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Nicotine-induced anxiety-like behavior in a rat model of the novelty-seeking phenotype is associated with long-lasting neuropeptidergic and neuroplastic adaptations in the amygdala: Effects of the cannabinoid receptor 1 antagonist AM251

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Abstract

A rat model of the novelty-seeking phenotype predicts vulnerability to the expression of behavioral sensitization to nicotine, where locomotor reactivity to novelty is used to screen experimentally-naïve rats for high (HR) versus low (LR) responders. The present study examines the long-term neuropeptidergic and neuroplastic adaptations associated with the expression of locomotor sensitization to a low dose nicotine challenge and social anxiety-like behavior following chronic intermittent nicotine exposure during adolescence in the LRHR phenotype. Our data show that the expression of behavioral sensitization to nicotine and abstinence-related anxiety are detected in nicotine pre-exposed HRs even across a long (3 wks) abstinence. Moreover, these behavioral effects of nicotine are accompanied by a persistent imbalance between neuropeptide Y and corticotrophin releasing factor systems, and a persistent increase in brain-derived neurotrophic factor (BDNF) and spinophilin mRNA levels in the amygdala. Furthermore, treatment with the cannabinoid receptor 1 antagonist, AM251 (5 mg/kg) during a short (1 wk) abstinence is ineffective in reversing nicotine-induced anxiety, fluctuations in BDNF and spinophilin mRNAs, and the neuropeptidergic dysregulations in the amygdala; although this treatment is effective in reversing the expression of locomotor sensitization to challenge nicotine even after a long abstinence. Interestingly, the identical AM251 treatment administered during the late phase of a long abstinence further augments anxiety and associated changes in BDNF and spinophilin mRNA in the basolateral nucleus of the amygdala in nicotine pre-exposed HRs. These findings implicate long-lasting neuropeptidergic and neuroplastic changes in the amygdala in vulnerability to the behavioral effects of nicotine in the novelty-seeking phenotype.

Keywords

Behavioral sensitization; Social anxiety; Cannabinoid receptor 1; Nicotine abstinence; Amygdala; Neuroplasticity

1. Introduction

The novelty-seeking phenotype has been shown to predict individual differences in vulnerability to psychostimulant sensitization in rodents (Hooks et al., 1991; Dietz et al., 2005; Cain et al., 2009). This phenotype is identified by the level of locomotor reactivity to a novel environment in an experimentally-naïve outbred population of rats. Specifically, some rats exhibit high locomotor response to novelty and are identified as high responders,

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HRs; whereas some display low locomotor response to novelty and are identified as low responders, LRs. Our laboratory previously reported expression of locomotor sensitization to a low dose nicotine challenge following a chronic intermittent nicotine exposure during the peripubertal–juvenile period and 1 wk of abstinence in the HR, but not LR rats that is reversed by a cannabinoid receptor 1 (CB1R) antagonist, AM251, administration (Bhatti et al., 2009); implicating CB1R signaling in sensitizing effects of nicotine.

Clinical studies demonstrated effectiveness of a commercially available CB1R antagonist, rimonabant, in increasing prolonged abstinence rates compared to placebo (Le Foll et al., 2008). However, due to its neuropsychiatric side effects, such as anxiety, rimonabant has been disapproved by the FDA (US Food and Drug Administration, 2007; Bermudez-Silva et al., 2010; Cahill and Ussher, 2011). In confirmation of the clinical findings, studies on rodents showed that rimonabant reduces nicotine self-administration (Cohen et al., 2002), as well as the expression of locomotor sensitization to low dose nicotine challenge (Bhatti et al., 2009), but also increases anxiogenic-like responses to nicotine (Balerio et al., 2006). These data suggest involvement of CB1R signaling in nicotine-induced anxiogenesis. Indeed, we have recently reported a decrease in CB1R mRNA levels in the amygdala along with emergence of social anxiety-like behavior in HRs following repeated nicotine exposure in the peripubertal–juvenile period (Aydin et al., 2012), suggesting that a deficit in CB1R function in the amygdala may be a substrate for nicotine-induced anxiety-like behavior.

Previous reports from our laboratory showed that a behaviorally-sensitizing nicotine regimen results in increased abstinence-related social anxiety-like behavior, which is accompanied by a deficit in neuropeptide Y (NPY) mRNA levels in the medial nucleus of amygdala (MeA) together with increased corticotrophin releasing factor (CRF) mRNA levels in the central nucleus of amygdala (CeA; Aydin et al., 2011a, 2011b). Moreover, our data also showed that systemic antagonization of the presynaptic Y2 receptors during 1 wk of abstinence by way of peripheral injections of a novel, brain penetrant Y2 receptor antagonist, JNJ-31020028 reestablishes the critical homeostatic balance between the amygdalar NPY and CRF systems and reverses the expression of behavioral sensitization to a low dose nicotine challenge and associated social anxiety-like behavior in the nicotine vulnerable HR rats (Aydin et al., 2011b). These findings suggest a relationship between the emergence of anxiety-like behavior and the neuropeptidergic changes within the amygdala in behavioral sensitization to nicotine in the novelty-seeking phenotype. However, the role of CB1R signaling in the nicotine-induced negative affective state and the neuropeptidergic adaptations in the amygdala remain to be elucidated.

In addition to NPY and CRF, amygdalar BDNF also mediates anxiety-like behavior. Data from BDNF overexpressing mice show that these animals have upregulated BDNF levels in the basolateral nucleus of amygdala (BLA), and display increased anxiety-like behavior in the open field and elevated plus maze (EPM) tests, compared to controls (Govindarajan et al., 2006), suggesting a modulatory role for amygdalar BDNF in anxiety-like behavior. Moreover, BDNF promotes dendritic and spine growth (Ji et al., 2005) and the observed increase in anxiety-like behavior in the BDNF overexpressing mice strongly correlates with increased spine density in the BLA, suggesting that increased spinogenesis in the amygdala may be a cellular substrate for enhanced anxiety (Govindarajan et al., 2006). Indeed, such correlation between increased anxiety and BLA spinogenesis has also been reported following chronic stress (Bennur et al., 2007). Additionally, an upregulation in levels of spinophilin in the amygdala is reported following a behaviorally-sensitizing amphetamine regimen and 4 wks of abstinence (Boikess and Marshall, 2008), suggesting a long-lasting dendritic plasticity in the amygdala with psychostimulant administration. However, the role of BDNF and subsequent neuroplasticity in the amygdala has not received attention in a nicotine-induced anxiety paradigm.

In the present study, we have investigated if there are long-lasting behavioral, neuropeptidergic and neuroplastic adaptations following repeated nicotine exposure during the peripubertal–juvenile period in the LRHR rats. In doing so, we assessed whether nicotine exposure during the peripubertal juvenile period can induce long-lasting changes in the expression of behavioral sensitization to nicotine, social anxiety-like behavior, dysregulations in the amygdalar NPY and CRF as well as fluctuations in the molecular markers of neuroplasticity (i.e., BDNF and spinophilin mRNAs) in the amygdala in LRHR rats. To determine long-lasting nature of these parameters, we employed a short (i.e., 1 wk) and a long (i.e., 3 wks) abstinence spanning into young adulthood following nicotine training. We also assessed if AM251 administered during the short or late phase (i.e., last week) of the long abstinence sufficiently reverses the nicotine-induced behavioral and neural adaptations in the LRHR rats.

2. Materials and methods

2.1. Drugs

Nicotine hydrogen tartrate was obtained from a commercial supplier (Sigma), dissolved in 0.9% NaCl and the pH was adjusted to 7.4. AM251 was obtained from Tocris Bioscience and dissolved in a vehicle solution consisting of Tween 80, DMSO and 0.9% NaCl with the proportion of 1:2:7. This specific proportion has been used to formulate the vehicle solution to dissolve AM251 in a number of studies in the literature (Hoffman et al., 2007; Rubino et al., 2008). The doses for nicotine and AM251 were chosen based on effective doses used in the literature in several reports by others (Miller et al., 2001; Suto et al., 2001; Le Foll and Goldberg, 2004), and by us (Bhatti et al., 2007, 2009; Aydin et al., 2011a, 2011b, 2012).

2.2. Animal housing and the LRHR phenotype screening

Animals were treated in accordance with the National Institute of Health guidelines on laboratory animal use and care. All efforts were made to minimize animal suffering and to reduce the number of animals used. A grand total of 216 male Sprague-Dawley rats (Charles River, Wilmington, MA) arrived at weaning (postnatal day, PN 22), were housed 3 per cage in 43 × 21.5 × 25 cm^3 Plexiglas cages and were kept on a 12 h light/dark cycle (lights on at 7:00 A.M.). Food and water were available *ad libitum*. Animals were allowed to habituate to the housing conditions and were handled daily for 2 days. On PN 25, animals were screened for locomotor reactivity to the mild stress of a novel environment for 1 h using commercially available locomotion chambers (San Diego Instruments, San Diego, CA). Briefly, locomotor reactivity to novelty was tested in 43 × 43 × 24.5-cm^3 (high) clear Plexiglas cages with stainless steel grid flooring. Activity was monitored by means of photocells (a total of $X = 16$ by $Y = 16$ photocells) 2.5 cm above the grid floor and equally spaced along the sides of the box. Horizontal locomotion was monitored by this lower bank of photocells. Each locomotor count recorded a minimum of 3-cm traversing of the cage. Additional photocells were located 11.5 cm above the grid floor and 9 cm above the lower bank of photocells. Rearing (i.e., locomotion in the Z plane) was monitored by this upper bank of photocells. At the end of the screening session, total locomotor activity (i.e., X, Y, and Z locomotion) was pooled and the rats were ranked as HRs (i.e., rats that exhibited locomotor scores in the highest third of the sample; $n = 72$) or LRs (i.e., rats that exhibited locomotor scores in the lowest third of the sample $n = 72$). The intermediary responders were used as residents rats in the social interaction (SI) test described below.

