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Long-term effects of juvenile nicotine exposure on abstinence-related social anxiety-like behavior and amygdalar cannabinoid receptor 1 (CB1R) mRNA expression in the novelty-seeking phenotype

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Abstract

A rat model of novelty-seeking phenotype predicts vulnerability to nicotine relapse where locomotor reactivity to novelty is used to rank high (HR) versus low (LR) responders. Present study investigates implication of cannabinoid receptor 1 (CB1R) in the basolateral (BLA) and the central (CeA) nuclei of amygdala in behaviorally-sensitizing effects of nicotine and accompanying social anxiety following juvenile nicotine training and a 1 or 3-wk injection-free period in the novelty-seeking phenotype. Sprague Dawley rats were phenotype screened, and received four, saline (1 ml/kg; s.c) or nicotine (0.35 mg/kg; s.c) injections, followed by a 1 or 3-wk injection-free period. Subsequently, animals were challenged with a low dose of nicotine (0.1 mg/kg; s.c.), subjected to the social interaction test and sacrificed. *In situ* hybridization histochemistry was used to assess CB1R messenger RNA (mRNA) levels in the amygdala. Nicotine pre-trained HRs displayed expression of locomotor sensitization to nicotine challenge along with enhanced social anxiety compared to saline pre-trained controls following a 1 or 3-wk injection-free period. HR-specific behavioral effects were accompanied by decreased CB1R mRNA levels in the CeA and the BLA following a 1-wk injection-free period. Decreased CB1R mRNA levels in both compartments of the amygdala were also observed following nicotine challenge in saline pre-trained HRs after a 3-wk injection-free period compared to HRs after a 1-wk injection-free period. These findings show robust, long-lasting expression of behavioral sensitization to nicotine in HRs associated with changes in amygdalar CB1R mRNA as a potential substrate for abstinence-related anxiety.

Keywords

Basolateral nucleus of amygdala; central nucleus of amygdala; behavioral sensitization; social anxiety; cannabinoid receptor 1; nicotine abstinence

Individual differences in vulnerability to psychostimulants are studied in an outbred rodent model of the novelty-seeking phenotype [1, 2, 3], where locomotor response to a novel environment is used to determine high (HR, highest 1/3) versus low (LR, lowest 1/3) responders. We have shown that a chronic intermittent nicotine training during the

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peripubertal-juvenile period (postnatal day, PN 28-36) followed by a 1-wk injection-free period and a low dose nicotine challenge results in expression of locomotor sensitization to nicotine and abstinence-related social anxiety in HRs [4, 5, 6] assigning a predictive role for the LRHR phenotype in vulnerability to the behavioral adaptations induced by chronic nicotine.

The endocannabinoid system mediates the effects of drugs of abuse, including nicotine [7] by binding to cannabinoid receptor 1 (CB1R). Systemic administration of a CB1R antagonist blocks expression of nicotine sensitization following abstinence [8, 9]. Moreover, the expression of locomotor sensitization in HRs is reversed by a CB1R antagonist administration during the 1-wk injection-free period [4], linking CB1R to the phenotype-specific vulnerability to nicotine. Previously, intra-BLA injections of a CB1R antagonist following abstinence is shown to reduce cue-induced nicotine-seeking behavior in rats trained to self-administer nicotine [10], associating CB1R in the BLA with relapse. In addition, CeA is also implicated in nicotine abstinence-related anxiety [11, 5, 6], a major factor in vulnerability to nicotine relapse [12].

Present study investigates implication of CB1R in the BLA and the CeA in the expression of behavioral sensitization to a low dose nicotine challenge and accompanying social anxiety following juvenile nicotine training and a short (1-wk) or a long (3-wk) injection-free period in the LRHR animals. We hypothesize that observed locomotor sensitization to nicotine challenge and abstinence-related increase in social anxiety-like behavior in HRs will last into young adulthood. Moreover, the CB1R mRNA levels in the amygdala will be assessed in relation to long-term behavioral effects of nicotine in the HR rats.

