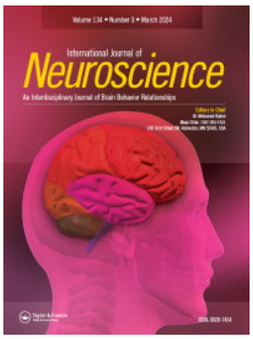

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RESEARCH ARTICLE

Examination of empathy-like behaviour in nicotine-preferring rat lines

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ABSTRACT

Aim: Addiction is an important global health issue, impacting also addicts environment and society. Empathy plays crucial role in establishing successful social relationships and is a fundamental component of social life. The aim of this study is to investigate how nicotine preferring (NP) strain and oral forced nicotine administration affects empathy-like behaviour in rats, with gender differences.

Materials and Methods: Sprague-Dawley NP rats (10 males/10 females) and wild-type control rats (10 males/10 females) were used. Behavioural tests were administered to all rats before and after oral forced nicotine administration. The behavioural tests were completed in the fourth week of nicotine administration. Anxiety levels that could affect empathy-like behaviour were evaluated with open field, elevated plus maze tests and with blood cortisol levels. Oxytocin receptor and arginine vasopressin (AVP) levels, which have been shown to be related to empathy-like behaviour, were examined in the prefrontal cortex and amygdala regions using the enzyme-linked immunoassay method.

Results: It was observed that males from the NP strain showed less empathy-like behaviour than all other groups, and nicotine administration did not cause a significant change in the results. Higher levels of locomotor activity (LA) were found in control females than in all other groups. Blood nicotine and corticosterone levels were higher in NP rats. No significant differences were found in AVP and oxytocin receptor levels in the prefrontal cortex and amygdala.

Conclusions: It was found that coming from an addicted strain particularly reduces empathy-like behaviour in males.

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Empathy; behaviour; addiction; nicotine; genetic

Introduction

Empathy is the ability to comprehend and relate to the emotions, thoughts, and intentions of others, and appropriately respond to them. From an evolutionary perspective, it is expected that empathy would initially be directed towards offspring, then partners and relatives, and eventually strangers for facilitating the survival and reproductive success of the species [1]. The presence of high or low empathy can lead to difficulties in social interactions and mental health. Differences in empathic abilities have been observed in many neuropsychiatric conditions, including autism, schizophrenia, and major depressive disorder [2].

Researchers have adopted two general approaches to measuring empathy-like behaviour. The first involves experimental setups based on fear and pain, while the second focuses on setups measuring prosocial behaviour, such as rescuing a cage-mate from a stressful situation.

In a study by Bartal et al. [3], it was shown that rodents act intentionally to end the distress of another conspecific rat which is experiencing non-painful psychological restraint stress [4]. They evaluated helping behaviours in rats at the expense of overcoming their innate fear of open spaces. In this experiment, rats learned to open a door containing a stressed cage-mate. But they only opened the door if it contained a trapped cage-mate, not if it was empty or had an inanimate object. The delay in opening the door was greatly reduced over time by training [5].

It is known that free rats open the restrainer door to rescue their stressed peers. Even in the presence of a tangible food reward as an alternative to helping a soaked cage-mate, they continued to open the door to rescue their cage-mates [5,6]. Empathy is believed to be the main motivation for prosocial behaviour [7].

Selective breeding is an important research method used in animal behaviour genetics. In this method,

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individuals displaying a chosen behaviour are selected and bred among themselves to create a strain exhibiting that behaviour. Sprague Dawley rats are divided into three groups based on their oral nicotine consumption: high, medium, and low. High- and low-nicotine-consuming rats were interbred to produce nicotine-preferring and non-nicotine-preferring rat strains. In a study conducted by Nesil and colleagues, in the early stages of these lineages, demonstrated that generations and nicotine preferences have significant interactions when nicotine use for six weeks is taken as a dependent variable, and generations and nicotine preferences as independent variables. When average nicotine use was taken as the dependent variable and generations were evaluated as factors, a significant difference was found between high- and low-nicotine-preferring generations [8]. The maximum nicotine-preferring rats (NP) used in our study are the 24th-generation offspring of these strains.

