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Effects of a selective Y2R antagonist, JNJ-31020028, on nicotine abstinence-related social anxiety-like behavior, neuropeptide Y and corticotropin releasing factor mRNA levels in the novelty-seeking phenotype

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Abstract

An outbred rat model of novelty-seeking phenotype has predictive value for the expression of locomotor sensitization to nicotine. When experimentally naïve rats are exposed to a novel environment, some display high rates of locomotor reactivity (HRs, scores ranking at top 1/3rd of the population), whereas some display low rates (LRs, scores ranking at bottom 1/3rd of the population). Basally, HRs display lower anxiety-like behavior compared to LR rats along with higher neuropeptide Y (NPY) mRNA in the amygdala and the hippocampus. Following an intermittent behavioral sensitization to nicotine regimen and 1 wk of abstinence, HRs show increased social anxiety-like behavior in the social interaction test and robust expression of locomotor sensitization to a low dose nicotine challenge. These effects are accompanied by a deficit in NPY mRNA levels in the medial nucleus of the amygdala and the CA3 field of the hippocampus, and increases in Y2R mRNA levels in the CA3 field and corticotropin releasing factor (CRF) mRNA levels in the central nucleus of the amygdala. Systemic and daily injections of a Y2R antagonist, JNJ-31020028, during abstinence fully reverse nicotine-induced social anxiety-like behavior, the expression of locomotor sensitization to nicotine challenge, the deficit in the NPY mRNA levels in the amygdala and the hippocampus, as well as result an increase in Y2R mRNA levels in the hippocampus and the CRF mRNA levels in the amygdala in HRs. These findings implicate central Y2R in neuropeptidergic regulation of social anxiety in a behavioral sensitization to nicotine regimen in the LRHR rats.

Keywords

hippocampus; medial amygdala; central nucleus of amygdala; behavioral sensitization; negative affect; Y2R antagonist

1. Introduction

We have previously shown that the novelty-seeking phenotype in the outbred rat has a predictive value for individual differences in the expression of locomotor sensitization to nicotine [2, 5, 6]. The phenotype is determined in an experimentally-naïve outbred

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population of rats by the level of locomotor reactivity to a novel environment. Some rats exhibit high locomotor response to novelty (i.e., top 1/3rd of the population) and are identified as high responders, HRs; whereas some display low locomotor response to novelty (i.e., bottom 1/3rd of the population) and are identified as low responders, LR. Chronic intermittent nicotine training followed by 1 wk of abstinence and a low dose nicotine challenge results in robust expression of locomotor sensitization to nicotine in HRs but not LR, and this behavior is associated with distinct, phenotype-specific morphological and neurochemical alterations [2, 5, 6], validating this model as a tool for studying individual differences in behavioral and neural mechanisms that mark vulnerability to nicotine.

Present study aims to further elaborate on behavioral and neurobiological underpinnings of such vulnerability to nicotine in the HR rats with an emphasis on identifying therapeutic molecular targets. Withdrawal from drugs of abuse has been associated with a negative affective state such as anxiety, which may significantly contribute to maintenance of drug addiction [23]. In the laboratory rats, psychostimulant withdrawal-induced anxiety-like behavior is shown to involve augmentation of the brain stress system mediated by CRF in the central nucleus of the amygdala [CeA; 9, 27, 37]. Furthermore dysregulations in NPY signaling have been reported during development of dependence in the amygdala [27], where NPY and CRF produce opposing actions to conserve a balanced emotional state [15]. Although alterations in central NPY signaling in anxiety-like behavior following alcohol withdrawal has been extensively studied [12, 34, 35, 47, 49, 54], the role of NPY signaling in psychostimulant, specifically nicotine, abstinence-related negative affect is largely unexplored. We have recently shown that intermittent nicotine training followed by 1 wk of abstinence induces social anxiety-like behavior in HRs and a deficit in NPY signaling in the hippocampus and the amygdala along with increased CRF mRNA levels in the CeA [2]. Our findings are in confirmation of another report showing that nicotine abstinence leads to a long-lasting increase in anxiety-like behavior and a decrease in ratio of NPY to CRF in the amygdala [46]. All together, these findings suggest that neurobehavioral changes associated with nicotine exposure may be related to allostatic changes in stress (e.g., CRF) and anti-stress (e.g., NPY) neuropeptide systems [46]. In addition to amygdala, hippocampal NPY is also implicated in anxiolytic effects in traditional anxiety paradigms such as the elevated plus maze (EPM) [48]. In our hands, the hippocampus is implicated in the expression of behavioral sensitization to nicotine in the HR rats [5, 6], and a deficit in the hippocampal NPY is shown following intermittent nicotine training and 1 wk of abstinence in the HR phenotype [2]. However, a causal relationship between the nicotine-associated neuropeptidergic (i.e., NPY and CRF) changes and abstinence-related anxiety-like behavior has not been established.

The biological effects of NPY are mediated by five classes of receptors with Y1 and Y2 being the major subtypes expressed in the rat brain [20]. The postsynaptic Y1 receptor is found in brain regions such as the hippocampus, the hypothalamus, and the amygdala where its activation is associated with neurobiological responses to ethanol [33, 42] and psychostimulants [19, 39]. Unlike Y1 receptors, Y2 receptors are located presynaptically and support a virtual antagonism to Y1 receptors [36]. Activation of Y2 receptors on NPY-ergic terminals inhibits release of endogenous NPY [22], hence decrease availability of NPY in the synaptic cleft. Originally, Nakajima et al. demonstrated anxiogenic-like effects of Y2 receptors in the EPM [32]. Antagonizing central Y2 receptors results in decreased operant responding for ethanol in animals with a history of alcohol dependence, and reverses the anxiogenic effects of withdrawal from a single dose of alcohol on the EPM [38, 49, 10]. Unlike Y1 receptors, Y2 receptors have not been studied within the context of behavioral effects of psychostimulants.

