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Vulnerability to nicotine abstinence-related social anxiety-like behavior: Molecular correlates in neuropeptide Y, Y2 receptor and corticotropin releasing factor

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ABSTRACT

An outbred rat model of the novelty-seeking phenotype is used to study nicotine vulnerability, where experimentally naïve rats were phenotype screened as high or low responders (HRs or LRs, ranking in the upper or lower one-third of the population respectively) based on locomotor activity displayed in a novel environment. Following nicotine training and abstinence, HR animals pre-trained with nicotine showed expression of locomotor sensitization to nicotine challenge along with enhanced social anxiety-like behavior in the social interaction test compared to saline pre-trained controls. HR rats also showed a downregulation in neuropeptide Y (NPY) mRNA levels in the medial nucleus of amygdala and the CA1 field of the hippocampus, an upregulation in Y2 mRNA levels in the CA3 field of the hippocampus, and an upregulation in the corticotropin releasing factor (CRF) mRNA levels in the central nucleus of the amygdala. These findings implicate dysregulations in the NPY-CRF systems in the HR hippocampus and amygdala associated with the emergence of social anxiety-like behavior, and a novel Y2R-mediated pathway in nicotine relapse.

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The novelty-seeking phenotype is an outbred rat model of individual differences that has predictive value for nicotine vulnerability. Some rats display high locomotor reactivity in novel environments and are identified as high responders or HRs, whereas some display low locomotor reactivity in novel environments and are identified as low responders or LRs. HR rats are known to acquire nicotine self-administration more readily than LR rats and work more to obtain the drug when tested under the progressive ratio schedule of reinforcement [23]. Our laboratory reported that adolescent HRs develop locomotor sensitization to a low dose nicotine challenge following chronic intermittent nicotine training and 1 week of abstinence [1,2], and even a single nicotine training with a mild dose is sufficient for the expression of locomotor sensitization to a low dose challenge [1], further validating this phenotype in the study of individual differences in vulnerability to nicotine.

Nicotine withdrawal-associated anxiety has been hypothesized as an important factor contributing to smoking maintenance in humans [11], and is studied in rodents using several tests of anxiety-like behavior [4,15]. In the present experiment we assess whether repeated nicotine training in the nicotine vulnerable HR phenotype leads to the emergence of negative affect in the form of anxiety-like behavior in response to a low dose nicotine challenge following abstinence. We are particularly interested in anxiety measured on the social interaction test as this test is different from traditional indices of anxiety such as performance on the elevated plus maze (EPM) or the light/dark box (LDB) in that it measures a behavioral correlate of social anxiety akin to social phobia [5].

Repeated drug exposure and withdrawal mediate adaptations in the extended amygdala that is hypothesized to control the switch from homeostasis to pathophysiology associated with drug abuse [13]. Nicotine withdrawal following chronic nicotine exposure increases CRF release in the central nucleus of amygdala (CeA), which is shown to mediate defensive burying [6], suggesting that increased CRF function in the amygdala may underlie behavioral aspects of anxiety associated with nicotine abstinence [12]. In addition to CRF, hippocampal and amygdalar NPY also play an important role in the neurocircuitry controlling anxiety [8]. In the amygdala, NPY and CRF produce opposing actions to conserve a balanced emotional state with CRF being anxiogenic and NPY being anxiolytic [7]. Brain anti-anxiety system, in particular NPY in the amygdala, is dysregulated during the development of dependence, which com-

Abbreviations: BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; CRF, corticotropin releasing factor; DG, dentate gyrus; EPM, elevated plus maze; HR, high responder; LR, low responder; LDB, light dark box; MeA, medial nucleus of the amygdala; Nacc, nucleus accumbens; NPY, neuropeptide Y; SI, social interaction; VTA, ventral tegmental area.

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promises a mechanism for restoring homeostasis [14]. In line with this, rats exposed to chronic nicotine during adolescence show a decreased ratio of NPY to CRF immunoreactivity in the amygdala long after discontinuation of the drug [21]. Administration of NPY or a Y1 receptor agonist during acute nicotine withdrawal is sufficient to suppress somatic withdrawal signs [18]; however, unlike the Y1 receptors, Y2 receptors have not been previously implicated in psychostimulant taking behavior.

Present study investigates the regulation of the brain stress (e.g., CRF) and its opponent anti-stress/anti-anxiety (e.g., NPY) systems following an intermittent behavioral sensitization to nicotine regimen in the LRHR phenotype. We assess if there are phenotype-specific alterations in mRNA levels of CRF, NPY and its receptors Y1 and Y2 in the hippocampus and the amygdala following an intermittent behavioral sensitization to nicotine regimen in the LRHR rats. The central hypothesis is that the emergence of a nicotine-induced dysregulation in the CRF and NPY systems in the HR rats is associated with the development of social anxiety-like behavior during nicotine abstinence.

