of CRF in the BNST. (b) Mecamylamine did not affect the latencies but (d) removal of the nicotine pumps led to a small increase in response latencies. In panel a, asterisks (* P<0.05, ** P<0.01) indicate elevated ICSS thresholds compared to the saline group that received the same viral vector and were treated with the same dose of mecamylamine. Plus signs (++) P<0.01 indicate lower ICSS thresholds compared to GFP-nicotine animals that received the same dose of mecamylamine. Pound signs (# P<0.05, ## P<0.01) indicate higher ICSS thresholds compared to the same experimental group treated with a lower dose of mecamylamine (3 vs. 1 and 1 vs 0.33 mg/kg). In panel c, asterisks (** P<0.01) indicate elevated ICSS thresholds compared to the saline-GFP group and plus signs (++) P<0.01 indicate lower ICSS thresholds compared to the nicotine-GFP group. In panel d, asterisks (** P<0.01) indicate longer latencies than the saline-GFP group. Data expressed as means $\pm$ SEM.

Figure 4.

Overexpression of CRF in the BNST increases CRF₁ and CRF₂ receptor gene expression.

(a) The AAV2/5-CRF vector increased CRF protein and CRF mRNA levels in the BNST. (b)

Representative Western blots of CRF and the normalization control GAPDH. (c) The

overexpression of CRF increased CRF₁ and CRF₂ receptor mRNA levels, and (d) increased

the CRF₂/CRF₁ receptor ratio. Asterisks (*P<0.05, ** P<0.01) indicate difference from GFP-control group. Data expressed as means ± SEM (n = 11–13 per group).

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Figure 5.

Nicotine withdrawal increases anxiety-like behavior in the elevated plus maze test. (a) Nicotine withdrawal decreased the percentage of time on the open arms and (b) the number of head dips into the open arms. The asterisk (* $P < 0.05$) indicates fewer head dips into the open arms compared to the saline-GFP group. The overexpression of CRF in the BNST did not affect behavior in the elevated plus maze test. Data expressed as means $\pm$ SEM ($n = 12$ per group).

Table 1

Effect of nicotine withdrawal and overexpression of CRF in the BNST on behavior in the small and large open field.

Pump	Saline		Nicotine	
Vector	AAV2/5-GFP	AAV2/5-CRF	AAV2/5-GFP	AAV2/5-CRF
Small open field				
Total distance (cm)	2974 ± 190	2691 ± 166	2616 ± 129	2295 ± 157
Rearing (beam breaks)	547 ± 28	599 ± 23	460 ± 50	345 ± 24
Large open field				
Total distance (cm)	3492 ± 187	3267 ± 180	3055 ± 216	2828 ± 174
Border distance (cm)	3183 ± 154	3014 ± 156	2885 ± 196	2638 ± 153
Center distance (cm)	309 ± 75	253 ± 49	170 ± 35	190 ± 64
Center entries (n)	10 ± 2	8 ± 1	5 ± 1	6 ± 1