Present study investigates the effects of daily systemic administration of a novel, brain-penetrant Y2 receptor antagonist, JNJ-31020028, during 1 wk of abstinence in an intermittent behavioral sensitization to nicotine regimen on anxiety-like profile and locomotor sensitization to the low dose nicotine challenge in the LRHR rats, and subsequent neuropeptidergic effects on the CRF and the NPY (Y1R, Y2R) levels in the amygdala and the hippocampus. We hypothesize that the expression of locomotor sensitization to nicotine develops in the HR phenotype in conjunction with emergence of social anxiety-like behavior, a dysregulation in the amygdalar stress (e.g., CRF) and anti-stress (e.g., NPY) systems, along with a deficit in the Y2R in the hippocampus. Furthermore, Y2R antagonist is hypothesized to reverse these behavioral and neuropeptidergic adaptations in the nicotine vulnerable HR phenotype. We will use the novel Y2R antagonist agent, JNJ-31020028, which is recently described as selective against Y1, Y4 and Y5 receptors with the ability to penetrate into the brain and dose dependently occupy Y2R binding sites upon systemic delivery [44].

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. The Y2R antagonist, JNJ-31020028, was generously donated by Johnson & Johnson Pharmaceutical Research and Development, L.L.C., and dissolved in 20% 2-hydroxypropyl-beta-cyclodextrin.

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. A grand total of 234 male Sprague-Dawley rats (Charles River, Wilmington, MA) arrived at weaning (postnatal day, PN 22), were housed 3 per cage in 43 X 21.5 X 25 cm³ 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 a novel environment for 1 hr using commercially available locomotion chambers (San Diego Instruments, San Diego, CA). Briefly, locomotor reactivity to novelty was tested in 43 X 43 X 24.5-cm³ (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=78) or LR (i.e., rats that exhibited locomotor scores in the lowest third of the sample n=78).

2.3. Anxiety paradigms

Animals were individually tested on all three anxiety tests (5 min each), and the order of testing was counterbalanced.

2.3.1. Elevated plus maze (EPM)—The apparatus was constructed of black-painted Plexiglas, with four elevated arms (70 cm from the floor, 45-cm long and 12-cm wide). The arms were arranged in a cross, with two opposite arms being enclosed by 45-cm high walls. The two other arms were open, having at their intersection a central 12 X 12 cm square

platform giving access to all arms. The illumination above the central platform was 85lux. Each rat was placed in the central square facing an open arm, and the time spent (with the four paws) in every arm, number of open arm entries and total number of arm entries were recorded for 5 min.

2.3.2. Light dark box (LDB)—The test was conducted in a 30 X 60 X 30 cm Plexiglas shuttle box that was divided into two equal size compartments by a wall with an open door. One compartment was brightly illuminated while the other compartment was painted black with very dim light. The rats' locomotor activity and time spent in each compartment were monitored for 5 min.

2.3.3. Social interaction test (SI)—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.

2.4. Experiment 1: Baseline LRHR differences in anxiety-like behavior, mRNA expressions of CRF, NPY, Y1R and Y2R in the amygdala and the hippocampus

Thirty-six male Sprague-Dawley rats (PN 22), following phenotype screening into LR and HRs (n= 12/group) as described above, were allowed to rest for 22 days to age match the animals used in Experiment 2, handled twice a week and on PN 44 were tested on the LDB and the SI tests as described above; and upon completion of testing, were rapidly decapitated. Brain tissue was harvested, sectioned and processed for *in situ* hybridization histochemistry as described below.

2.5. Experiment 2: Effects of systemic Y2R antagonism on nicotine-related behavioral and neuropeptidergic adaptations in the LRHR rats

One hundred and forty four male Sprague-Dawley rats (PN 22) were allowed to rest until PN 28 after phenotype screening, and were assigned to saline (1 ml/kg; s.c.) or nicotine (0.35 mg/kg; s.c.) training injection groups. On injection days, rats were given 1 hr 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 training injection, rats were further assigned to vehicle (1 ml/kg, i.p.) or JNJ-31020028 (20 mg/kg, i.p.) therapeutic injection groups, and underwent 1 wk of nicotine-free period where they received daily vehicle or JNJ-31020028 injections. At the end of the abstinence period, all LRHR rats were challenged either with saline (1.0 ml/kg, s.c.) or with a low dose of nicotine (0.1 mg/kg, s.c.), and their locomotor response was monitored for 45 min. Experimental groups and corresponding sample sizes are summarized in Table 1. Upon completion of the challenge session, all animals were tested in 5 min sessions on the EPM, LDB and SI tests for assessing anxiety-like behavioral profile as described above. Once testing was concluded, animals were rapidly decapitated and brain tissue was harvested for *in situ* hybridization histochemistry.

2.6. In situ hybridization histochemistry

Brains tissue was 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 hr,

followed by three washes in 2x SSC (1x 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, Y1R, Y2R and CRF were antisense linearized by using the restriction enzymes Hind III, Sal I, BamH I and Xba I respectively; transcribed by using T3 RNA polymerase (NPY and Y1R) or T7 RNA polymerase (Y2R and CRF), and were ³⁵S labeled separately in reaction mixtures consisting of 1 ml of linearized plasmid, 1x transcription buffer, 125 mCi [³⁵S]UTP, 125 mCi [³⁵S]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, 2x SSC, 50 mM sodium phosphate buffer, pH 7.4, 1x Denhardt's solution, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol) to yield 10⁶ dpm/70 ml. Sections were hybridized with probe mixture inside a humidified chamber over night at 55°C. Next day, sections were washed in 3x SSC for 5 min each, incubated for 1 hr in RNAase (20 mg/ml in Tris buffer containing 0.5 M NaCl, pH 8) at 37°C. Sections were washed with 2x, 1x and 0.5x SSC, and incubated for 1 hr in 0.1x 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 3.5X 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 NPY, Y1 and Y2 receptors were quantified in the dorsal hippocampus, the medial nucleus of the amygdala (MeA) and the basolateral amygdala (BLA). CRF mRNA was quantified in the CeA. Optical density values were corrected for background and averaged to produce one data point for each brain region for each animal. Integrated density values were calculated from optical density measures by multiplying signal by area and averaged per group and compared statistically.