2.3. Behavioral sensitization to nicotine and abstinence-related anxiety-like behavior

The behavioral sensitization to nicotine procedure is similar to Table 1 without the challenge phase. Male Sprague-Dawley rats arrived on PN 22 and were phenotype screened on PN 25. Following phenotype screening, the LRHR animals were assigned to saline (1 ml/kg; s.c.) or

nicotine (0.35 mg/kg; s.c.) injection groups. On injection days, rats were given 1 h to habituate to the locomotor chambers before they received an injection of the assigned drug. Their locomotor response was recorded for 90 min. This procedure was repeated four times at a 3-d interval. Following the fourth injection, half of the animals in each group underwent 1 wk of abstinence, while the remaining half underwent 3 wks of abstinence (n = 5–6/group). In the present study, the term "abstinence" is used to refer to the period starting after the 4th nicotine injection and lasting until the day of low dose nicotine challenge, during which nicotine is withdrawn by the experimenter. At the end of the abstinence periods, all animals were tested on the SI test for assessing anxiety-like behavioral profile as described below, in the absence of nicotine.

2.4. AM251 treatment and low dose nicotine challenge-induced anxiety

The behavioral sensitization to nicotine procedure and the experimental groups used in the present study are outlined in Tables 1 and 2, respectively. Male Sprague-Dawley rats arrived on PN 22 and were allowed to rest until PN 25, at which time they were phenotype screened. Following phenotype screening, the LRHR animals were assigned to saline (1 ml/kg; s.c.) or nicotine (0.35 mg/kg; s.c.) injection groups. On injection days, rats were given 1 h to habituate to the locomotor chambers before they received an injection of the assigned drug. Their locomotor response was recorded for 90 min. This procedure was repeated four times at a 3-d interval. Following the fourth injection, rats were further assigned to vehicle (1 ml/kg, i.p.) or AM251 (5 mg/kg, i.p.) treatment groups. Half of the animals in each group underwent 1 wk of abstinence when they received vehicle or AM251 injections every other day (n = 5–6/group, Table 1A). The remaining half underwent 3 wks of abstinence and received vehicle or AM251 during the last week of this period, every other day (n = 5–6/group, Table 1B). At the end of the abstinence periods, all animals were challenged with a low dose of nicotine (0.1 mg/kg, s.c.), and their locomotor response was monitored for 45 min. Upon completion of the challenge session, rats were tested on the SI test for assessing anxiety-like behavioral profile as described below.

2.5. Social interaction (SI) test

Testing took place under a constant, medium illumination condition in an open topped, rectangular, transparent social interaction box. The resident rat was placed in the box 8 min prior to placement of the experimental rat. The resident rat was of similar weight that was housed under identical conditions as the experimental rat, which had no previous contact with the experimental rat. The experimental rat was placed in the box and the amount of time the experimental rat spent initiating social interaction (i.e., grooming, sniffing, following, crawling over or under) with the resident was determined for 5 min. Upon completion of testing, animals were rapidly decapitated, brain tissue were harvested and processed for *in situ* hybridization histochemistry as described below.

2.6 In situ hybridization histochemistry

Brain tissues were collected and immediately frozen in isopentane cooled to −30 °C. Brains were then sectioned on a cryostat and 20-μm-thick coronal sections were mounted on electrostatically charged slides. These slides were kept at −80 °C until processed. On the day of hybridization, sections were fixed in 4% paraformaldehyde at room temperature for 1 h, followed by three washes in 2× SSC (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate). Sections were placed in a solution containing acetic anhydride (0.25%) in triethanolamine (0.1 M, pH 8) for 10 min, rinsed in distilled water, dehydrated through graded alcohols (50%, 75%, 85%, 95% and 100%) and air-dried. cDNA probes for rat NPY, BDNF and CRF were obtained from Dr. Stanley J. Watson (University of Michigan), while spinophilin was cloned in our laboratory (accession #: NM_053474). Probes were antisense linearized and were ^{35}S labeled separately in reaction mixtures consisting of 1 ml of

linearized plasmid, 1× transcription buffer, 125 mCi [^{35}S]UTP, 125 mCi [^{35}S]CTP, 150 mM each of ATP, and GTP, 12.5 mM dithiothreitol, 20 U RNAase inhibitor and 6 U polymerase. Reactions were incubated for 90 min at 37 °C, and separated from unincorporated nucleotides over Micro Bio-Spin chromatography columns (Bio-Rad, CA). Probes were diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, 50 mM sodium phosphate buffer, pH 7.4, 1× Denhardt's solution, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol) to yield 10^6 dpm/70 ml. Sections were hybridized with probe mixture inside a humidified chamber over night at 55 °C. Next day, sections were washed in 3× SSC for 5 min each, incubated for 1 h in RNAase (20 mg/ml in Tris buffer containing 0.5 M NaCl, pH 8) at 37 °C. Sections were washed with 2×, 1× and 0.5× SSC, and incubated for 1 h in 0.1× SSC at 65 °C. After rinsing in distilled water, sections were dehydrated, air dried and exposed to a Kodak XAR film (Eastman Kodak, NY). Section images were captured digitally from x-ray films with a CCD camera, and relative optical densities were determined. Only pixels with gray values exceeding 3.5× above background were included in the analyses. Optical density measurements were sampled from templates made for each brain region from both hemispheres and in the rostro–caudal axis using the Scion Image software. Levels of mRNA for spinophilin and BDNF were quantified in the BLA. NPY mRNA levels were quantified in the MeA and the BLA, while CRF mRNA levels were quantified in the CeA. Optical density values were corrected for background and then averaged to produce one data point for each brain region for each animal. In addition, integrated density was calculated as optical density multiplied by area and included in the analyses. These data points were averaged per group and compared statistically.

2.7. Statistical analyses

Behavioral data pertaining to repeated nicotine injections were analyzed by a repeated-measures ANOVA: Phenotype (LR, HR) × Pre-exposure (SAL, NIC) × Injection Days (INJ 1, INJ 2, INJ 3, INJ 4). Locomotor reactivity to challenge nicotine, percent time spent engaged in social interaction at challenge, as well as mRNA levels obtained from *in situ* hybridization histochemistry from these animals were assessed by four-way ANOVAs: Phenotype (LR, HR) × Pre-exposure (SAL, NIC) × Treatment (AM251, VEH) × Abstinence (1 wk, 3 wks). Percent time the experimental animal spent interacting with the resident rat following 1 wk or 3 wks of abstinence in the absence of challenge nicotine, and the mRNA levels obtained from these experimental animals were assessed by three-way ANOVAs: Phenotype (LR, HR) × Pre-exposure (SAL, NIC) × Abstinence (1 wk, 3 wks). Significant main effects and interactions were followed by post-hoc comparisons using Fisher's protected least significant difference tests, and significance was set at $p < 0.05$.

3. Results

Fig. 1 shows total locomotor reactivity to four intermittent nicotine or saline injections (A) and the subsequent low dose nicotine challenge following 1 wk or 3 wks of abstinence in LRs and HRs (B). Repeated measures ANOVA revealed a significant interaction between Injection Days (INJ 1, INJ 2, INJ 3, INJ 4) and Pre-exposure [SAL, NIC; $F_{(3, 129)} = 3.48$, $p = 0.018$], and significant main effects of Pre-exposure [SAL, NIC; $F_{(1, 43)} = 7.97$, $p = 0.007$] and Injection Days [INJ 1, INJ 2, INJ 3, INJ 4; $F_{(3, 129)} = 3.03$, $p = 0.032$]. Specific post-hoc comparisons showed that at INJ 4, nicotine pre-exposure resulted in increased locomotor reactivity in both LRs [$p = 0.06$] and HRs [$p = 0.011$] compared to saline pre-exposed controls. Moreover, a four-way ANOVA also showed significant main effects of Phenotype [LR, HR; $F_{(1, 80)} = 11.84$, $p = 0.0009$] and Treatment [VEH, AM251; $F_{(1, 80)} = 7.84$, $p = 0.006$] in locomotor response to the challenge nicotine. Furthermore, post-hoc comparisons showed that nicotine pre-exposed HRs, but not LRs, exhibited increased locomotor reactivity to the low dose nicotine challenge compared to saline pre-exposed controls following 1 wk [$p = 0.041$] and 3 wks [$p = 0.046$] of abstinence. These effects were

reversed by AM251 administration during both abstinence phases [$p_s \leq 0.025$] in nicotine pre-exposed HRs back to vehicle control levels.