Many studies have been conducted on animal models related to empathy-like behaviour in the literature. However, no studies have been found on how addiction mechanisms directly affect empathy-like behaviour. In our study, we thought that revealing the possible effects of addiction, coming from an addicted strain, and addictive agents on empathy-related behaviours and hormones could be crucial for a better understanding of empathy mechanisms and the development of addiction treatment strategies.

Methods

Sprague Dawley rats (born March 2020) from the 24th generation with maximum nicotine preference (NP) strain ($n=20$), and wild-type control group ($n=20$), obtained from Ege University Experimental Animals Application and Research Center, were used. All experimental animals were housed in pairs with access to standard pellet feed and water ad libitum at a temperature of 23 ± 2 °C with a 12-h light/dark cycle. Handling was performed during the last week of the three-week adaptation period. One female rat from the control group died during the experiments. This study was approved by the Ege University Medical School Animal Experimentation Local Ethics Committee (EU TF-HADYEK) on July 31, 2019 (application number 2019-059).

Experimental design

After the rats were arrived to the laboratory, adaptation and handling process completed and behavioral tests were started gradually. After the initial experiments were completed, the rats were exposed to forced oral nicotine for 4 weeks to establish a chronic nicotine addiction model. Empathy reminder training was carried out once a week during the nicotine application. Empathy-like behaviour and anxiety tests were repeated for the second time in the 5th week of nicotine application for rats with the established addiction model. Video recordings of the behavioural experiments were taken, and evaluations were made by a researcher blind to the groups.

Immediately after the completion of empathy-like behaviour and anxiety tests, rats were decapitated, trunk blood was collected, and their serums were separated by centrifugation. The prefrontal cortex (PFC) and amygdala regions were dissected on ice and frozen at −80 °C along with the serums. After all the experiments were completed, analyses were performed on blood and tissue samples using enzyme-linked immunoassay (ELISA) tests.

Empathy-like behaviour test

To measure empathy-like behaviour in animals, a setup measuring 100x450x450 mm made of black plexiglass side walls was used (Figure 1). In the middle of the box, there is a transparent partition dividing the box into two equal parts. The floor of the rescuer rat's compartment is 100 mm higher than the pool compartment. Water is placed in the pool compartment at a height of 90 mm. In the center of the transparent wall, there is a 60 mm diameter hole that allows passage between the compartments and an 80 mm diameter round shape door that can be opened by the rat. The behaviour of each rat to rescue its cage-mate from the water stress was observed. Rats were monitored for 5 min, and door-opening times were recorded. The experiment ended when each rat demonstrated door-opening behaviour or when five minutes were completed. This experiment was repeated for 12 days [6].

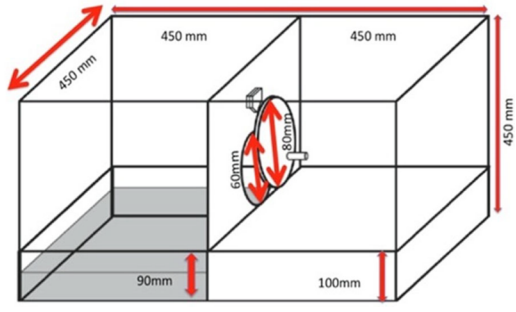

Figure 1. Empathy-like behaviour experiment setup.

Open field test (of)

The open field test is commonly used to evaluate locomotor activity and anxiety. The open field apparatus consists of an 80×80 cm area surrounded by a 45 cm high wall and a video camera mounted 2.5 m above the apparatus and computer system. The apparatus is in a soundproof observation room illuminated by controlled light (100 lx). Each rat is placed in the center of the open field and their movements are recorded for 5 min. Total travelled distance data were calculated by a computer-based video tracking system (HVS Image HVS Image, Hampton, UK). The time spent in the middle area and the number of lines passed and the time spent in the side area, the number of lines passed, and the number of crossings in the middle area were recorded.