2.7. Corticosterone (CORT) levels to acute exposures of SI and EPM

In order to assess if there are differences in the intensity of stress imposed by the SI test compared to a spontaneous exploration task such as EPM, CORT responses to an acute exposure of the two anxiety paradigms are examined. Fifty-four male Sprague-Dawley rats (PN 38) were allowed to rest until PN 44 following phenotype screening to age-match the animals used in Experiment 2 and were assigned to SI or EPM exposure groups (n= 7–9/group). On the testing day, animals were transferred into the testing area where they were subjected to a 5-min SI or EPM exposure as described in 2.3. Blood samples were collected by tail vein nick at 0, 15, 30, 45 and 60 min from the onset of anxiety testing. Blood samples were separated by centrifugation; plasma was removed and stored at –80 °C until assayed. Plasma CORT levels were determined by using a corticosterone enzyme linked immunoassay kit (Assay Designs, Ann Arbor, MI) according to manufacturer's specifications. The level of non-specific binding was 2% and the sensitivity was 22 pg/mL.

2.8. Statistical analyses

Baseline data on measures of anxiety-like behavior (percent time spent in light compartment, LDB; percent time spent in social interaction, SI) and mRNA levels in Experiment 1 were assessed by two-tailed t-tests. Locomotor reactivity to nicotine training in Experiment 2 and plasma CORT concentrations were analyzed by repeated-measures ANOVAs: Phenotype (LR, HR) X Pre-training (SAL, NIC) X Injection Days (INJ 1, INJ 2, INJ 3, INJ 4) and Phenotype (LR, HR) X Time Points (0, 5, 15, 30, 45, 60), respectively. Data pertaining to locomotor reactivity to challenge nicotine and behavioral measures on

anxiety tests in Experiment 2 were analyzed by four-way ANOVAs: Phenotype (LR, HR) X Pre-training (SAL, NIC) X Therapy (Y2, VEH) X Challenge (SAL, NIC). Significant main effects and interactions were followed by post-hoc comparisons, where significance was set at $p = 0.05$. The mRNA quantification results in Experiment 2 were also analyzed by four-way ANOVAs: Phenotype (LR, HR) X Pre-training (SAL, NIC) X Therapy (Y2, VEH) X Challenge (SAL, NIC) and significant main effects and interactions were followed by post-hoc comparisons using the Bonferroni/Dunn corrections.

3. Results

3.1. Experiment 1: Baseline LRHR differences in anxiety-like behavior, mRNA expressions of CRF, NPY, Y1R and Y2R in the amygdala and the hippocampus

Figure 1 shows differences in anxiety-like behavior in the SI (*G*) and LDB (*H*) tests respectively, in the LRHR animals. Significant phenotype differences were observed in anxiety-like behavior in the SI test [$t = 6.57, p = 0.016$], where HRs spent more time interacting with the resident rat; and in the LDB [$t = 5.368, p = 0.037$], where HRs spent significantly more time in the light compartment compared to LRs.

Figure 1 also demonstrates expressions of NPY mRNA in the CA1 and CA3 fields of the hippocampus (*A*), in the MeA and the BLA (*C*), and Y1R mRNA in the CA1 and CA3 fields of the hippocampus (*E*) in the LRHR rats. Significant phenotype differences were observed in NPY mRNA in the CA1 [$t = 6.43, p = 0.032$] and CA3 [$t = 8.12, p = 0.019$] fields of the hippocampus, as well as in MeA [$t = 10.14, p = 0.011$] and the BLA [$t = 8.30, p = 0.0418$]. Compared to LRs, HR animals expressed higher levels of NPY mRNA in the above mentioned brain regions. Significant phenotype differences were also observed in Y1R mRNA levels in the CA1 [$t = 8.19, p = 0.019$] and the CA3 fields [$t = 19.37, p = 0.002$] of the hippocampus where, HRs expressed lower levels of Y1R mRNA compared to LRs. Y2R mRNA in the hippocampus and amygdala did not show significant differences between phenotypes (data not shown).

3.2. CORT levels to acute exposures of SI and EPM

Plasma CORT concentrations in response to an acute 5 min SI or EPM exposure in the LRHR animals are shown in Figure 2D. Repeated measures ANOVA revealed a significant main effect of phenotype [LR, HR; $F = 8.72, p = 0.008$]. Specific post-hoc comparison showed that while there were no significant differences in plasma CORT levels between the SI and EPM tests within or between phenotypes at 0, 15, 30 and 45 min time points, HRs exposed to EPM had lower plasma CORT levels at 60 min following the onset of testing compared to LR animals [$p = 0.030$].