Fig. 1C shows percent time the experimental animals spent interacting with the resident rats in a social context following 1 wk or 3 wks of abstinence without nicotine on board during testing. A three-way ANOVA revealed significant interactions between Pre-exposure (SAL, NIC) and Phenotype [LR, HR; $F_{(1, 46)} = 25.30$, $p = 0.0001$] in the SI test. Specific post-hoc comparisons showed a significant phenotype difference in percent time spent in social interaction, where saline pre-exposed HRs spent significantly more time interacting with the resident rat compared to LR counterparts following a short [1 wk; $p = 0.048$] or a long [3 wks; $p = 0.002$] injection-free period, suggesting an anxiolytic-like state in nicotine-naïve HRs compared to LR counterparts. Moreover, decreased levels of social interaction was observed in nicotine pre-exposed HRs compared to saline controls [$p_s \leq 0.015$], affirming that repeated nicotine results in an anxiogenic state in both short and long abstinence conditions in HRs. Furthermore, a chronic intermittent nicotine exposure during the peripubertal–juvenile period resulted in increased anxiolytic-like behavior in young adult LRs after long abstinence compared to saline pre-exposed counterparts [$p = 0.016$], suggesting a delayed, nicotine-induced phenotype switch in the LR phenotype in anxiety-like behavior.

Fig. 1D shows percent time the experimental animals spent interacting with the resident rats in a social context with the challenge nicotine on board. A four-way ANOVA revealed significant interactions between Pre-exposure (SAL, NIC) and Abstinence [1 wk, 3 wks; $F_{(1, 80)} = 6.78$, $p = 0.011$] and between Pre-exposure (SAL, NIC) and Phenotype [LR, HR; $F_{(1, 80)} = 12.02$, $p = 0.0008$] in the SI test. Main effects of Phenotype [LR, HR; $F_{(1, 80)} = 12.97$, $p = 0.0005$] and Pre-exposure [SAL, NIC; $F_{(1, 80)} = 5.13$, $p = 0.026$] were also significant in the same test. Specific post-hoc comparisons showed a significant phenotype difference in percent time spent in social interaction, where saline pre-exposed, vehicle-treated HRs spent significantly more time interacting with the resident rat compared to LR counterparts in response to the challenge nicotine following a short [1 wk; $p = 0.004$] and a long [3 wks; $p = 0.0001$] abstinence, suggesting suppressed social anxiety-like behavior in the HR animals compared to LRs independent of repeated nicotine training. Moreover, a low dose nicotine challenge resulted in decreased levels of social interaction in nicotine pre-exposed, vehicle-treated HRs compared to saline controls [$p_s \leq 0.042$], affirming that repeated nicotine training results in an anxiogenic state in both short and long abstinence conditions in HRs. However, while AM251 treatment during 1 wk of abstinence was ineffective in reversing nicotine-induced social anxiety-like behavior in HRs [$p = 0.096$], the same treatment during the late phase of the long (3 wks) abstinence further decreased social interaction in the same animals [$p = 0.035$], suggesting that AM251 in this period augments nicotine-induced social anxiety-like behavior.

Fig. 2 shows expression of NPY mRNA in the MeA and BLA (A–F), and CRF mRNA expression in the CeA (G–L) in LRHR brains that were collected on the last day of abstinence, without challenge nicotine on board during testing. In this experiment, a transformed data set in the form of a ratio of the NPY mRNA in the MeA (M) or the BLA (N) to CRF mRNA in the CeA has been used. As amygdalar NPY and CRF work in opposition to regulate a balanced emotional state (Heilig et al., 1994), the ratio of NPY to CRF in the amygdala has been used as a molecular indicator of anxiety state of rats in a previously published report (Slawecki et al., 2005), hence was adopted in these analyses. Three-way ANOVAs revealed significant interactions between Phenotype (LR, HR), Pre-exposure (SAL, NIC) and Abstinence (1 wk, 3 wks) in the ratio of NPY mRNA in the MeA to CRF mRNA in the CeA [$F_{(1, 35)} = 6.65$, $p = 0.014$], and in the ratio of NPY mRNA in the BLA to CRF mRNA in the CeA [$F_{(1, 35)} = 8.62$, $p = 0.006$]. The same analysis also showed

significant interactions between Phenotype (LR, HR) and Pre-exposure (SAL, NIC) in the ratio of NPY mRNA in the MeA to CRF mRNA in the CeA [$F_{(1, 35)} = 26.46$, $p = 0.0001$] and in the ratio of NPY mRNA in the BLA to CRF mRNA in the CeA [$F_{(1, 35)} = 21.05$, $p = 0.0001$]. Significant main effects of Phenotype (LR, HR) in the ratio of mRNA levels of NPY in the MeA to CRF in the CeA [$F_{(1, 35)} = 14.66$, $p = 0.0005$] and NPY in the BLA to CRF in the CeA [$F_{(1, 35)} = 6.92$, $p = 0.012$] were also revealed. Moreover, significant main effects of Pre-exposure (SAL, NIC) in the same ratio with NPY mRNA levels in the MeA [$F_{(1, 35)} = 10.24$, $p = 0.003$], and of Abstinence (1 wk, 3 wks) in NPY mRNA in the BLA to CRF mRNA in the CeA [$F_{(1, 35)} = 15.03$, $p = 0.0004$] were observed. Specific post-hoc comparisons showed that saline pre-exposed HRs had a higher ratio of mRNA levels of NPY in the MeA to CRF in the CeA [$p = 0.032$] and NPY in the BLA to CRF in the CeA [$p = 0.0001$] compared to LR counterparts 1 wk following fourth saline injection. Moreover, the ratio of NPY mRNA in the MeA to CRF mRNA in the CeA [$p = 0.008$] and the same ratio with NPY mRNA in the BLA [$p = 0.016$] were lower in young adult HRs compared to juvenile counterparts. Furthermore, repeated nicotine during the peripubertal–juvenile period resulted in decreased ratio of mRNA levels of NPY in the MeA to CRF in the CeA [$p = 0.0001$] and NPY in the BLA to CRF in the CeA [$p = 0.0001$] in HRs compared to saline controls following 1 wk of abstinence. The decreased ratio of NPY mRNA to CRF mRNA was preserved in the MeA, but not in the BLA, following 3 wks of abstinence in the HR rats [$p = 0.011$].

Fig. 3A–D shows expression of BDNF mRNA in the BLA in HR brains that were collected on the last day of abstinence, without challenge nicotine on board during testing. A three-way ANOVA revealed significant interactions between Phenotype (LR, HR) and Pre-exposure [SAL, NIC; $F_{(1, 36)} = 9.95$, $p = 0.003$], and between Phenotype (LR, HR) and Abstinence [1 wk, 3 wks; $F_{(1, 36)} = 5.94$, $p = 0.020$] in BDNF mRNA levels in the BLA. The same analysis also showed significant main effects of Phenotype [LR, HR; $F_{(1, 36)} = 6.06$, $p = 0.019$] and Pre-exposure [Sal, NIC; $F_{(1, 36)} = 11.84$, $p = 0.001$]. Specific post-hoc comparisons showed that in nicotine pre-exposed HRs, BDNF mRNA levels in the BLA were significantly upregulated following 1 wk [$p = 0.001$] and 3 wks [$p = 0.003$] of abstinence, compared to saline controls.

Spinophilin mRNA expression in the BLA of HR brains that were collected on the last day of abstinence, without challenge nicotine on board during testing is shown in Fig. 4A–D. A three-way ANOVA revealed significant interactions between Phenotype (LR, HR) and Pre-exposure [SAL, NIC; $F_{(1, 36)} = 18.95$, $p = 0.0001$], and between Phenotype (LR, HR) and Abstinence [1 wk, 3 wks; $F_{(1, 36)} = 4.39$, $p = 0.004$] in spinophilin mRNA levels in the BLA. The same analysis also showed significant main effects of Phenotype [LR, HR; $F_{(1, 36)} = 41.78$, $p = 0.0001$] and Abstinence [1 wk, 3 wks; $F_{(1, 36)} = 8.50$, $p = 0.006$]. Specific post-hoc comparisons showed an upregulation of spinophilin mRNA levels in the BLA in nicotine pre-exposed HRs following 1 wk [$p = 0.0001$] and 3 wks [$p = 0.006$] of abstinence, compared to saline controls.