Elevated plus maze (EPM)

This test is a widely used experimental model to evaluate anxiety. The elevated plus maze consists of a central platform (10×10 cm) with two open arms (50×10 x 0.5 cm) and two closed arms (50×10 x 40 cm), each elevated 50 cm above the floor. Rats are placed facing an open arm in the center area and observed for 5 min. The first entry time (latency) to the closed arm, the total number of entries to the open and closed arms, and the total time spent in both open and closed arms were recorded.

Forced oral nicotine administration

In this study, all animals in all groups were pair-housed and exposed to forced nicotine administration. Two bottles containing 400 ml of nicotine water were placed in each cage, and rats were forced to drink only nicotine water for 5 weeks. Gradually, the amounts of saccharin and nicotine were adjusted as follows: 0.2% saccharin + 25 mg/ml nicotine water (days 1–7), 0.2% saccharin + 50 mg/ml nicotine (days 8–10), 0.1% saccharin + 50 mg/ml nicotine (days 11–14), and 50 mg/ml nicotine (day 15 and thereafter) [9]. Nicotine consumption was not calculated as rats were pair-housed.

Biochemical analysis

Immediately after the behavioural tests, each rat was decapitated, and trunk blood was collected. The collected blood samples were centrifuged at 1000 g for 15 min to separate the serum, which was then stored at −20 °C for analysis. The amygdala and PFC were dissected, weighed using a precision balance, and stored at −80 °C. Serum corticosterone and cotinine levels, oxytocin receptor and vasopressin levels in PFC and amygdala were measured using ELISA (BTLAB ELISA kit) method. Total protein analysis was performed on tissue homogenates using spectrophotometric methods. All ELISA measurements using tissue homogenates were expressed per total tissue protein.

Statistical analysis

All statistical procedures were performed using SPSS software for Windows, Version 20.0 (SPSS, Chicago, IL). Behavioural experiments were analysed using repeated measures or multivariate ANOVA. Differences between groups were determined using one-way ANOVA and post-hoc Duncan's test. Blood and tissue samples were evaluated using one-way ANOVA.

Results

Behavioural experiments

During the first 11 days of training, the time it took for the rats to open the door was found to be significantly different at $F_{(10,27)} = 5.648$, $p = 0.000$, decreasing as the days progressed (Figure 2). Interaction between days and strains was observed ($F_{(10,27)} = 2.771$, $p = 0.017$). According to between factor results of ANOVA for the repeated measures, strain $F_{(1,36)} = 20.007$, $p = 0.000$, and gender $F_{(1,36)} = 6.446$, $p = 0.016$ were found to be significant, while no interaction was found between the two variables.

During the 11-day training period, the difference between groups was determined at $F_{(1,36)} = 187.642$, $p = 0.000$. In the post hoc Duncan's test, it was observed that males from the nicotine-preferring strain did not

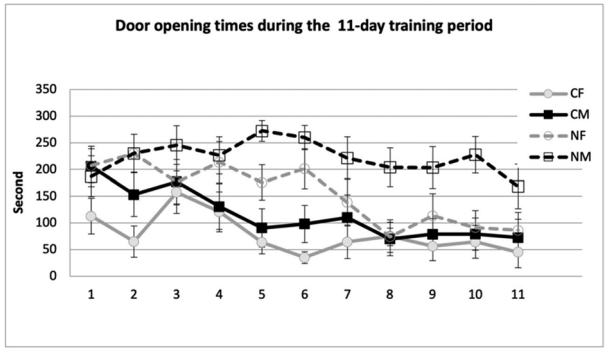

Figure 2. Changes in rats' door opening times during the 11-day training period, control female (CF), control male (CM), nicotine preference female (NF), nicotine preference male (NM).

exhibit empathy behaviour, unlike all other groups. It was also found that empathy responses of females from the nicotine strain were significantly lower compared to control females.

The results of empathy experiments conducted before and during nicotine administration were compared using repeated measures ANOVA test. Nicotine application did not cause a significant change in the results. However, strain ($F_{(1,36)}=25.480$, $p=0.000$) and gender ($F_{(1,36)}=23.776$, $p=0.000$) were found to be significant. A significant interaction between the two factors ($F_{(1,36)}=14.061$, $p=0.001$) was also observed. Being male and from the nicotine-preferring strain increased door opening times, and the interaction between the two factors emerged due to the similarity between control male and NP female groups. When the same test was conducted with the group as an independent variable, the groups were found to be different ($F_{(3,36)}=21.106$, $p=0.000$) (Figure 3), and in Duncan's test, males from the nicotine strain were identified as different from other groups.