3.3. Experiment 2: Effects of systemic Y2R antagonism on nicotine-related behavioral and neuropeptidergic adaptations in the LRHR rats

Figure 2 shows total locomotor reactivity to four intermittent nicotine or saline training injections (*A*) and the subsequent low dose nicotine challenge following 1 wk of nicotine-free period in LRs and HRs (*B*). Repeated-measures ANOVA revealed a significant interaction between Phenotype (LR, HR) and Pre-training [SAL, NIC; $F = 12.75, p = 0.0006$]; and significant main effects of Phenotype [LR, HR; $F = 42.31, p = 0.0001$] and Pre-training [SAL, NIC; $F = 68.15, p = 0.0001$]. Specific post-hoc comparisons showed that at all injection days, HRs that received nicotine training exhibited higher locomotor reactivity compared to saline pre-trained controls, while in LRs such nicotine-induced elevations in locomotor reactivity were observed on INJ 3 [$p = 0.05$] and on INJ 4 [$p = 0.001$] compared to saline pre-trained controls. Post-hoc comparisons further showed that nicotine pre-training resulted in increased locomotor reactivity to the low dose nicotine

challenge compared to saline pre-trained controls in HRs [$p = 0.041$] but not in LR, and daily Y2R antagonist treatment during the nicotine-free period reversed this effect in nicotine pre-trained HRs back to levels observed in saline pre-trained controls [$p = 0.014$].

Figure 2 also shows percent time the experimental animals spent interacting with the resident rats in a social context (C). A four-way ANOVA revealed significant interactions between Phenotype (LR, HR), Challenge (SAL, NIC) and Therapy [Y2, VEH; $F = 7.53$, $p = 0.007$], and between Pre-training (SAL, NIC) and Therapy [Y2, VEH; $F = 64.78$, $p = 0.031$]. Specific post-hoc comparisons showed a significant phenotype difference in percent time spent in social interaction, where HRs that are saline pre-trained, vehicle-treated and saline challenged spent significantly more time interacting with the resident rat compared to LR counterparts [$p = 0.036$]. Moreover, nicotine pre-training and a low dose nicotine challenge resulted in decreased percentage of time spent in social interaction in HRs compared to saline pre-trained controls [$p = 0.033$], and this nicotine-induced social anxiety-like behavior was fully reversed by the Y2R antagonist treatment in HRs back to levels observed with saline pre-trained controls [$p = 0.0008$]. No significant effects were observed in anxiety-like behavior in the LDB or EPM test (data not shown).

Expression of NPY mRNA in the hippocampus is shown in Figure 3A–C. Four-way ANOVAs showed a significant interaction between Phenotype (LR, HR), Pre-training (SAL, NIC), Challenge (SAL, NIC) and Therapy [Y2, VEH; $F = 5.66$, $p = 0.020$] in NPY mRNA levels in the CA3 field of the hippocampus. Significant interactions were also observed between Phenotype (LR, HR), Therapy (Y2, VEH) and Pre-training [SAL, NIC; $F = 6.10$, $p = 0.016$], between Phenotype (LR, HR) and Therapy [Y2, VEH; $F = 6.24$, $p = 0.015$] and between Phenotype (LR, HR) and Pre-training [SAL, NIC; $F = 14.65$, $p = 0.0003$] in the same analysis. Post-hoc comparisons showed that nicotine pre-trained HRs exhibited a downregulation in NPY mRNA levels in the CA3 (D) field of the hippocampus compared to saline pre-trained controls [$p = 0.0003$], and this effect was reversed with Y2R antagonist treatment back to levels seen with saline pre-trained controls [$p = 0.0001$]. No significant differences were detected in NPY mRNA levels in the CA1 and the dentate gyrus (data not shown).

NPY mRNA expression in the MeA and the BLA in LRHR rats is shown in Figure 4A–C. A four-way ANOVA revealed a significant interaction between Phenotype (LR, HR), Pre-training (SAL, NIC), Challenge (SAL, NIC) and Therapy [Y2, VEH; $F = 4.54$, $p = 0.037$] in NPY mRNA levels in the MeA. The same analysis also showed significant interactions between Therapy (Y2, VEH) and Challenge [SAL, NIC; $F = 27.30$, $p = 0.0001$], between Phenotype (LR, HR) and Therapy [Y2, VEH; $F = 6.15$, $p = 0.016$], and between Phenotype (LR, HR) and Pre-training [SAL, NIC; $F = 13.95$, $p = 0.0004$]. Along with significant interactions, significant main effects of Phenotype [LR, HR; $F = 7.38$, $p = 0.008$], Pre-training [SAL, NIC; $F = 7.38$, $p = 0.008$] and Challenge [SAL, NIC; $F = 18.16$, $p = 0.0001$] were also detected in NPY mRNA in the MeA. Post-hoc comparisons showed that following 1 wk of abstinence, nicotine pre-trained HRs had downregulation in NPY mRNA levels in the MeA compared to saline pre-trained controls [D , $p = 0.0001$]. Moreover, post-hoc comparisons also showed that the observed downregulation in NPY mRNA expression in the MeA in nicotine pre-trained HRs was normalized back to saline pre-trained control levels with the Y2R antagonist treatment [$p = 0.0002$]. No significant effects were detected in NPY mRNA levels in the BLA (data not shown).

Figure 5A–C shows Y2R mRNA expression in the CA3 field of the hippocampus in nicotine or saline pre-trained animals that received Y2R antagonist or vehicle treatment during the nicotine-free period. A four-way ANOVA revealed a significant interaction between Phenotype (LR, HR), Pre-training (SAL, NIC), Challenge (SAL, NIC) and Therapy [Y2,

VEH; $F=6.88$, $p=0.011$). Moreover, significant interactions were observed between Pre-training (SAL, NIC), Phenotype (LR, HR) and Therapy [Y2, VEH; $F=6.88$, $p=0.011$], between Challenge (SAL, NIC), Therapy (Y2, VEH) and Pre-training [SAL, NIC; $F=10.05$, $p=0.002$], between Phenotype (LR, HR) and Therapy [Y2, VEH; $F=4.41$, $p=0.039$], between Pre-training (SAL, NIC) and Therapy [Y2, VEH; $F=8.40$, $p=0.0001$], and between Pre-training (SAL, NIC) and Phenotype [LR, HR; $F=8.57$, $p=0.005$]. A significant main effect of Phenotype [LR, HR; $F=4.33$, $p=0.041$] was also observed in Y2R mRNA in the CA3 field of the hippocampus. Specific post-hoc comparisons showed that a chronic intermittent nicotine regimen resulted in an upregulation in Y2R mRNA levels in the CA3 field of the hippocampus in HRs compared to saline pre-trained controls [Figure 5D, $p=0.0001$], which was in turn reversed by the Y2R antagonist treatment during the nicotine-free period [$p=0.0001$]. No significant effects were detected in the Y1 mRNA levels in the hippocampus (data not shown).