Fig. 5 shows expression of NPY mRNA in the MeA and BLA (A–F), and CRF mRNA expression in the CeA (G–L) in LRHR animals that were tested with the challenge nicotine on board. In the present experiment, four-way ANOVAs revealed significant interactions between Phenotype (LR, HR) and Pre-exposure (SAL, NIC) in the ratio of NPY mRNA in the MeA to CRF mRNA in the CeA [$F_{(1, 80)} = 28.55$, $p = 0.0001$] and in the ratio of NPY mRNA in the BLA to CRF mRNA in the CeA [$F_{(1, 80)} = 8.03$, $p = 0.006$]. The same analysis also showed a significant interaction between Phenotype (LR, HR) and Abstinence (1 wk, 3 wks) in the ratio of NPY mRNA in the MeA to CRF mRNA in the CeA [$F_{(1, 80)} = 7.83$, $p = 0.009$] along with a significant main effect of Phenotype [LR, HR; $F_{(1, 80)} = 5.37$, $p = 0.023$]. Moreover, a significant main effect of Pre-exposure (SAL, NIC) was also

observed in the same ratio with NPY mRNA levels in the MeA [$F_{(1, 80)} = 13.38$, $p = 0.0005$] and in the BLA [$F_{(1, 80)} = 10.07$, $p = 0.002$]. Specific post-hoc comparisons showed that saline pre-exposed, vehicle-treated HRs had a higher ratio of NPY mRNA in the MeA to CRF mRNA in the CeA [$p = 0.0001$] and higher ratio of NPY mRNA in the BLA to CRF mRNA in the CeA [$p = 0.017$] compared to LR counterparts 1 wk following repeated injections. Moreover, the ratio of NPY mRNA in the MeA to CRF mRNA in the CeA [$p = 0.011$] and the same ratio with NPY mRNA in the BLA [$p = 0.007$] were lower in young adult HRs compared to juvenile counterparts. Furthermore, nicotine exposure during the peripubertal–juvenile period resulted in decreased ratio of NPY mRNA in the MeA to CRF mRNA in the CeA [$p = 0.0001$] and NPY mRNA in the BLA to CRF mRNA in the CeA [$p = 0.0007$] in HRs compared to saline controls following 1 wk of abstinence. The decreased ratio of NPY mRNA to CRF mRNA was preserved in the MeA, but not in the BLA, following 3 wks of abstinence in the HR rats [$p = 0.043$].

Fig. 6A–F shows expression of BDNF mRNA in the BLA of HR rats that were tested with the challenge nicotine on board. A four-way ANOVA revealed a significant interaction between Phenotype (LR, HR), Pre-exposure (SAL, NIC), Treatment (VEH, AM251) and Abstinence [1 wk, 3 wks; $F_{(1, 80)} = 4.77$, $p = 0.032$] in BDNF mRNA levels in the BLA. The same analysis also showed significant interactions between Phenotype (LR, HR), Pre-exposure (SAL, NIC) and Abstinence [1 wk, 3 wks; $F_{(1, 80)} = 6.97$, $p = 0.010$], and between Phenotype (LR, HR) and Pre-exposure [SAL, NIC; $F_{(1, 80)} = 4.69$, $p = 0.033$]. A four-way ANOVA also revealed significant main effects of Phenotype [LR, HR; $F_{(1, 80)} = 18.13$, $p = 0.0001$] and Pre-exposure [$F_{(1, 80)} = 5.78$, $p = 0.019$] in BDNF mRNA levels in the BLA. Specific post-hoc comparisons showed that in nicotine pre-exposed, vehicle-treated HRs, BDNF mRNA levels in the BLA were significantly upregulated following 1 wk of abstinence, compared to saline pre-exposed, vehicle-treated controls [$p = 0.028$]. Moreover, this effect was reversed by AM251 administration [$p = 0.010$]. Furthermore, a significant upregulation in BDNF mRNA levels was also observed in nicotine pre-exposed, vehicle-treated HRs compared to saline pre-exposed, vehicle treated controls following the long (3 wks) abstinence [$p = 0.047$]. In contrast, this effect was further augmented by AM251 administration during the last week of the long (3 wks) abstinence above the levels observed with nicotine injections alone [$p = 0.039$].

Spinophilin mRNA expression in the BLA of representative HR animals that were tested with the challenge nicotine on board is shown in Fig. 7A–F. A four-way ANOVA revealed a significant interaction between Phenotype (LR, HR), Pre-exposure (SAL, NIC) and Abstinence [1 wk, 3 wks; $F_{(1, 80)} = 9.49$, $p = 0.003$] in spinophilin mRNA levels in the BLA. Significant interactions between Phenotype (LR, HR) and Abstinence [1 wk, 3 wks; $F_{(1, 80)} = 38.48$, $p = 0.0001$] and between Phenotype (LR, HR) and Pre-exposure [SAL, NIC; $F_{(1, 80)} = 21.08$, $p = 0.0001$], as well as significant main effects of Pre-exposure [SAL, NIC; $F_{(1, 80)} = 37.96$, $p = 0.0001$] and Treatment [VEH, AM251; $F_{(1, 80)} = 4.00$, $p = 0.049$] were also revealed. Specific post-hoc comparisons showed that spinophilin mRNA levels were significantly upregulated in nicotine pre-exposed, vehicle-treated HRs compared to saline pre-exposed, vehicle-treated controls following 1 wk of abstinence [$p = 0.0001$]. Moreover, this effect persisted following 3 wks of abstinence [$p = 0.039$]. AM251 administration during the last week of the long (3 wks) abstinence resulted in increased spinophilin mRNA levels in the BLA in nicotine pre-exposed HRs above the levels observed in vehicle-treated counterparts [$p = 0.048$].

4. Discussion

In conformation of our previous findings (Aydin et al., 2012), the present study shows that intermittent nicotine exposure during the peripubertal–juvenile period results in a sustained

increase in social anxiety-like behavior and a robust expression of locomotor sensitization to a low dose nicotine challenge regardless of the length of abstinence in the HR animals. In addition, the present study reveals four major, novel findings: 1) Regardless of the length of abstinence, AM251 treatment can successfully reverse the expression of locomotor sensitization to the low dose nicotine challenge in HRs, providing evidence for the efficacy of the CB1R antagonists in suppressing the sensitizing effects of nicotine in the HR phenotype. 2) While AM251 treatment during 1 wk of abstinence is ineffective in reversing the nicotine-induced social anxiety-like behavior in HRs, the identical treatment regimen further augments social withdrawal when administered during the late phase (i.e., last week) of a long (3 wks) abstinence in nicotine pre-exposed HRs compared to vehicle-treated controls. This suggests that AM251 treatment, especially during a long abstinence may not be an effective agent to combat nicotine-induced negative affective state in the HR phenotype. 3) Independent of the challenge nicotine exposure and regardless of the length of abstinence, nicotine pre-exposed HRs display increased social anxiety-like behavior and a decreased ratio of NPY mRNA in the MeA to CRF mRNA in the CeA in comparison to saline controls. This suggests a shift in the NPY to CRF balance in the HR amygdala toward a deficit in NPY that persists even after 3 wks of abstinence. 4) The anxiogenic state induced by repeated nicotine during the peripubertal–juvenile period in HRs accompanies increased BDNF and spinophilin mRNA levels in the BLA, independent of the challenge nicotine exposure and regardless of the length of abstinence, suggesting increased neuronal plasticity with juvenile nicotine exposure in this phenotype. While AM251 treatment during the short (1 wk) abstinence reverses the nicotine-induced increase in BDNF mRNA levels in the BLA, it fails to normalize the neuropeptidergic dysregulations between NPY and CRF in the amygdala, or reverse the upregulation of spinophilin mRNA in the BLA. This is in agreement with our finding that AM251 is unable to reverse the nicotine-induced social anxiety-like behavior in HRs when applied during 1 wk of abstinence following repeated nicotine. Surprisingly, AM251 treatment during the late phase of a long (3 wks) abstinence further augments BDNF as well as spinophilin mRNA levels in the BLA above those observed with nicotine alone, while leaving neuropeptidergic changes induced by nicotine unaffected. This suggests that AM251 may exacerbate the nicotine-induced synaptic plasticity in the BLA when applied during a long (3 wks) abstinence, and that could be a mechanism by which nicotine-induced anxiogenic state is enhanced in AM251 treated HRs above levels observed with nicotine pre-exposure alone. In sum, the present findings suggest possible time-dependent alterations in the neurocircuitry controlling anxiety responses during abstinence, which may lead to differential outcomes in the expression of social anxiety-like behavior following AM251 treatment in the HR rats.

