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Effect of acupuncture on anxiety-like behavior during nicotine withdrawal and relevant mechanisms

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Abstract

Acupuncture, likely the most well-known 'alternative' medical treatment, has been shown to have effects in several types of animal model of drug dependence, including nicotine addiction. We investigated the effect of acupuncture on anxiety-like behavior and corticotrophin-releasing factor (CRF) and neuropeptide Y (NPY) mRNA expression in the amygdala during nicotine withdrawal. Rats were given repeated nicotine injections (0.1 mg/kg s.c., once daily for 7 days) or saline. Acupuncture groups were treated with acupuncture at acupoint HT7 or ST36 during withdrawal. The anxiogenic response was measured at 72 h after the termination of nicotine injection using an elevated plus maze. CRF and NPY mRNA levels were also evaluated using reverse transcription polymerase chain reaction (RT-PCR) analysis at this time. Rats undergoing nicotine withdrawal (NW) were less likely to explore the open arms of the plus maze compared with the saline-treated controls. The percentage of open arm entries in the HT7 acupuncture group, but not in the ST36 acupuncture group, was significantly increased compared with the NW group. Consistent with this behavior, CRF mRNA levels in the NW group were increased compared with the control group. CRF mRNA levels in the HT7 acupuncture group were significantly decreased compared with the NW group. However, NPY mRNA levels were not different among the groups. These findings indicate that increases in CRF may be involved in the negative affect state associated with nicotine withdrawal and that acupuncture may attenuate anxiety-like behavior following nicotine withdrawal by modulating CRF in the amygdala.

Keywords: Acupuncture; Amygdala; Anxiety; CRF; Nicotine addiction

It has been estimated that 80% of all regular smokers want to stop smoking; a majority of them have tried and failed to quit. The most commonly reported reason for relapsing to smoking during smoking cessation attempts is the desire to relieve the discomfort of smoking withdrawal, including irritability, anxiety, depression, weight gain, and a craving for tobacco [15]. Increased anxiety has been highlighted as an affective aspect of nicotine withdrawal that may lead to relapse. Much evidence suggests that nicotine withdrawal results in anxiety-like behavior in various kinds of tests of anxiety in humans [10] and animals [3,6].

The amygdala is one of the major brain structures involved in the generation, expression, and maintenance of anxiety responses [13]. Corticotrophin-releasing factor (CRF) and neuropeptide Y (NPY) play physiological roles in the excitability of the amygdala, as mediators of anxiety [5]. Dysregulation of the CRF and NPY systems contributes to the motivational basis of continued drug-seeking behavior [17]. Anxiety-like behavior induced by adolescent nicotine exposure occurs in concert with changes in CRF and NPY [16]. Alterations in the CRF and NPY systems in the amygdala may be important in the regulation of anxiety during nicotine withdrawal.

Acupuncture, which is likely the best-known complementary or alternative medical treatment, has gained popularity and greater acceptance as a treatment option. Although recent systematic reviews have not provided consistent evidence for

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the use of acupuncture in nicotine addiction, acupuncture and related techniques have been applied in smoking cessation, in the belief that they may reduce nicotine withdrawal symptoms [18]. Recent experiments have demonstrated that acupuncture or electroacupuncture has modulatory effects in several kinds of animal models of drug addiction, including nicotine, alcohol, and morphine addiction [2,4,7,19,21,22]. Acupuncture attenuates c-Fos expression in the central nucleus of the amygdala during morphine withdrawal [8]. Furthermore, acupuncture at acupoint HT7 reduced anxiety-like behavior by enhancing neuropeptide Y expression in the basolateral amygdala of maternally separated rats [9]. Because many studies have suggested that acupuncture plays an important role in the regulation of anxiety via the amygdala, it is possible that the effects of acupuncture on nicotine dependency may reflect alterations in CRF and NPY gene expression in the amygdala.

We investigated whether acupuncture can effect functional alterations in the CRF and NPY systems, which are involved in anxiety during drug dependence. First, we determined the most effective acupuncture point in anxiety-like behavior during nicotine withdrawal using the elevated plus maze (EPM). Second, we evaluated CRF and NPY mRNA expression in the amygdala using reverse transcription polymerase chain reaction (RT-PCR) analysis to assess their possible involvement in the inhibition of anxiety during nicotine withdrawal by acupuncture in rats.