Effects of nicotine pre-training and subsequent Y2R antagonist treatment on the CRF mRNA expression in the CeA are shown in Figure 6A–C. A four-way ANOVA revealed a significant interaction between Phenotype (LR, HR), Pre-training (SAL, NIC), Challenge (SAL, NIC) and Therapy [Y2, VEH; $F=5.16$, $p=0.026$]. Moreover, significant interactions were observed between Pre-training (SAL, NIC), Phenotype (LR, HR) and Challenge [SAL, NIC; $F=5.04$, $p=0.028$], between Phenotype (LR, HR), Therapy (Y2, VEH) and Pre-training [SAL, NIC; $F=24.85$, $p=0.0001$], between Phenotype (LR, HR) and Therapy [Y2, VEH; $F=17.28$, $p=0.0001$], between Pre-training (SAL, NIC) and Therapy [Y2, VEH; $F=38.64$, $p=0.0001$], between Pre-training (SAL, NIC) and Phenotype [LR, HR; $F=19.31$, $p=0.0001$], and between Pre-training (SAL, NIC) and Challenge [SAL, NIC; $F=6.27$, $p=0.015$]. Significant main effects of Phenotype [LR, HR; $F=50.93$, $p=0.0001$], Pre-training [SAL, NIC; $F=21.47$, $p=0.0001$] and Challenge [SAL, NIC; $F=5.72$, $p=0.020$] were also observed in CRF mRNA levels in the CeA. Post-hoc comparisons showed that a behaviorally-sensitizing nicotine regimen resulted in augmented CRF mRNA expression in the CeA in HRs compared to saline pre-trained controls [Figure 6D, $p=0.0001$], which was fully reversed back to levels observed in saline pre-trained controls following the Y2R antagonist treatment [$p=0.0001$].

4. Discussion

In basal, drug-free condition, HRs but not LRs show inhibited anxiety-like behavior in the social interaction and the light/dark box tests. Experimentally naïve HRs also show increased NPY mRNA levels in the CA1 and CA3 fields of the hippocampus and the MeA, and decreased Y1R mRNA levels in both pyramidal cell fields of the hippocampus. Interestingly, following an intermittent nicotine training regimen and 1 wk of abstinence, HRs exhibit locomotor sensitization to a low dose nicotine challenge together with increased social anxiety-like behavior in the social interaction test compared to saline pre-trained controls. The phenotype selective switch from an anxiolytic to an anxiogenic state on the social interaction test, an index of anxiety induced in a social context, with a behaviorally-sensitizing regimen of nicotine in the HRs is accompanied by a downregulation of NPY mRNA levels in the CA3 field of the hippocampus and the MeA and an upregulation of CRF mRNA levels in the CeA compared to saline pre-trained controls. Hence a link is established between NPY deficit in the hippocampus and the amygdala together with increased CRF levels in the CeA with the emergence of social anxiety-like behavior following nicotine training and abstinence in the HR phenotype. Additionally, an upregulation of the Y2R mRNA levels is also observed in the CA3 field of the hippocampus with the same training and abstinence regimen in the HRs, implicating this autoreceptor in the nicotine-induced regulation of the hippocampal NPY. Furthermore, daily systemic injections of a potent selective Y2R antagonist, JNJ-31020028, during the abstinence reverse

challenge nicotine-induced hyperlocomotion and abstinence-related social anxiety-like behavior in nicotine pre-trained HRs to the levels displayed in saline pre-trained controls. This treatment regimen also reverses the nicotine-induced deficit in NPY levels in the amygdala and the hippocampus as well as the upregulation of Y2R mRNA levels in the CA3 field of the hippocampus in the nicotine pre-trained HRs to the levels observed in saline pre-trained controls. In addition to reversing nicotine-induced NPY deficit, the Y2R antagonist treatment also results in a downregulation of the CRF mRNA in the CeA in nicotine pre-trained HRs back to the levels seen in saline pre-trained controls. Hence an opposite regulatory relationship may exist between the brain stress (i.e., CRF) and anti-stress (i.e., NPY) systems in the HR amygdala that appears critical for the modulation of negative affect associated with abstinence in the HR phenotype.