We have recently shown that dysregulations in amygdalar NPY and CRF systems are implicated in the expression of behavioral sensitization to nicotine and associated social anxiety-like behavior observed in HRs after repeated, intermittent nicotine injections followed by 1 wk of abstinence and a low dose nicotine challenge (Aydin et al., 2011a, 2011b). The present study extends these findings to a long (3 wks) abstinence, and show that the imbalance between amygdalar NPY and CRF systems accompanies the nicotine-induced behavioral adaptations in HRs, even after 3 wks of abstinence. Moreover, the present data also show that social anxiety and associated neuropeptidergic changes in the amygdala are detectable in HR animals on the last day of abstinence without nicotine on board, suggesting that these behavioral and molecular effects are likely abstinence-related. Overall, the present results confirm the role of neuropeptidergic adaptations in the HR amygdala in nicotine-induced behavioral effects. Interestingly, our results show that while the ratio of NPY in both nuclei of the amygdala (i.e., MeA and BLA) to CRF in the CeA is decreased in nicotine pre-exposed HRs compared to saline controls following a short (1 wk) abstinence, this effect is sustained only in the ratio of NPY in the MeA to CRF in the CeA, across a long (3 wks) abstinence, suggesting that long-term deficit in the NPY is restricted to the MeA. Given the

distinct role of the MeA in social behaviors (Duxon et al., 1997; Ferguson et al., 2001) and our previous findings showing that although in basal conditions HRs manifest inhibited anxiety in the SI, EPM and the LDB tests compared to LRs (Kabbaj et al., 2000; Aydin et al., 2011b), following the intermittent behavioral sensitization regimen they exhibit increased anxiety-like behavior only in the SI test (Aydin et al., 2011b), the present data may suggest that social component of anxiety may be critical in long-lasting negative affect in nicotine pre-exposed HRs that may be particularly mediated by a sustained NPY deficit in the MeA. In addition, our results also show that AM251 administration during abstinence does not alter the nicotine-induced effects on NPY and CRF mRNA levels in the amygdala and cannot significantly alter social anxiety-like behavior in nicotine pre-exposed HRs. These results suggest that the imbalance in amygdalar NPY and CRF systems is robust and irreversible with AM251 treatment and may be associated with long-lasting abstinence-related social anxiety in HR animals that received nicotine during the juvenile period. However, it should be noted that in addition to amygdalar NPY and CRF, other neurotransmitter systems (e.g. GABA, benzodiazepines, cholecystokinin, orexin) in the amygdala and/or other brain regions (e.g. hippocampus, hypothalamus, locus coeruleus) where NPY and CRF are expressed may be implicated in the social anxiety-like behavior observed in HRs. Nonetheless, given the reciprocal relationship between NPY and CRF in the amygdala in regulation of anxiety (Heilig et al., 1994; Sheriff et al., 2001; Sajdyk et al., 2004, 2006; Britton et al., 2000) and specifically in nicotine-induced anxiety (Slawecki et al., 2005; Aydin et al., 2011b), the ratio of the mRNA levels of these two neuropeptides in the amygdala has been used as a marker of anxiety in the present study.

In addition to the neuropeptidergic mechanisms in the amygdala, the present study investigates the alterations in amygdalar neuroplasticity in HRs as a potential substrate for the behavioral adaptations induced by nicotine, using BDNF and spinophilin as molecular markers of neuronal plasticity. It has been shown that chronic nicotine treatment promotes facilitation of synapses (Huang et al., 2008) and alters dendritic morphology in the BLA (Bergstrom et al., 2010) following abstinence, implicating BLA as a target for nicotine-induced synaptic plasticity. Furthermore, data from BDNF overexpressing mice has shown that these mice have dendritic plasticity in the form of increased dendritic material and spinogenesis in the BLA which results in an anxiogenic phenotype on the EPM (Govindarajan et al., 2006), associating BDNF-mediated dendritic remodeling in the BLA with emergence of anxiety-like behavior. Spinophilin has been shown to be enriched in spines (Allen et al., 1997; Feng et al., 2000) and its expression correlates with changes in dendritic spine density (Zhou et al., 2010; Allen, 2004; Sarrouilhe et al., 2006; Prange-Kiel et al., 2009), verifying the role of spinophilin as a marker of dendritic remodeling, and structural plasticity (Sarrouilhe et al., 2006). Within this context, our results show a long lasting increase in spinophilin, along with BDNF mRNA levels in the BLA both with or without nicotine on board following repeated nicotine injections and abstinence, in HRs compared to saline controls, suggesting an increase in dendritic remodeling and/or spinogenesis, possibly mediated by BDNF in amygdala, albeit a causal relationship is beyond the scope of the current experiment. Confirmatory reports show that induction of spinogenesis in the BLA is associated with development of anxiety displayed on the EPM (Mitra et al., 2005), and blockage of dendritic expansion in the BLA results in decreased anxiety-like behavior on the same test (Mitra and Sapolsky, 2010). Together these findings suggest that abstinence-related social anxiety-like behavior observed in HRs may be associated with enhanced synaptic plasticity within the BLA that may be mediated by BDNF.

Our data show that AM251 treatment reverses the nicotine-induced increase in BDNF levels in HRs when applied during a short (1 wk) abstinence, whereas the same treatment amplifies BDNF mRNA levels in the BLA when administered during the late phase (i.e., last week) of

a long (3 wks) abstinence. Moreover, while spinophilin mRNA levels remain unchanged in this region following AM251 treatment during 1 wk of abstinence, the same treatment augments the levels of spinophilin mRNA when administered during the late phase (i.e., last week) of a long (3 wks) abstinence. These results suggest that AM251 treatment may reverse the immediate effects of nicotine on BDNF expression in the amygdala, but may fail to reverse the structural changes that occur at the synaptic level, as indexed by elevations in spinophilin mRNA. Moreover, AM251 treatment following a long (3 wks) abstinence augments anxiety in HRs compared to vehicle-treated controls and results in upregulation of BDNF and spinophilin mRNAs, potentially implicating enhanced structural plasticity in the BLA as a substrate for exacerbated negative affect in HRs.

The present data suggest that the use of CB1R antagonists as agents for nicotine cessation has to be critically evaluated for lasting therapeutic success in order to avoid exacerbation of the negative affective state in nicotine vulnerable individuals. Indeed, rimonabant, a selective CB1R antagonist/inverse agonist has been disapproved by the FDA, due to its neuropsychiatric side effects including depressed mood and anxiety (US Food and Drug Administration, 2007; Bermudez-Silva et al., 2010; Cahill and Ussher, 2011). In line with the clinical findings, anxiogenic-like effects of CB1R antagonism have been reported in rodents (Arévalo et al., 2001; McGregor et al., 1996; Navarro et al., 1997; Haller et al., 2004; Rodgers et al., 2005). For example, it has been shown that injections of AM251 at 2, 4 or 8 mg/kg doses prior to maze exposure results in a significant increase in anxiety-like behavior in rats on the EPM compared to vehicle injected controls (Sink et al., 2010). However, our results show no significant effects of AM251 alone at the dose of 5 mg/kg on anxiety-like behavior or molecular indices of anxiety in saline pre-exposed LRHR animals compared to their vehicle-treated counterparts. This discrepancy with the literature may be attributable to the different animal model utilized and the experimental history of the animals used in our study i.e., repeated saline injections, as this has been shown to affect anxiety-like behavioral profile following CB1R antagonist administration before (Rodgers et al., 2003; Viveros et al., 2007).

The present data also showed a significant decrease in the ratio of NPY mRNA levels in the MeA and the BLA to CRF mRNA levels in the CeA in saline pre-exposed, young adult HRs compared to juvenile counterparts. Moreover, this effect was seen in both nicotine challenged and nicotine-naïve HRs that were pre-exposed to saline, suggesting that it is independent of the low dose nicotine exposure. Furthermore, since the only variable between saline pre-exposed juvenile HRs and saline pre-exposed young adult HRs was the time elapsed after the last saline injection (i.e., 1 wk as opposed to 3 wks), the decrease in the ratio of the mRNA levels of NPY to CRF in the amygdala between these two groups may be attributable to developmental or maturational mechanisms, specifics of which are speculative at this point. However, together with our recently published findings showing a drastic decrease in CB1R mRNA levels in the CeA and BLA in saline pre-exposed HRs as they matured into young adulthood (Aydin et al., 2012), the present data may suggest ongoing developmental processes in the HR amygdala, which may contribute to its vulnerability to the neuroplastic effects of nicotine.

Our results also showed that in LRs, the identical nicotine pre-exposure during the peripubertal–juvenile period results in an increase in anxiolytic-like behavior compared to saline controls 3 wks after the 4th nicotine injection. Moreover, this nicotine-induced increase in anxiolysis in young adult LRs return back to the levels observed in saline controls following challenge nicotine injection. However, the observed behavioral changes in nicotine pre-exposed LRs are not accompanied by neuropeptidergic and neuroplastic changes in the amygdala, suggesting that other neurochemical systems and/or brain regions may be involved in these alterations in the LR phenotype that remain to be investigated.

In summary, these findings show that the long-lasting expression of behavioral sensitization to a low dose nicotine challenge and increased social anxiety-like behavior observed in HRs is accompanied by a persistent increase in BDNF and spinophilin mRNAs in the BLA coupled with a shift in the balance between amygdalar NPY and CRF toward a deficit in NPY levels, which may contribute to perpetuation of negative affect in nicotine vulnerable HRs. Moreover, a CB1R antagonist, AM251 treatment given during the late phase of a long (3 wks) abstinence exacerbates the nicotine-induced negative affective state, possibly by augmenting synaptic plasticity in the BLA in HR rats. These findings suggest that although in clinical settings CB1R antagonism may increase the chance of quitting approximately 1½-fold (Cahill and Ussher, 2011); it is ineffective in reversing the abstinence-related negative affective state and depending on the timeline of the treatment, may even interfere with maintenance of abstinence due to intensified symptoms of social anxiety in a subset of vulnerable individuals.