Seventy-two male Sprague-Dawley rats (Charles River, Wilmington, MA) arrived at weaning (PN 22), were housed 3 per cage in $43 \times 21.5 \times 25$ cm³ Plexiglas cages and were kept on a 12 h light/dark cycle (lights on at 7:00 A.M.). On PN 25, animals were screened for locomotor reactivity to a novel environment as described before [6]. Total locomotor activity (i.e., X, Y, and Z locomotion) was pooled and the rats were ranked as HRs (the highest third of the sample; n=24) or LR (the lowest third of the sample; n=24).

Phenotype pre-screened rats were assigned to saline (1 ml/kg; s.c.) or nicotine (0.35 mg/kg; s.c.) training on PN 28. Nicotine hydrogen tartrate was (Sigma) dissolved in 0.9% NaCl and the pH was adjusted to 7.4. On injection days, rats were given 1 hr to habituate to 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 training injection, half of the animals in each group (n= 6/group) underwent a 1-wk injection-free period (PN 37-44), while the remaining half underwent a 3-wk injection-free period (PN 37-58). 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. Testing took place 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 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 the SI test animals were rapidly decapitated and brain tissue was harvested. The *in situ* hybridization protocol is identical to our published protocol [13]. Rat CB1R cDNA was cloned in our laboratory (accession #: U40395), antisense linearized, transcribed, and ³⁵S labeled. Section images were captured digitally from x-ray films with a CCD camera, and optical densities were determined relative to the background by subtracting any labeling below 3.5X the background for the

BLA and the CeA using the Scion Image software. Eight sections per animal were used to capture the entire rostro-caudal length of the CeA and the BLA. Integrated density values were calculated as optical density multiplied by area and averaged to produce one data point for each brain region for each animal, and grand means were compared statistically between groups.

Figure 1 shows total locomotor reactivity to four intermittent nicotine or saline training injections (A) and the subsequent low dose nicotine challenge following a 1-wk or a 3-wk injection-free period in LRHR rats (B). Repeated-measures ANOVA revealed a significant interaction between Injection Days (INJ 1, INJ 2, INJ 3, INJ 4) and Pre-training [SAL, NIC; $F = 3.48$, $p = 0.018$], and significant main effects of Pre-training [SAL, NIC; $F = 7.97$, $p = 0.007$] and Injection Days [INJ 1, INJ 2, INJ 3, INJ 4; $F = 3.03$, $p = 0.032$]. Post-hoc comparisons showed that at INJ 4, nicotine pre-training resulted in increased locomotor reactivity in both LR [p = 0.006] and HR [p = 0.011] compared to saline pre-trained controls. A three-way ANOVA for the locomotor reactivity to challenge nicotine revealed a significant interaction between Phenotype (LR, HR) and Pre-training [SAL, NIC; $F = 3.91$, $p = 0.05$], along with main effects of Phenotype [LR, HR; $F = 4.33$, $p = 0.044$] and Pre-training [SAL, NIC; $F = 4.67$, $p = 0.037$]. Post-hoc comparisons showed that nicotine pre-trained HRs exhibited increased locomotor reactivity to the low dose nicotine challenge compared to saline pre-trained controls following a 1- wk [p = 0.05] and a 3-wk [p = 0.044] injection-free period.

Figure 1 also shows percent time spent interacting with the resident rats in a social context (C). A three-way ANOVA revealed significant interactions between Phenotype (LR, HR) and Pre-training [SAL, NIC; $F = 11.96$, $p = 0.001$], and a main effect of Phenotype [LR, HR; $F = 13.06$, $p = 0.001$]. Post-hoc comparisons showed that saline pre-trained juvenile [p = 0.007] and young adult [p = 0.0002] HRs spent significantly more time engaged in social interaction compared to saline pre-trained LR. Moreover, following a behaviorally-sensitizing nicotine regimen HRs spent significantly less time interacting with the resident rat compared to controls following a 1- wk [p = 0.05] and a 3-wk [p = 0.004] injection-free period.