4.1. Effects of Y2R antagonist on abstinence-related anxiety and expression of behavioral sensitization to nicotine

Our data show that HRs express locomotor sensitization to a low dose nicotine challenge along with increased social anxiety-like behavior following four intermittent nicotine training injections and 1 wk of nicotine-free period, both of which are reversed by daily systemic Y2R antagonist treatment during abstinence. Clinical studies have shown that in humans, one of the manifestations of behavioral sensitization to psychostimulants such as cocaine [43] is a progressive increase in anxiety [18] that can result in social pathologies [11, 29] which can be precipitated by a single drug use long after discontinuation of the drug [40, 41]. These findings indicate a distinct role for social anxiety in behaviorally sensitizing effects of psychostimulants. Moreover, studies on rodents have also shown that following a chronic nicotine regimen and a subsequent withdrawal, rats exhibit increased anxiety-like behavior in the social interaction test [16, 17], suggesting a role for social anxiety in nicotine withdrawal. Our results concur that although in basal conditions HRs manifest an inhibited anxiety profile in the SI and the LDB tests compared to LR, following the intermittent behavioral sensitization regimen, they exhibit increased challenge-induced anxiety-like behavior only in the SI test. Moreover, plasma CORT assessments showed that acute (5 min) exposure to the EPM or SI tests result in comparable physiological CORT response within each phenotype across time, suggesting that these two anxiety paradigms are equally stressful and differences in nicotine-induced effects in anxiety may not be attributable to differences in stress induced by the two types of tests. However HR animals achieved a significant decrease in the CORT response at the 60 min time point compared to LR in the EPM test, indicating a phenotype difference in the shutting down of the CORT response in the EPM as opposed to SI exposures. Overall, these findings suggest that a distinct aspect of negative affect, that of anxiety induced by a social context, may be critical in the course of developing expression of behavioral sensitization to nicotine in the HR phenotype. Our mRNA expression assessments revealed that NPY deficit, possibly through a Y2R mediated pathway, may be implicated in the abstinence-related social anxiety-like behavior in the HR phenotype. In this vein, systemic antagonization of the Y2 receptors with a potent selective Y2R antagonist during the 1 wk of abstinence was effective in fully reversing social anxiety-like behavior and the expression of locomotor sensitization to challenge nicotine in HRs. These findings assign a critical role for the Y2R as a novel therapeutic drug target for reversing nicotine-induced negative affect. Several studies in the literature have shown involvement of Y2R in regulation of anxiety-like behavior in rodents, albeit none of these were drug-induced anxiety paradigms. For example, i.c.v. injections of NPY13-36, a Y2R agonist, resulted in increased anxiety-like behavior in the EPM [32], whereas injections of a Y2R antagonist, BIIE0246, resulted in decreased anxiety-like behavior in the same test [3], suggesting that deactivating central Y2R reduces anxiety-like behavior in rodents. Our behavioral findings constitute the first evidence showing that antagonizing Y2R during nicotine abstinence reduces social anxiety-like behavior and vulnerability to the behavioral

effects of nicotine; hence identifying this receptor as a drug target for alleviating nicotine-associated behavioral pathology.

Unlike BIIE-0246 which is administered intracerebrally, JNJ-31020028, a novel, selective Y2R antagonist used in this study, can cross the blood brain barrier upon systemic delivery [44]. Microdialysis studies showed that JNJ-31020028 significantly increases norepinephrin (NE) release in the hypothalamus, where BIIE0246 has been previously shown to increase NE release from synaptosomes [7]. The maximum effect in hypothalamic NE release is seen 30 min after systemic delivery at both 10 and 20 mg/kg doses [44]. Moreover, at both doses, JNJ-31020028 decreases plasma CORT levels in stressed animals; and at 20 mg/kg dose, significantly increases food intake in stressed animals compared to vehicle treated counterparts [44], suggesting that 20 mg/kg dose of JNJ-31020028 administration is able to regulate CORT-dependent effects of NPY on food intake. On the other hand, injections of JNJ-31020028 did not result in anxiolytic activity in rats with no previous exposure to stressful stimuli in several anxiety-like behavior tests [44]. Although, these results seem controversial considering that NPY is an anxiolytic neuropeptide, they may be explained by the findings of another study showing that during baseline conditions, NPY overexpressing rats do not differ in their anxiety state from the wild type controls in the EPM, but when the EPM test is preceded by a 1 hr stress session they exhibit anxiolytic-like behavior compared to wild type counterparts [48]; suggesting that NPY effects are activity-dependent. Our data are in agreement with this assessment in that Y2R antagonist treatment results in behavioral effects in the social anxiety-like paradigm in response to the low dose nicotine challenge following repeated nicotine but not saline training in the HR animals, suggesting that the efficacy of JNJ-31020028 on anxiety-like behavior depends on the prior drug state of the HR animal. In this study, we selected the 20 mg/kg dose to ensure the drug's efficacy to maximally occupy central Y2R that has been shown to occur in a dose-dependent manner, where JNJ-31020028 almost completely inhibits [¹²⁵I]PYYY labeling in hippocampus as demonstrated via *in vitro* receptor autoradiography [44]. Indeed, in our hands, daily treatment with the 20 mg/kg dose of JNJ-31020028 during abstinence did result in full reversal of social anxiety-like behavior and the expression of locomotor sensitization to nicotine, confirming the ability of this drug dose to effectively yield anxiolytic behavioral influence corresponding with increases in the NPY mRNA levels in the amygdala and the hippocampus of the behaviorally responsive animals.

4.2. Effects of Y2R antagonist on hippocampal NPY

Our results showed that while basally HRs express higher levels of the NPY mRNA in the CA1 and CA3 fields of the hippocampus compared to LRs, following the behavioral sensitization regimen, they show enhanced expression of the Y2R mRNA levels and downregulated NPY mRNA levels in the CA3 field of the hippocampus. Moreover, daily Y2R antagonist treatment during abstinence not only reverses the behavioral effects of nicotine observed in nicotine pre-trained HRs but also normalizes hippocampal NPY and Y2R mRNA levels. Y2 receptors, within the hippocampus, are predominantly expressed at the mossy fiber terminals comprised of axons originating from the dentate granule neurons and synapsing onto the CA3 cells, [21, 31, 45, 53]. Mossy fiber sprouting following acute pilocarpine-induced epileptic seizures is accompanied by activation of the presynaptic Y2 receptors and reduced probability of glutamate release [51, 52], suggesting that NPY activity at the mossy fiber terminals may regulate glutamate release in a Y2R-dependent manner. We have previously shown that the identical behavioral sensitization to nicotine regimen used in this study results in increased mossy fibre terminal field size in HR but not LR rats compared to saline pre-trained controls [5]. Present findings demonstrate that nicotine-induced mossy fiber sprouting in HRs is accompanied by upregulated Y2R mRNA expression in the CA3 field of the hippocampus, and perhaps through a similar