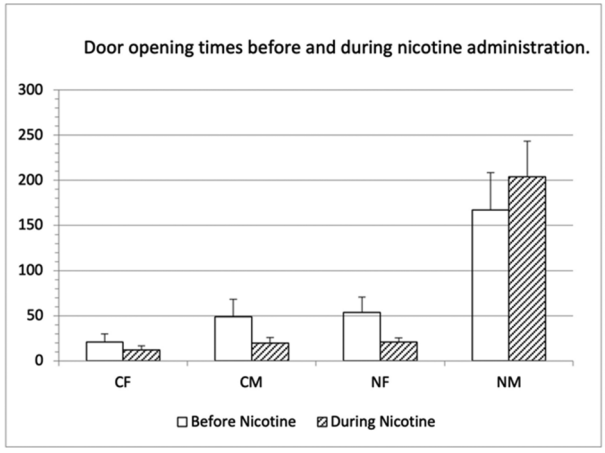

Figure 3. Door opening times before and during nicotine administration.

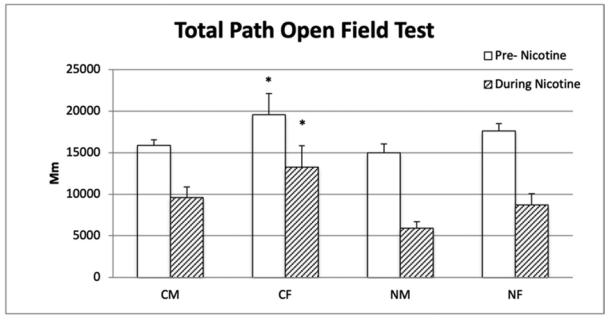

Figure 4. Average distance traveled by groups before and during nicotine administration (*=$p \leq 0.01$).

In the open field test, the total distance travelled by rats before and during nicotine administration was recorded using the HVS image system and used as a locomotor activity indicator. When the application days were evaluated as a factor, it was found to be significant at $F_{(1,35)}=110.132$, $p=0.000$. The total travelled distance decreased during nicotine administration. When the group was taken as an independent variable, control females were found to be more active than all other groups ($F_{(3,35)}=9.413$, $p=0.000$) (Figure 4). Both before and during nicotine administration, the locomotor activity of control female rats was found to be significantly higher than the other groups. Another locomotor activity indicator, the total number of lines crossed, also showed that the locomotor activity of control females was higher than the other groups.

In the open field test, nicotine application was found to reduce the time spent in the central area ($F_{(1,33)}=21.139$, $p=0.000$), the number of entries into the central area ($F_{(1,33)}=41.072$, $p=0.000$), the number of lines crossed in the central area ($F_{(1,33)}=33.554$, $p=0.000$), the time spent in the peripheral area ($F_{(1,35)}=7.221$, $p=0.011$), and the number of lines crossed in