Experimental procedures were carried out according to the animal care guidelines of the NIH and the Korean Academy of Medical Sciences to minimize the number of animals used and their suffering. The subjects were 38 male Sprague–Dawley rats that weighed 250–270 g at the start of the experiment. Upon arrival, the animals were randomly divided into several groups and housed for at least 1 week prior to experimental procedures. Rats were kept under constant temperature ($22 \pm 2\,^{\circ}C$) and lighting (12-h light/12-h dark cycle; lights on at 07:00 h).

The repeated nicotine groups were pretreated with nicotine (Sigma, St. Louis, MO, 0.1 mg/kg, s.c., free base dissolved in saline at pH 7.2) once daily for seven consecutive days. No drugs were injected during the 72 h after the last nicotine injection. The control group ($n = 11$) received saline for 7 days and was tested on the tenth day. The acupuncture group received nicotine for 7 days and received acupuncture at acupoint HT7 ($n = 9$) or ST36 ($n = 9$) for 3 days during the withdrawal period. The nicotine withdrawal group (NW, $n = 11$) received nicotine for 7 days, but received no other treatment except for handling during the withdrawal period. The anxiogenic response was measured at 72 h after the termination of nicotine injection in all groups using the elevated plus maze (EPM).

For acupuncture stimulation, stainless steel needles (0.2 mm in diameter) were inserted approximately 3 mm into the left and right side of the selected acupuncture points. The needles were twisted twice a second for 30 s and then removed. The stimulated areas corresponded to acupuncture points in humans [20]. HT7 is located at the end of the transverse crease of the ulnar wrist of the forepaw; ST36 is located near the knee joint of the hind limb, 2 mm lateral to the anterior tubercle of the tibia. Acupuncture point HT7 has been used to ameliorate mental disorders, whereas acupuncture point ST36 has been used to regulate gastrointestinal function and relieve pain [9]. All groups were both slightly immobilized for 30 s. All treatments were repeated during the withdrawal phase, i.e., from the eighth to the tenth day.

Rats were housed individually before behavioral testing. Locomotor activity was measured in a rectangular container (40 cm × 40 cm × 45 cm) equipped with a video camera above the center of the floor. The walls and floor were made of clear Plexiglas painted black. Locomotor activity was monitored using a video tracking system, using the SMART program (PanLab, Barcelona, Spain). Animals were habituated for 1 h in the box and the distance traveled was then recorded during 1 h of baseline and 1 h of treatment.

The elevated plus maze test has been used to assess internal conflict between voluntary approach and withdrawal tendencies as a rodent model for human anxiety [12]. Because this test is based on a natural fear of open and elevated spaces, the number of entries into open arms and the time spent in open arms are negatively correlated with the anxiety level of the subject. The experimental apparatus consisted of a plus-shaped maze that was elevated 50 cm above the ground. The four arms were each 40 cm long and 10 cm wide. Two opposing arms were enclosed by black wood walls 30 cm high (closed arms), whereas the other two arms were devoid of walls (open arms). A total of 38 rats were tested individually on the EPM, without any pretest handling in adulthood. Each rat was placed in the center of the maze, after which the cumulative time spent on each arm and the numbers of entries into the open or closed arms were recorded during a 5-min test session at 72 h after the last injection of nicotine or saline. The area inside the center portion (10 cm × 10 cm) was not considered. Entry by an animal into an arm was defined as beginning when the animal had placed all four paws in that arm. The maze was cleaned with water after each rat had been tested. Exploration of the open arms was encouraged by testing under indirect dim light (2 × 60 W). The behavior in the maze was recorded using a video camera mounted on the ceiling above the center of the maze and relayed to the SMART program (PanLab, Barcelona, Spain). The data were recorded as time spent in open arms, expressed as a percentage of total time spent in the arms.

Five rats from each group were sacrificed, and the entire brain was obtained and dissected using the coordinates of Paxinos and Watson to obtain amygdala tissue samples [11]. The amygdala was dissected by a cut at the lateral borders of the lateral hypothalamus (Bregma −2.12 mm) and ventral of the rhinal fissure, with cortical tissue then being removed at the lateral edges of the dissected slice. The caudal border of the amygdalar dissection was the rostroventral border of the CA3 subfield of Ammon's horn (−4.16 mm). Microdissected tissues were snap-frozen on dry ice and stored at $-80\,^{\circ}C$ until RNA isolation. RNA was isolated using the RNeasy Mini kit, according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). Isolated RNA was redissolved in diethylpyrocarbonate-treated water, treated with DNase I, and stored at $-80\,^{\circ}C$.

The cDNA was synthesized using M-MLV reverse transcriptase and amplified using specific oligonucleotide primers for CRF and NPY by the PCR method. The cDNA was amplified in 20 μL of reaction mix that included 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM each of the