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Abbreviations

BLA	basolateral nucleus of the amygdala
BDNF	brain-derived neurotrophic factor
CB1R	cannabinoid receptor 1
CeA	central nucleus of the amygdala
CRF	corticotrophin releasing factor
EPM	elevated plus maze
HR	high responder
LR	low responder
MeA	medial nucleus of the amygdala
NPY	neuropeptide Y
SI	social interaction

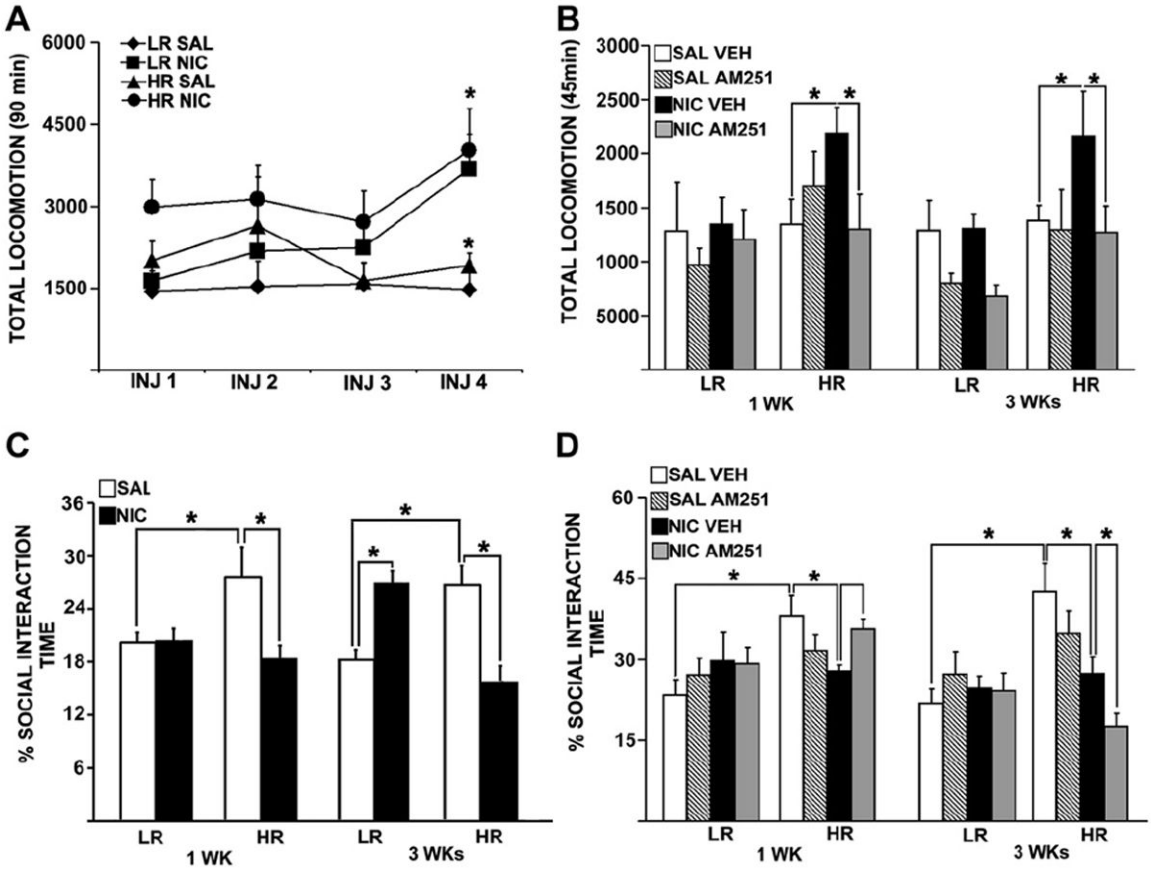

Fig. 1.

Total locomotor reactivity to four intermittent nicotine (0.35 mg/kg, s.c.) or saline (1.0 ml/kg, s.c.) injections (A), total locomotor reactivity to a low dose nicotine (0.1 mg/kg, s.c.) challenge (B), percent time spent interacting with the resident rat in the SI test on the last day of abstinence without challenge nicotine (0.1 mg/kg, s.c.) on board (C), and percent time spent interacting with the resident rat in the SI test with a low dose nicotine (0.1 mg/kg, s.c.) challenge on board (D) are shown. Group means± SEMs are plotted in line (A) and bar (B, C, and D) graphs (*: $p < 0.05$).

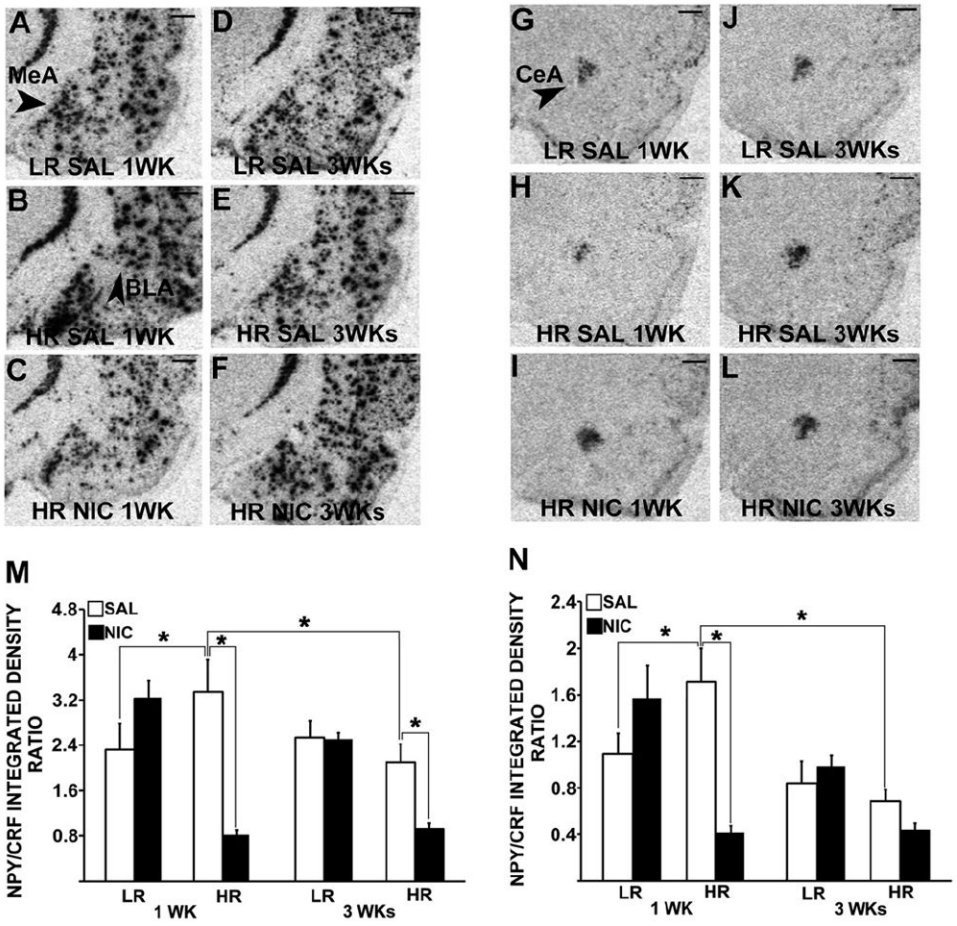

Fig. 2.

Images of coronal hemisections of the BLA and the MeA (A–F), and the C–A (G–L) of LRHR rats that were tested on the last day of abstinence, without challenge nicotine on board. The sections were radioactively labeled with an antisense cRNA probe against NPY (A–F) or CRF (G–L) and exposed on an x-ray film. Integrated density ratios of NPY mRNA in the MeA to CRF mRNA in the CeA (M) and the same ratio with NPY mRNA in the BLA to CRF mRNA in the CeA (N) are plotted with bar graphs for (*: $p < 0.05$). Scale bar = 250 μm.

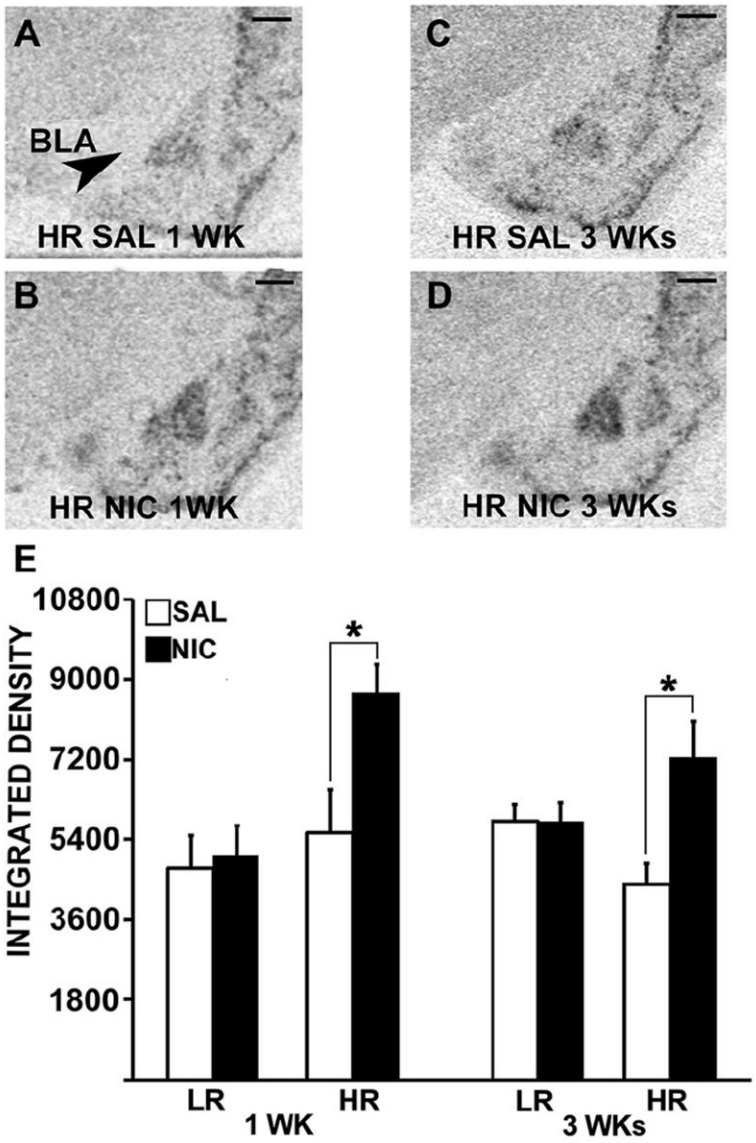

Fig. 3.