neuropeptidergic mechanism reported before [51, 52] could act to compensate for the level of excitation induced by this fibre pathway. By the same token, the same NPY mechanism in the hippocampus could simultaneously regulate anxiety-like behavior. Dorsal hippocampus is an important component of neuronal circuitry controlling anxiety-related behaviors [1, 14], where NPY overexpression is shown to induce an anxiolytic-like effect in the open field and the EPM tests [30]. Hence observed increase in abstinence-related social anxiety-like behavior in nicotine pre-trained HRs may be associated with an anxiogenic neuronal phenotype partly marked by a deficit in hippocampal NPY signaling. By the same token, blocking Y2R activation may disinhibit NPY activity in the hippocampus, which in turn could act to normalize the Y2R levels in the CA3 field. Correction of the NPY deficit in the hippocampus may significantly contribute to reversal of anxiogenic effects observed in the social anxiety-like behavior in nicotine pre-trained HRs, suggesting involvement of the hippocampal NPY signaling via a Y2R-mediated pathway in negative affect critical for the expression of behavioral sensitization to nicotine in the HR phenotype.

4.3. Effects of Y2R antagonist on amygdalar NPY and CRF

Identical to NPY effects observed in the hippocampus, HRs express higher basal NPY mRNA levels compared to LRs in the MeA, whilst a behaviorally-sensitizing nicotine regimen leads to downregulation of the NPY mRNA levels in this region in nicotine pre-trained HRs compared to saline pre-trained controls. Moreover, this nicotine regimen also induces an upregulation of the CRF mRNA levels in the CeA in HRs pre-trained with nicotine compared to saline pre-trained controls. Activation of the brain stress system is thought to be the key to the negative emotional state produced by dependence [26]. It has been shown that acute withdrawal from nicotine can produce an increase in circulating CORT [4], and sensitize CRF in the CeA, which is proposed to mark the withdrawal/negative affect stage of addiction [24, 25, 26, 28]. Nicotine abstinence has been shown to result in increased anxiety-like behavior in several tests which is associated with upregulated CRF signaling in the CeA [9, 13]. Moreover, brain anti-stress system, in particular NPY in the amygdala, may also be dysregulated during the development of dependence, which compromises a mechanism for restoring homeostasis [27]. It has been suggested that in a homeostatic state, NPY is released following an increase in CRF in the CeA and produce opposing actions to maintain a balanced emotional state [15]. Decreased amygdalar NPY to CRF ratio and increased anxiety-like behavior has been observed in nicotine exposed rats during withdrawal [46]. In line with these findings, our results showed upregulated CRF along with downregulated NPY mRNA levels in the amygdala that mark increased social anxiety-like behavior in response to a behaviorally-sensitizing nicotine regimen in HRs, suggesting a nicotine-induced imbalance between the CRF and the NPY in the amygdala. Systemic Y2R antagonist administration daily during abstinence reverses the deficit in the NPY mRNA expression in the MeA of HRs. As in the hippocampus, Y2R are predominantly located presynaptically at the NPY containing neurons in the amygdala [8, 50], and hence net effect of antagonizing them is possible enhancement of NPY signaling. Increase in NPY levels in the MeA in HRs following behavioral sensitization to nicotine regimen and Y2R antagonist treatment may indeed directly lead to dampened CRF response in the CeA in an attempt to restore the homeostatic balance as proposed previously [15, 27], stressing the regulatory relationship between the NPY and the CRF levels in the HR amygdala. Moreover, reestablishing the balance between the CRF and NPY levels in the amygdala may result in inhibition of social anxiety-like behavior in nicotine pre-trained HRs. These findings implicate Y2R deactivation during abstinence as a possible mechanism for correcting abstinence-associated social anxiety partly by restoring the balance between stress (i.e., CRF) and anti-stress (i.e., NPY) systems in the HR amygdala.

5. Conclusions

Present study constitutes the first report demonstrating the efficacy of a novel, selective Y2R antagonist, JNJ-31020028, in reversing the expression of behavioral sensitization to a low dose nicotine challenge and associated social anxiety-like behavior in the nicotine vulnerable HR rats. A switch from a basally anxiolytic neuronal phenotype to an anxiogenic one, identified with a hyperactive brain stress (CRF) and a hypoactive anti-stress/anti-anxiety (NPY) systems in the amygdala of the HR phenotype marks the nicotine-induced emergence of reported behavioral vulnerabilities. Moreover, a deficit in the NPY levels in the hippocampus possibly through a presynaptic Y2R-mediated pathway is also linked to the development of nicotine-induced behavioral effects. Our results assign a role for Y2R in neurocircuitry controlling nicotine-induced negative affect, and provide evidence that JNJ-31020028; a novel, brain penetrant Y2R antagonist, may support nicotine cessation by combating adverse effects of nicotine abstinence.

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Abbreviations

BLA	basolateral nucleus of the amygdala
CeA	central nucleus of the amygdala
CRF	corticotropin releasing factor
CORT	corticosterone
EPM	elevated plus maze
HR	high responder
LR	low responder
LDB	light dark box
MeA	medial nucleus of the amygdala
NPY	neuropeptide Y
SI	social interaction
Y1R	Y1 receptor
Y2R	Y2 receptor

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Figure 1.