Figure 2 shows CB1R mRNA expression in the BLA and the CeA. A three-way ANOVA revealed significant interactions between Injection-free Period (1wk, 3wks) and Pre-training [SAL, NIC; $F = 7.30$, $p = 0.010$] along with significant main effects of Pre-training [SAL, NIC; $F = 4.28$, $p = 0.046$] and Phenotype [LR, HR; $F = 5.70$, $p = 0.022$] in the BLA. Additionally, a three-way ANOVA revealed significant interactions between Injection-free Period (1wk, 3wks) and Pre-training [SAL, NIC; $F = 7.02$, $p = 0.012$] along with significant main effects of Pre-training [SAL, NIC; $F = 4.15$, $p = 0.049$] and Injection-free Period [1wk, 3wks; $F = 7.38$, $p = 0.010$] in the CeA. Post-hoc comparisons showed that saline pre-trained juvenile HRs (i.e., following a 1-wk injection-free period) had higher CB1R mRNA levels compared to young adult counterparts (i.e., following a 3-wk injection-free period) in the BLA [p = 0.004] and in the CeA [p = 0.0002]. Moreover, during young adulthood, saline pre-trained HRs had lower CB1R mRNA levels in the BLA compared to LR counterparts [p = 0.041]. Moreover, a behaviorally-sensitizing nicotine regimen resulted in significant downregulation of the CB1R mRNA levels following a 1-wk injection-free period in HRs compared to saline pre-trained controls in the BLA [p = 0.003] and in the CeA [p = 0.009].

Our findings show that chronic nicotine training during the peripubertal-juvenile period followed by a short (1wk) or a long (3 wks) injection-free period results in augmented locomotor response to the low dose nicotine challenge and decreased duration of social interaction in HRs compared to saline pre-trained controls. Moreover these HR-specific behavioral effects of nicotine are accompanied by decreased CB1R mRNA levels in the CeA

and the BLA following a 1-wk injection-free period, implicating a deficit in amygdalar CB1R in abstinence-related negative affective state. In addition, a decrease in CB1R mRNA levels in both compartments of the amygdala are observed after the low dose of nicotine challenge in saline pre-trained HRs following a 3-wk injection-free period compared to HRs following a 1-wk injection-free period, suggesting that CB1R levels fluctuate as a factor of developmental age at the time of low dose nicotine challenge in HRs.

Rodent studies have shown that repeated juvenile nicotine exposure results in increased self-administration of nicotine in adulthood, compared to equivalent exposure at adulthood in rats [14], suggesting enhanced vulnerability to develop nicotine addiction in juvenile rats. Present data show that regardless of the length of the injection-free period, HRs pre-trained with nicotine during the peripubertal-juvenile period express behavioral sensitization to challenge nicotine and accompanying social withdrawal compared to saline pre-trained controls, emphasizing that behavioral effects of juvenile nicotine in the HRs are long-lasting.

Behavioral sensitization to psychostimulants such as cocaine [15] is marked by a progressive increase in anxiety [16] associated with social pathologies [17, 18] that can be precipitated by a single drug use long after discontinuation of the drug [19, 20]. In rodents, chronic nicotine administration and a subsequent withdrawal results in increased social anxiety-like behavior [21, 22]. Our lab showed that a behaviorally-sensitizing nicotine regimen results in increased social anxiety in the HR phenotype compared to saline pre-trained controls following a 1-wk injection-free period. Moreover, antagonizing the Y2 receptors of the anxiolytic neuropeptide Y during abstinence inhibits social anxiety and reverses the expression of locomotor sensitization to nicotine challenge, suggesting a role for social anxiety in vulnerability to nicotine relapse in HRs [5, 6]. Present data show that increased social withdrawal induced by juvenile nicotine pre-training and a 1-wk injection-free period persists following a 3-wk injection-free period spanning into young adulthood, along with the expression of locomotor sensitization to nicotine in HRs, suggesting that social anxiety may be critical in the course of developing a long-lasting vulnerability to relapse in HRs.