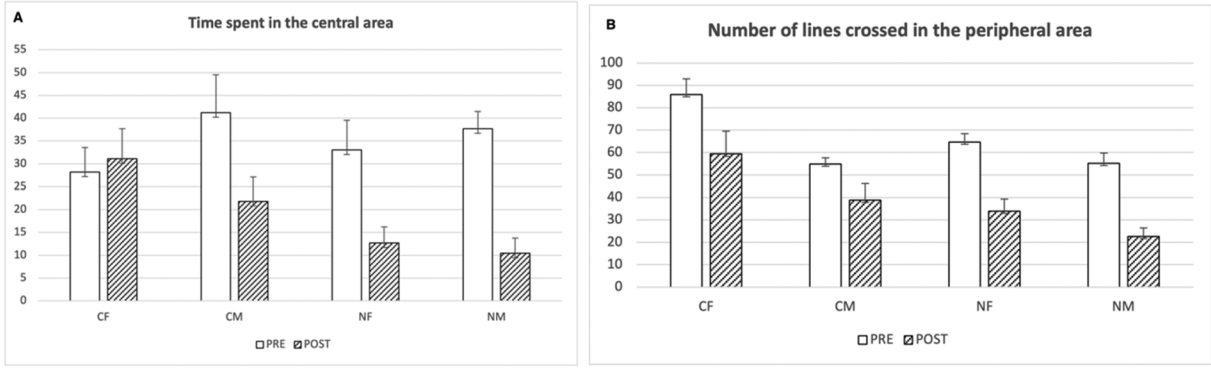

Figure 5. Time spent in the central area (A) and number of lines crossed in the peripheral area (B) and by groups in the open field test before (pre) and during (post) nicotine administration.

the peripheral area ($F_{(1,34)}=70.338$, $p=0.000$). The results of the open field test before nicotine administration were evaluated with one-way ANOVA and no statistically significant group difference was found in any data except for peripheral line crossing ($F_{(1,38)}=8.908$, $p=0.000$). During nicotine administration, significance was found in the time spent in the central area ($F_{(3,36)}=3.822$, $p=0.019$) (Figure 5A), the number of entries into the central area ($F_{(3,37)}=10.222$, $p=0.000$), the number of lines crossed in the central area ($F_{(3,37)}=5.873$, $p=0.002$), and the number of lines crossed in the peripheral area ($F_{(3,38)}=4.779$, $p=0.007$) (Figure 5B). Post hoc tests identified the control female group as different from the other groups, and the control male, nicotine-preferring male, and female groups were found to be similar.

When the elevated plus maze results were evaluated using the repeated measures ANOVA test, nicotine administration significantly changed the number of first entries into the closed arm ($F_{(1,29)}=11.299$, $p=0.002$), time spent in the open arm ($F_{(1,30)}=31.907$, $p=0.000$), number of entries into the open arm ($F_{(1,31)}=61.350$, $p=0.000$), time spent in the closed arm ($F_{(1,31)}=7.531$, $p=0.010$), and the number of entries into the closed arm ($F_{(1,31)}=17.401$, $p=0.000$). Strain ($F_{(1,31)}=12.147$, $p=0.001$) and gender ($F_{(1,31)}=6.433$, $p=0.016$) differences were found to be significant only in the closed arm entry data. The post hoc Duncan test showed that control females were different from all other groups. When all data were evaluated separately with one-way ANOVA, the closed arm entry data was found to be significantly different both before nicotine ($F_{(3,39)}=2.916$, $p=0.047$) and under nicotine effect ($F_{(3,34)}=4.507$, $p=0.010$). No statistical significance was found in other parameters.

When the pre-nicotine closed arm entry data were evaluated with Duncan, control females made similar number of entries to control males and more entries than females and males from the nicotine strain. However, under the nicotine effect, it was observed that control females had more entries than control males and males from the nicotine strain (Figure 6).

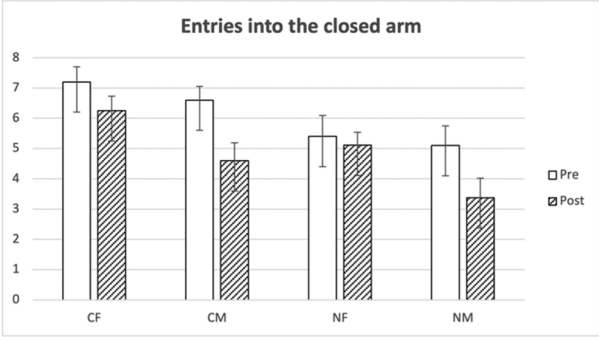

Figure 6. Number of entries into the closed arm in the elevated plus maze before and after nicotine administration by groups.

Biochemical analyses

The results of cotinine levels obtained from the serum were compared using one-way and univariate ANOVA tests. As a result of the one-way ANOVA, the strain was found to be significant at $F_{(1,24)}=5.567$, $p=0.028$. Cotinine levels of nicotine-preferring rats were found to be higher. There was no statistically significant difference based on gender (Figure 7A).