Fifty-four male Sprague–Dawley rats (Charles River, Wilmington, MA) arrived at weaning (postnatal day, PN 22), were housed 3 per cage 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 for 60 min using commercially available, square-shaped locomotion chambers (San Diego Instruments, San Diego, CA). 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. Total locomotor activity (i.e., *X*, *Y*, and *Z* locomotion) was pooled and the rats were ranked as HRs [i.e., rats with scores in the highest third of the sample, $n = 18$, mean = 3254.27 ± 136.61] or LRs [i.e., rats with scores in the lowest third of the sample, $n = 18$, mean = 1404.94 ± 97.56]. Following phenotype screening, rats were assigned to saline (1 ml/kg; s.c.; $n = 9$) or nicotine (0.35 mg/kg; s.c.; $n = 9$) training groups. Nicotine hydrogen (Sigma) was dissolved in 0.9% NaCl and the pH was adjusted to 7.4 using NaOH. On injection days, rats were given 60 min 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-day interval. Following 1 week of abstinence, on PN 44, all rats were challenged with a low dose of nicotine (0.1 mg/kg; s.c.) and their locomotor response was monitored for 45 min. Subsequently, all animals were tested on the LDB, EPM and the social interaction (SI) tests with the order of tests presented in a randomized fashion. Animals returned to their home cages between each test and were allowed to rest.

LDB: The test was conducted in a 30 cm × 60 cm × 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 (60 lx) while the other compartment was painted black with very dim light. Time spent in each compartment-having the four paws on the same side-was monitored for 5 min.

EPM: The apparatus is 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 cm × 12 cm square platform giving access to all arms. The illumination above the central platform was 85 lx. Each rat was placed in the central square facing an open arm, and the time spent (with the four paws) in every arm was recorded for 5 min.

SI: A modified version of a previously described protocol was used [19]. Testing took place in an open topped, rectangular and transparent box. The resident rat was placed in the box 8 min prior to placement of the experimental rat. The resident rat was a naive rat of similar weight that was received and housed under identical conditions as but had no previous contact with the experimental rat. No aggressive behaviors such as biting were detected. The amount of time the experimental rat spent engaging in social interaction (i.e., grooming, sniffing, following, crawling over or under) directed at the resident rat was measured for 5 min.

Following testing, rats were rapidly decapitated; brains were immediately frozen in isopentane at −30 °C. Brains were coronally sectioned on a cryostat at 20 μm and mounted on slides which were kept at −80 °C until processed. The *in situ* hybridization protocol is identical to our published protocol [9]. cDNA probes for rat NPY, Y1R, Y2R and CRF were antisense linearized and transcribed by using polymerases, 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. Section images were captured digitally from X-ray films with a CCD camera, and optical densities were determined relative to the background using a macro, which subtracts any labeling below 3.5X the background [9]. Background measurements were taken from cell soma free areas within the structure of interest (i.e., hippocampus) or from the neighboring regions, and were kept consistent across sections. Integrated density was calculated as optical density multiplied by the area of the signal. For each brain region, data from multiple sections (6–8) were averaged to obtain a mean integrated density for each animal; and grand means were compared statistically between groups. Messenger RNA levels for NPY, Y1 and Y2 receptors were quantified in the hippocampus, the medial nucleus of amygdala (MeA) and the basolateral amygdala (BLA). CRF mRNA was quantified in the CeA.

Nicotine training data 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). Locomotor response to nicotine challenge, percent time spent in social interaction, percent time spent in light compartment (LDB) and percent time spent in open arms (EPM) were analyzed by two-way ANOVAs with phenotype (LR, HR) X pre-training (SAL, NIC). Integrated density measures for mRNA quantification were also analyzed using two-way ANOVAs with phenotype (LR, HR) X pre-training (SAL, NIC). Significant main effects and interactions were followed by post hoc comparisons and significance was set at $p = 0.05$.

Fig. 1 shows total locomotor reactivity to four intermittent nicotine or saline training injections (1A) and the low dose nicotine challenge following 1 week of abstinence (1B) in LRHR rats. Repeated-measures ANOVA revealed a significant three-way interaction between phenotype, pre-training and injection days [$F_{(3,32)} = 12.11$, $p = 0.002$] and main effects of phenotype [LR, HR; $F_{(1,32)} = 29.99$, $p = 0.0001$] and pre-training [SAL, NIC; $F_{(1,32)} = 39.43$, $p = 0.0001$]. Specific post hoc comparisons showed that at all injection days, nicotine pre-trained HRs exhibited higher locomotor reactivity compared to saline pre-trained controls [$ps < 0.028$]; while in LRs, such nicotine-induced elevations in locomotor reactivity were observed only on INJ 3 [$p = 0.031$] and on INJ 4 [$p = 0.001$]. A two-way ANOVA showed a significant interaction between phenotype and pre-training in locomotor reactivity to challenge nicotine [1B; $F_{(1,32)} = 9.09$, $p = 0.029$], and a main effect of pre-training [SAL, NIC; $F_{(1,32)} = 7.76$, $p = 0.028$]. Post hoc comparisons further showed that HRs pre-trained with nicotine exhibited increased locomotor reactivity to the challenge dose of nicotine compared to saline pre-trained controls [$p = 0.0005$]. Such challenge nicotine-induced differences in locomotor reactivity were not observed between saline pre-trained LRs and HRs.

A significant interaction between phenotype and pre-training was observed in percent time spent in social interaction [Fig. 1C; $F_{(1,32)} = 10.01$, $p = 0.007$], along with significant main effects of phenotype [LR, HR; $F_{(1,32)} = 9.53$, $p = 0.012$] and pre-training [SAL, NIC; $F_{(1,32)} = 9.88$, $p = 0.05$]. A significant main effect of only phenotype