Panels A–D show images of coronal hemisections of the BLA of representative HR rats that were tested on the last day of abstinence, without challenge nicotine on board. The sections were radioactively labeled with an antisense cRNA probe against BDNF and exposed on an x-ray film. Means of quantification results for integrated densities ± SEMs are plotted with bar graphs for the BLA (E; *: $p < 0.05$). Scale bar = 250 μm.

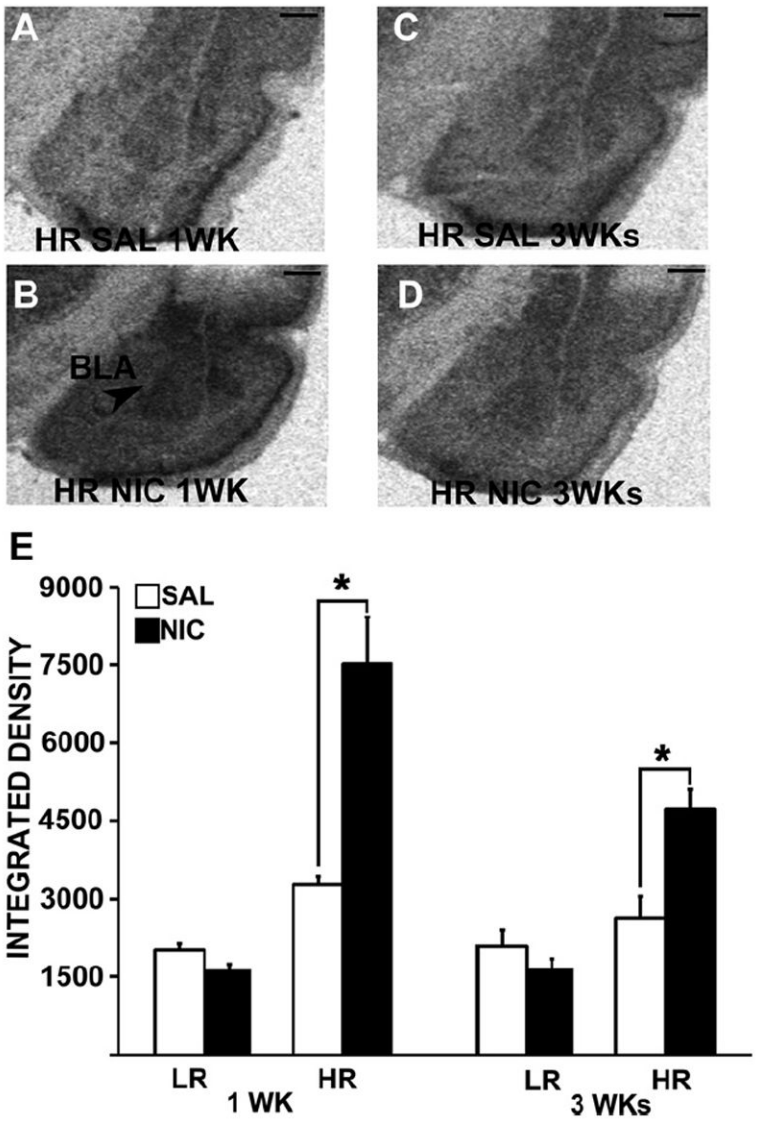

Fig. 4.

Panels A–D show images of coronal hemisections of the BLA of representative HR rats that were tested on the last day of abstinence, without challenge nicotine on board. The sections were radioactively labeled with an antisense cRNA probe against spinophilin and exposed on an x-ray film. Means of quantification results for integrated densities ± SEMs are plotted with bar graphs for the BLA (E; *: $p < 0.05$). Scale bar = 250 μm.

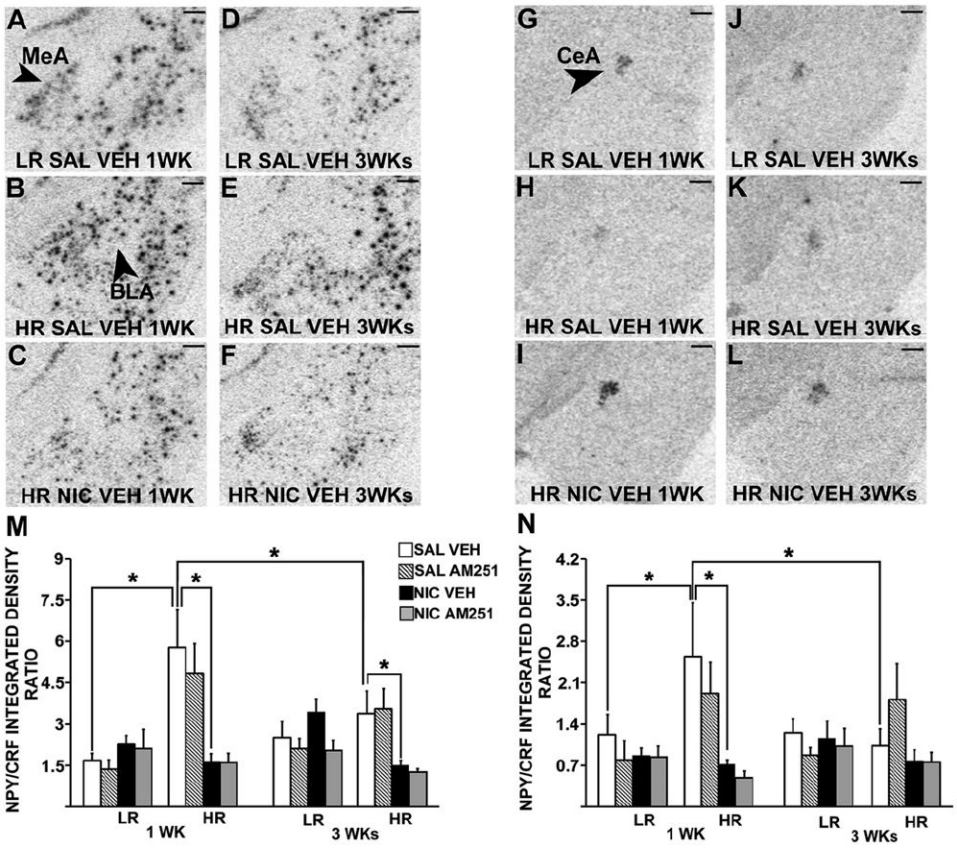

Fig. 5.

Images of coronal hemisections of the BLA and the MeA (A–F), and the CeA (G–L) of LRHR rats that were tested with a low dose nicotine challenge on board. The sections were radioactively labeled with an antisense cRNA probe against NPY (A–F) or CRF (G–L) and exposed on an x-ray film. Integrated density ratios of NPY mRNA in the MeA to CRF mRNA in the CeA (M) and the same ratio with NPY mRNA in the BLA to CRF mRNA in the CeA (N) are plotted with bar graphs for (*: $p < 0.05$). Scale bar = 250 μm.