When the results of corticosterone levels obtained from the serum were evaluated with univariate ANOVA, where strain and gender were taken as factors, strain was found to be significant at $F_{(1,39)}=7.172$, $p=0.011$. As with cotinine levels, corticosterone levels of nicotine-preferring rats were also found to be higher (Figure 7B). According to the post hoc Duncan test, control males were found to have lower levels than nicotine-preferring females and males

AVP levels in the prefrontal cortex and amygdala regions were evaluated using one-way ANOVA with

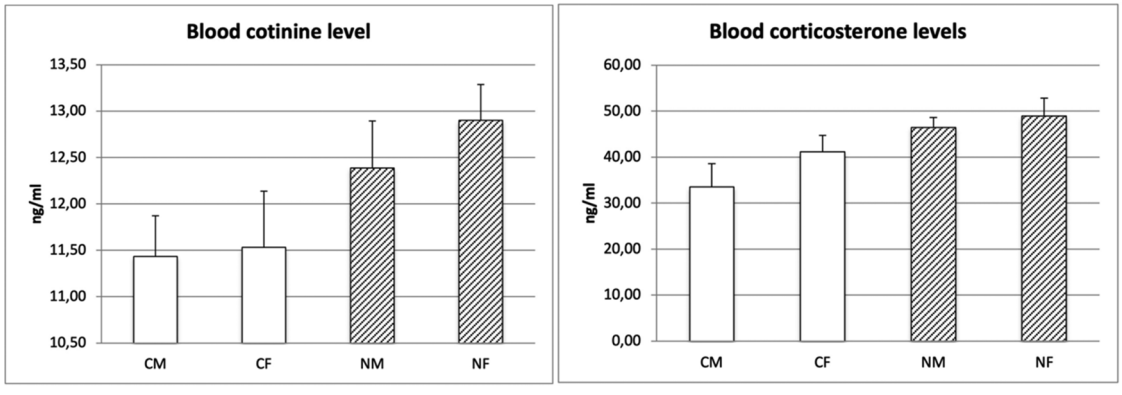

Figure 7. Blood cotinine (A) and corticosterone (B) levels, (*=$p \leq 0.05$).

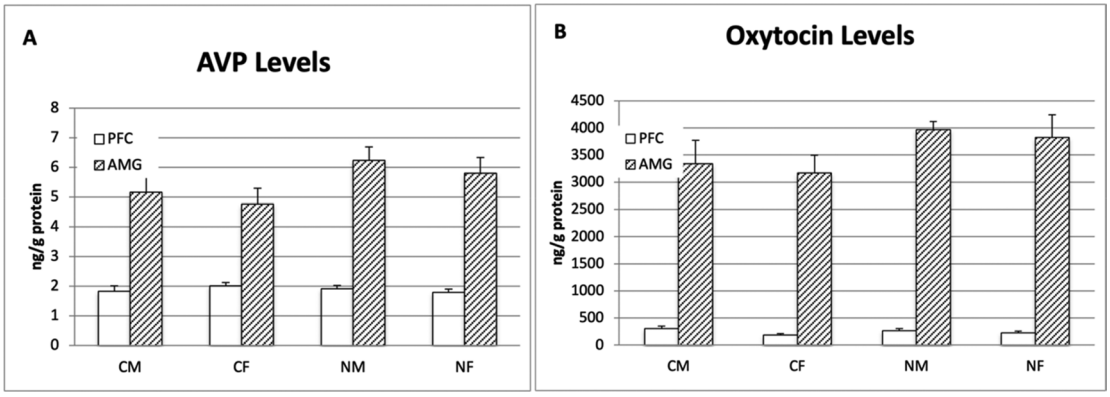

Figure 8. AVP (A) and oxytocin (B) receptor levels in brain regions.

strain and sex as independent variables. AVP levels were found to be higher in NP rat strains ($F(_{1.63}) = 4.889$, $p = 0.031$). No significant differences between groups were found when the same data were evaluated using one-way ANOVA (Figure 8A).