Baseline phenotype differences in NPY mRNA expression in the hippocampus (**A, B**) and the amygdala (**C, D**), Y1R mRNA expression in the hippocampus (**E, F**), and baseline phenotype differences in anxiety-like behavior in the social interaction (**G**) and the light/dark box (**H**) tests. Panels **A, C** and **E** show images of representative coronal hemisections of the dorsal hippocampus that were radioactively labeled with antisense cRNA probes against NPY (**A, C**) and Y1R (**E**) mRNA and exposed on x-ray films in LRHR rats. Panel **B** shows NPY mRNA integrated density in the CA1 (**Top**) and the CA3 fields (**Bottom**) of the hippocampus between LRHR rats. Panel **D** shows NPY mRNA integrated density in the MeA (**Top**) and the BLA (**Bottom**) between LRHR rats. Panel **F** shows Y1R mRNA

integrated density in the CA1 (*Top*) and the CA3 fields (*Bottom*) of the hippocampus between LRHR rats. Panel *G* shows percent time spent in social interaction between LRHR rats. Panel *H* shows percent time spent in the light compartment of the light/dark box between LRHR rats. Means of quantification results for integrated density as well as behavioral measures $\pm$ SEMs are plotted with bar graphs (*: $p \leq 0.05$). Scale bar = 250 μm .

Figure 2. Total locomotor reactivity to four intermittent nicotine (0.35 mg/kg, s.c.) or saline (1.0 ml/kg, s.c.) training injections (**A**), total locomotor reactivity to a low dose nicotine (0.1 mg/kg, s.c.) or saline (1.0 ml/kg, s.c.) challenge (**B**), percent time spent interacting with the resident rat in the SI test (**C**), and plasma CORT concentrations in response to a 5 min SI or EPM test (**D**) are shown. Group means $\pm$ SEMs are plotted in line (**A**, **D**) 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 LR compared to saline-injected controls. In Panel **D**, * represents a significant effect at 60 min time point in plasma CORT levels induced by a 5 min EPM exposure in HRs compared to LR. Significance is set at $p = 0.005$.

Figure 3.

NPY mRNA expression in the hippocampus of a representative HR rat that is pre-trained with saline, treated with vehicle and challenged with nicotine (**A**), HR rat that is pre-trained with nicotine, treated with vehicle and challenged with nicotine (**B**) and HR rat that is pre-trained with nicotine, treated with Y2R antagonist and challenged with nicotine (**C**). Panels **A**, **B** and **C** show images of coronal hemisections of the hippocampus that were radioactively labeled with an antisense cRNA probe against NPY and exposed on an x-ray film. Means of quantification results for integrated density $\pm$ SEMs are plotted with the bar graph (**D**; *: $p \leq 0.0004$). Scale bar = 250 μ m.

Figure 4. NPY mRNA expression in the MeA and BLA of a representative HR rat that is pre-trained with saline, treated with vehicle and challenged with nicotine (**A**), HR rat that is pre-trained with nicotine, treated with vehicle and challenged with nicotine (**B**) and HR rat that is pre-trained with nicotine, treated with Y2R antagonist and challenged with nicotine (**C**). Panels **A**, **B** and **C** show images of coronal hemisections of the MeA and the BLA that were radioactively labeled with an antisense cRNA probe against NPY and exposed on an x-ray film. Means of quantification results for integrated density $\pm$ SEMs are plotted with the bar graph for MeA (**D**; *: $p \leq 0.0004$). Scale bar = 250 μ m.

Figure 5.

Y2R mRNA expression in the hippocampal CA3 field of a representative HR rat that is pre-trained with saline, treated with vehicle and challenged with nicotine (**A**), HR rat that is pre-trained with nicotine, treated with vehicle and challenged with nicotine (**B**) and HR rat that is pre-trained with nicotine, treated with Y2R antagonist and challenged with nicotine (**C**). Panels **A**, **B** and **C** show images of coronal hemisections of the hippocampus that were radioactively labeled with an antisense cRNA probe against Y2R mRNA, and exposed on an x-ray film. Means of quantification results for integrated density $\pm$ SEMs are plotted with the bar graph (**D**; *: $p \leq 0.0004$). Scale bar = 250 μ m.

Figure 6.

CRF mRNA expression in the CeA of a representative HR rat that is pre-trained with saline, treated with vehicle and challenged with nicotine (**A**), HR rat that is pre-trained with nicotine, treated with vehicle and challenged with nicotine (**B**) and HR rat that is pre-trained with nicotine, treated with Y2R antagonist and challenged with nicotine (**C**). Panels **A**, **B** and **C** show images of coronal hemisections of the CeA that were radioactively labeled with an antisense cRNA probe against CRF mRNA, and exposed on an x-ray film. Means of quantification results for integrated density $\pm$ SEMs are plotted with the bar graph (**D**, *: $p \leq 0.0004$). Scale bar = 250 μ m.

Table 1
Behavioral Sensitization and Therapeutic Administration

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 in Experiment 2 (PN 28–37) during the training phase of the behavioral sensitization to nicotine procedure. Following the last saline or nicotine injection, all animals underwent 1 wk of abstinence period, where they received daily vehicle (1ml/kg; i.p) or JNJ-31020028 (Y2R antagonist; 20 mg/kg; i.p) injections. Animals were challenged with either saline (1.0 ml/kg, s.c.) or a low dose nicotine (0.1 mg/kg, s.c.) on PN 44 (i.e., each cell showing the two challenge drug condition). Total number of LRHR animals used for Experiment 2 was 96, excluding intermediary responders that were eliminated after phenotype screening.

N=96		Training			
		LR		HR	
Therapeutics in Abstinence	Y2R ANT.	SAL (n=6)	NIC (n=6)	SAL (n=6)	NIC (n=6)
	VEH	SAL (n=6)	NIC (n=6)	SAL (n=6)	NIC (n=6)

INJ 1-4	Therapeutics	Challenge
Training	Abstinence	(PN 44)
(PN 28-37)	(PN 37-43)	