Several studies have implicated amygdalar CB1R signaling in the modulation of anxiety-like behavior in rodents, although none of these were nicotine-induced anxiety paradigms. For example, CB1R antagonist microinjections into the BLA induce an anxiety-like state in the elevated plus maze [23]. Similarly, reduction of anxiety-like behavior is observed in the same test following a CB1R agonist administration into the CeA [24], suggesting a modulatory role for amygdalar CB1R in anxiety. Our results support previously published work, in that abstinence-related anxiety-like state in HRs following a 1-wk injection-free period is associated with decreased CB1R mRNA levels in the BLA and CeA in HRs compared to saline pre-trained controls. However, such chronic nicotine-induced downregulation in amygdalar CB1 mRNA expression is not observed in HRs after a 3-wk injection-free period, despite persistence of the negative affective state. This could be due an apparent downregulation in the CB1R mRNA levels observed in saline pre-trained HRs when challenged as young adult as opposed to juvenile rats. Amygdalar CB1R mRNA levels in saline pre-trained young adult HRs are similar to those observed in nicotine pre-trained HRs after a 1-wk injection-free period, possibly due to a floor effect in CB1R mRNA pool. It is plausible that a sustained decrease in amygdalar CB1R mRNA levels into young adulthood may act to exacerbate vulnerability to nicotine in HRs. Overall, these results show that changes in CB1R mRNA expression in the amygdala accompany a persistent nicotine abstinence-related social anxiety-like behavior in HRs, suggesting involvement of CB1R in nicotine-induced negative affect.

In conclusion, present findings implicate amygdalar CB1R in the development and maintenance of nicotine abstinence-related social anxiety-like behavior following a behaviorally-sensitizing nicotine regimen and suggest that changes in CB1R expression may contribute to perpetuation of nicotine relapse in vulnerable HRs. Further studies are in order to investigate a causal link between nicotine induced negative affect and the role of CB1R activation as well as the underlying neurotransmitter system(s) mediating this role in the novelty-seeking phenotype.

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Abbreviations

BLA	basolateral nucleus of the amygdala
CB1R	cannabinoid receptor 1
CeA	central nucleus of the amygdala
HR	high responder
LR	low responder
SI	social interaction

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Research Highlights

- Behavioral sensitization to nicotine is marked with increased social anxiety in HRs
- Behavioral effects of nicotine are long-lasting in HRs
- CB1R are implicated in nicotine abstinence-related social anxiety in HRs

Figure 1.

Total locomotor reactivity to four intermittent nicotine (0.35 mg/kg, s.c.) or saline (1.0 ml/kg, s.c.) training injections, with 3-d intervals (**A**), total locomotor reactivity to a low dose nicotine (0.1 mg/kg, s.c.) challenge following 1 wk (1 WK) or 3 wks (3 Wks) of injection-free period (**B**), and percent time spent interacting with the resident rat in the SI test following 1 wk (1 WK) or 3 wks (3 Wks) of injection-free period (**C**). Group means $\pm$ SEMs are plotted in line (**A**) and bar (**B**, **C**) graphs. In panel **A**, * represents significant effects in total locomotor reactivity to nicotine in HRs compared to saline-injected controls, whereas # represents significant effects in nicotine-injected LRs compared to saline-injected controls. Significance is set at $p = 0.005$.

Figure 2.

CB1R mRNA expression in the BLA and the CeA of a representative HR rat that is pre-trained with saline and underwent 1 wk of injection-free period (**A**), HR rat that is pre-trained with nicotine and underwent 1 wk of injection-free period (**B**), HR rat that is pre-trained with saline and underwent 3 wks of injection-free period (**C**), HR rat that is pre-trained with nicotine and underwent 3 wks of injection-free period (**D**). Panels **A**, **B**, **C** and **D** show images of coronal hemisections of the BLA and the CeA that were radioactively labeled with an antisense cRNA probe against CB1R and exposed on an x-ray film. Means of quantification results for integrated densities $\pm$ SEMs are plotted with bar graphs for the BLA (**E**) and the CeA (**F**; *: $p \leq 0.05$). Scale bar = 250 μ m