When oxytocin receptor protein levels in the two brain regions were evaluated using one-way ANOVA with strain and sex as independent variables, a significant increase was observed in strain at $F(_{1.63}) = 5.463$, $p = 0.023$. No significant differences between groups were found when the same data were evaluated using one-way ANOVA (Figure 8B).

No significant correlation was detected between both AVP and oxytocin receptor proteins and empathy scores (12th day and final test data).

Discussion

Empathy is the ability to identify others' emotions, thoughts, and intentions and to respond with an emotion appropriate to their mental states. This neuro-cognitive ability is crucial for our emotional experience and social interaction [2a).

We used the experimental setup measuring helping behaviour, which is the prosocial component of empathy, as applied by Sato and colleagues. Upon examining the 12-day empathy results in the experimental protocol, it was found that they opened the door faster with each passing day [6]. In this study, the behaviour of rescuing the cage mate from the stressful situation was evaluated separately according to the strain and sex of the rats when the day factor was taken into account.

In our study, we showed that being from a nicotine strain and being male disrupted the acquisition of empathic behaviour during the first 11-day training period. We also detected an interaction between days and strain factors during the acquisition period. This interaction is due to rats from the nicotine strain, particularly males, not reducing their door-opening times over the days. We aimed to demonstrate the effect of oral forced nicotine administration with single-session empathy experiments conducted in the 5th week of the application. We could not find a significant effect of nicotine administration in the results of these studies, but we observed that strain and sex altered empathic behaviour. In our study, we showed that females were better at empathic behaviour than males and that rats from the nicotine strain which had more limited empathic abilities.

There are studies reporting that empathy differs between genders in humans. It has been shown that women are stronger at empathizing [10]. These findings have been demonstrated in many studies conducted on humans where empathy was investigated [11]. Females have exhibited more successful performance in learning helping behaviour and generating faster responses. Female rats were found to have a higher probability of freeing their cage mates compared to males [1]. In our study, a clear gender difference has been demonstrated, and it was evident that the gender difference was prominent in male rats from the NP (nicotine-preferring) lineage. Male rats from the NP lineage exhibited distinct empathy-like behaviour compared to all other groups, taking longer to open the door. The empathy response of NP females was also reduced compared to control female rats.

Empathic behaviour in experimental animals is also affected by their stress/anxiety levels and locomotor activity. It is suggested that nicotine alters fear and anxiety-related behaviours and contributes to the development, maintenance, and recurrence of anxiety disorders [12]. Therefore, in our study, rats' locomotor activities were evaluated with the total path/line number in the Open Field (OF) test, and anxiety levels were assessed with the Elevated Plus Maze (EPM) test. Both before and during nicotine administration, the locomotor activity of control female rats was found to

be significantly higher compared to other groups, and nicotine administration reduced locomotor activity in all groups. Nicotine increases locomotor activity in rats when administered at appropriate doses. The locomotor activity stimulated by chronic nicotine administration is considered a behavioural indicator of increased dopamine secretion in the nucleus accumbens [13]. The first administration or high-dose nicotine intake suppresses locomotor activity. Interaction between locomotor activity and nicotine leads to different results depending on the nicotine dose and usage duration. While acute nicotine administration suppresses locomotor activity, chronic nicotine administration causes an increase in locomotor activity [14].

Since we used oral forced nicotine as our method, the rats were exposed to high doses of nicotine in order to obtain the required water intake. This resulted in a decrease in locomotor activity. We chose the forced oral nicotine administration method because it minimizes stress and individual consumption differences. However, being from the NP strain led to increased nicotine consumption with this method. Cotinine is the main biomarker used to distinguish tobacco users from non-users and reflects the degree of exposure [15]. In our study, cotinine levels in NP rats were found to be higher compared to controls. This can be considered as an indicator that the offspring of rats with a high preference for nicotine are more prone to prefer nicotine.

In the open field test results before nicotine administration, it was observed that there was no difference in parameters except for the number of lines crossed in the periphery, where females crossed more lines. We interpreted this difference as a result of the increase in females' locomotor activities and that there was no significant difference in anxiety levels between rats. In the results of the experiment conducted during nicotine administration, the higher scores in the number of lines crossed in the periphery and central area by the CF group were also considered as similar reflections of the increase in locomotor activity. However, a significant increase in the time spent in the central area was interpreted as a decrease in anxiety levels in control female rats compared to all other groups.