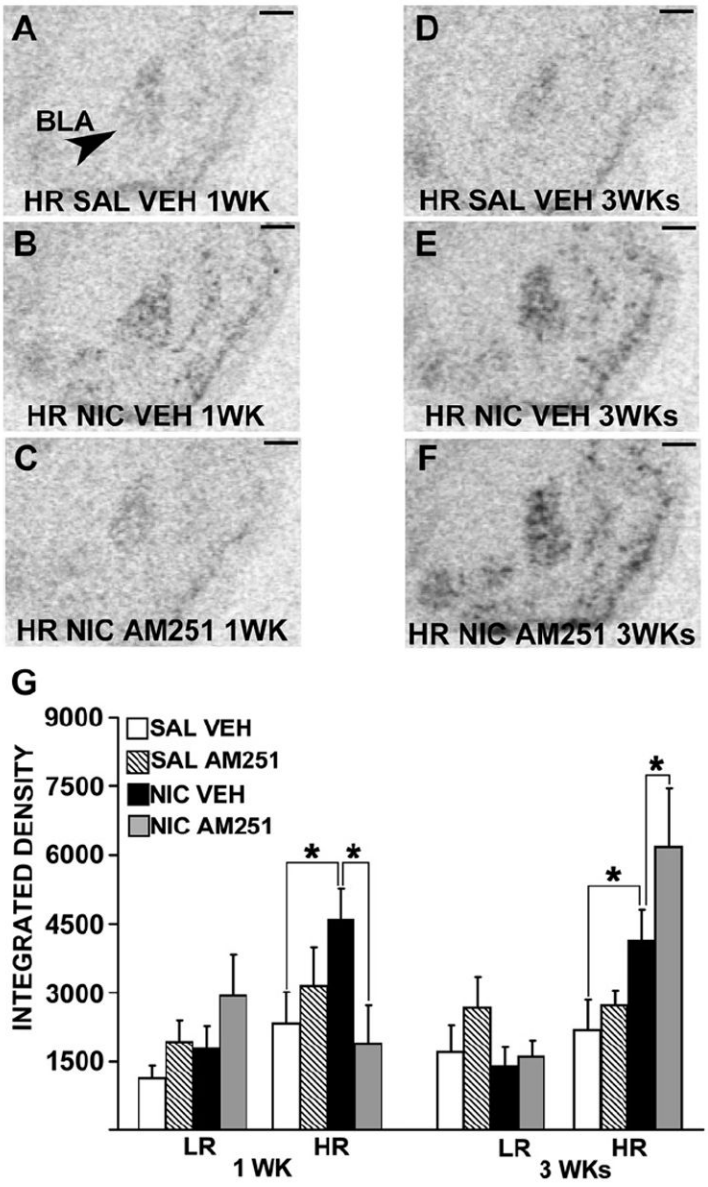

Fig. 6.

Panels A–F show images of coronal hemisections of the BLA of representative HR rats that were tested with a low dose nicotine challenge on board. The sections were radioactively labeled with an antisense cRNA probe against BDNF and exposed on an x-ray film. Means of quantification results for integrated densities ± SEMs are plotted with bar graphs for the BLA (G; *: $p < 0.05$). Scale bar = 250 μm.

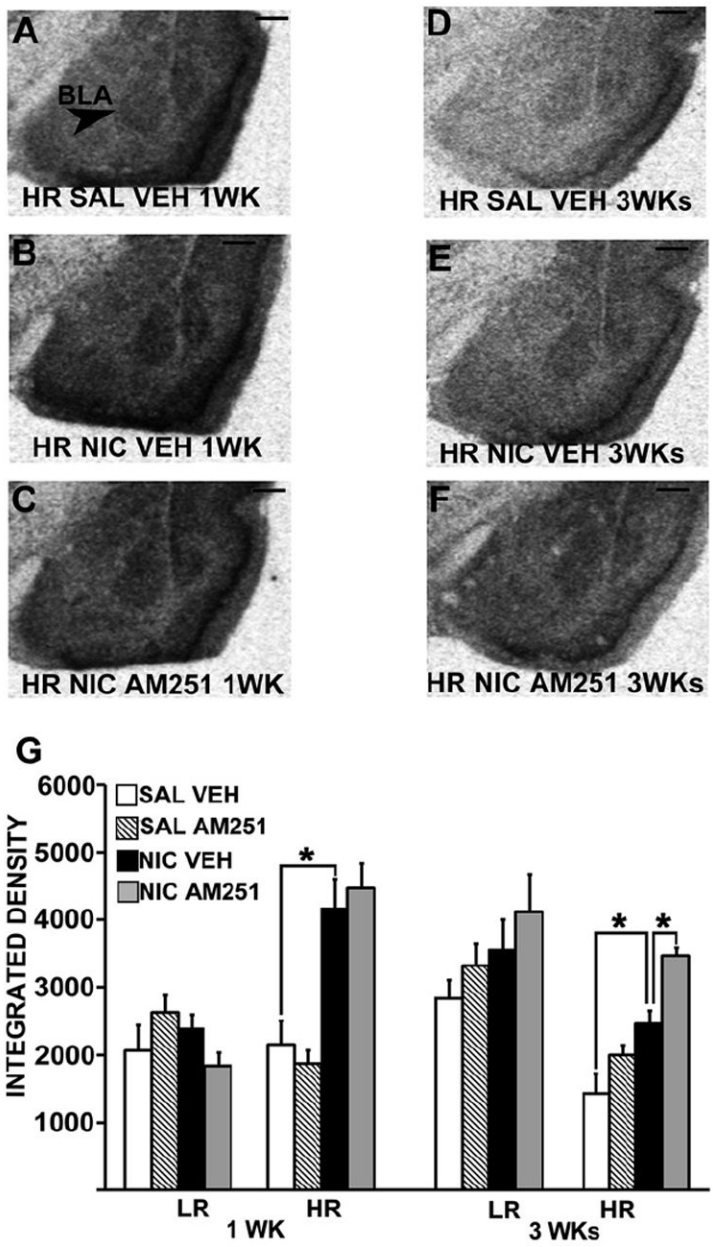

Fig. 7.

Panels A–F show images of coronal hemisections of the BLA of representative HR rats that were tested with a low dose nicotine challenge on board. The sections were radioactively labeled with an antisense cRNA probe against spinophilin and exposed on an x-ray film. Means of quantification results for integrated densities ± SEMs are plotted with bar graphs for the BLA (G; *: $p < 0.05$). Scale bar = 250 μm.

Table 1

Phenotype pre-screened LRHR rats received saline (1.0 ml/kg; s.c.) or nicotine (0.35 mg/kg; s.c.) injections four times at 3-day intervals (PN 28–37). Following the fourth injection, rats were further assigned to vehicle (1 ml/kg, i.p.) or AM251 (5 mg/kg, i.p.) treatment groups. Half of the animals in each group underwent 1 wk of abstinence when they received vehicle or AM251 injections every other day (A). The remaining half underwent 3 wks of abstinence and received vehicle or AM251 during the last week of this period every other day (B). All animals were tested on the SI test for anxiety assessment, with or without a low dose nicotine (0.1 mg/kg; s.c.) challenge on board.

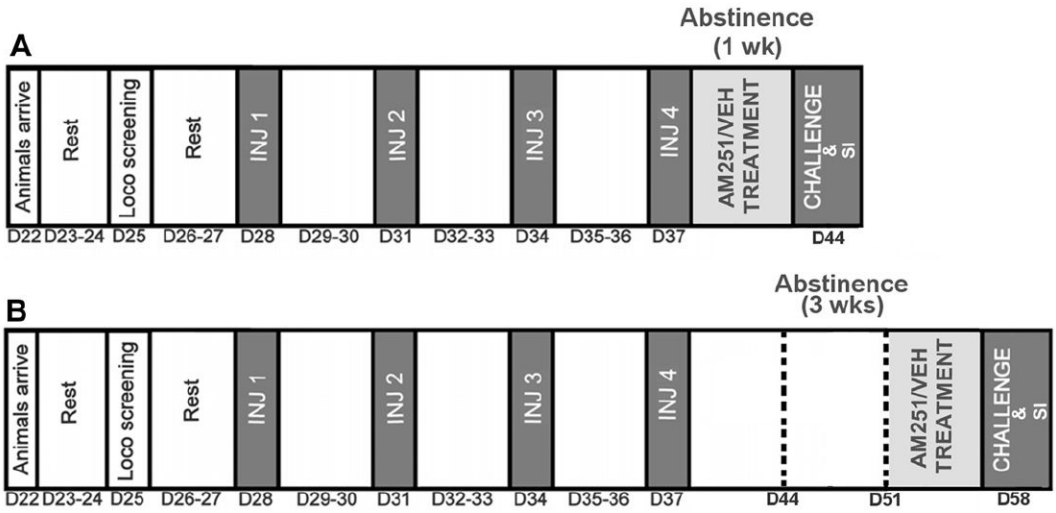

Table 2

Phenotype pre-screened LRHR rats received four saline (1.0 ml/kg; s.c.) or nicotine (0.35 mg/kg; s.c.) injections at a 3-day interval (*Sensitization*). Following the fourth injection, rats underwent 1 wk or 3 wks of abstinence
(*Abstinence*), during which they received vehicle (1 ml/kg, i.p.) or AM251 (5 mg/kg, i.p.; *Treatment*) injections every other day. At the end of the abstinence periods all animals were tested on the SI test for anxiety assessment, with or without a low dose nicotine (0.1 mg/kg; s.c.) challenge (*Challenge*) on board.

	Abstinence	
	1 wk	**3 wks**
LR	Sensitization + Abstinence + Treatment + Challenge	Sensitization + Abstinence + Treatment + Challenge
	Sensitization + Abstinence	Sensitization + Abstinence
HR	Sensitization + Abstinence + Treatment + Challenge	Sensitization + Abstinence + Treatment + Challenge
	Sensitization + Abstinence	Sensitization + Abstinence