In the EPM, both before nicotine and during nicotine administration, an increase was observed in the number of entries into the closed arm for females only. However, since this data was not supported by durations and open arm data, it was not considered an increase in anxiety levels but rather a result of higher locomotor activity. Nicotine administration caused a significant decrease in all data in the EPM. This was interpreted as a result of the suppression of locomotion.

When the open field and EPM data were jointly evaluated, it was concluded that there was no significant difference between the groups and that the strain difference observed in empathy experiments was not due to anxiety.

Social hormones, oxytocin and AVP, are associated with the modulation of social behaviour and affect a wide range of behaviours, including bonding, in-group and out-group relationships, and social communication [16]. There are numerous recent reports on the role of oxytocin in positive social behaviours in animals and humans [17]. Oxytocin also promotes social interaction between parents and children, partners, and peers. Studies have revealed that dopamine released from mesolimbic reward pathways, along with oxytocin, plays a significant role in bonding [18]. The mesolimbic dopamine (DA) circuit determines which behaviours are positively reinforced. It has been suggested that oxytocin (OT) plays a role in mediating natural rewards and in the projection of OT-synthesizing neurons to the ventral tegmental area (VTA) and nucleus accumbens (NAc) [19].

Studies have reported that the effect of oxytocin is dose-dependent. Low doses of oxytocin result in an anxiolytic-like effect [20]. Additionally, chronic intranasal oxytocin applications induce down-regulation of oxytocin receptors and impair social behaviour. This requires further investigation with different doses of oxytocin [21].

In a study by Kandis et al. examining the effects of paracetamol on empathy, they revealed the relationship between oxytocin, vasopressin, and empathic behaviour. They demonstrated a negative correlation between the delay in door-opening time and prefrontal cortex oxytocin levels [22]. In our study, no correlation was detected.

In our study, AVP and oxytocin receptor (OXTR) levels in the prefrontal cortex and amygdala regions were evaluated. The results showed that there was no significant difference between the groups for AVP levels. Additionally, the evaluation of oxytocin receptor protein levels in both brain regions revealed that the gender difference was at $p = 0.059$, which did not reach a significant level. This situation can be inferred as a result of the complexity of oxytocin's role in modulating social behaviours (both aggressive and supportive behaviours) and suggests that oxytocin may not be the sole determinant of empathy-like helping behaviour.

Women are at greater risk for relapse following withdrawal [23]. The oxytocin system may have a stronger influence on drug-taking behaviour in females [24]. The lack of significant differences in oxytocin

levels in our study suggests that the empathy-like behaviour deficiency in NP males may be related to nicotine-associated pathways that are not directly linked to oxytocin.

Nicotine-preferring (NP) rats have also been found to have higher corticosterone levels. This finding is consistent with studies revealing the interaction between addiction and stress-related hormones. Control males were found to be different from nicotine-preferring females and males. Control females were observed to have similar levels to both control males and nicotine-preferring females and males. This supports studies explaining the vulnerability of women to addiction affinity or relapse due to the effects of stress. Similarly, in our study, the absence of a difference in corticosterone levels between control males and NP males and females suggests that nicotine eliminates the gender difference and leads females to develop behaviours similar to males. The lack of empathy observed in NP males also indicates that it cannot be attributed to changes in corticosterone levels.

Our study can provide a different perspective for future studies aimed at understanding the pathophysiology of addiction and developing potential treatment strategies by examining the effects of coming from an addicted lineage and the direct effects of an addictive agent on empathy-like behaviour. The existing studies in the literature provide evidence for the relationship between social behaviour and the interactions of systems involved in modulating addiction and stress.

Our research has confirmed previous studies with strong evidence that rodents display empathy-related behaviours. Additionally, it has clearly demonstrated the disruptive effect of coming from an addictive lineage on both the potential for addiction formation and empathic-like behaviours, particularly in males. Further research studies are needed to understand the neural mechanisms by which addiction impacts empathy and to develop potential treatment strategies.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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