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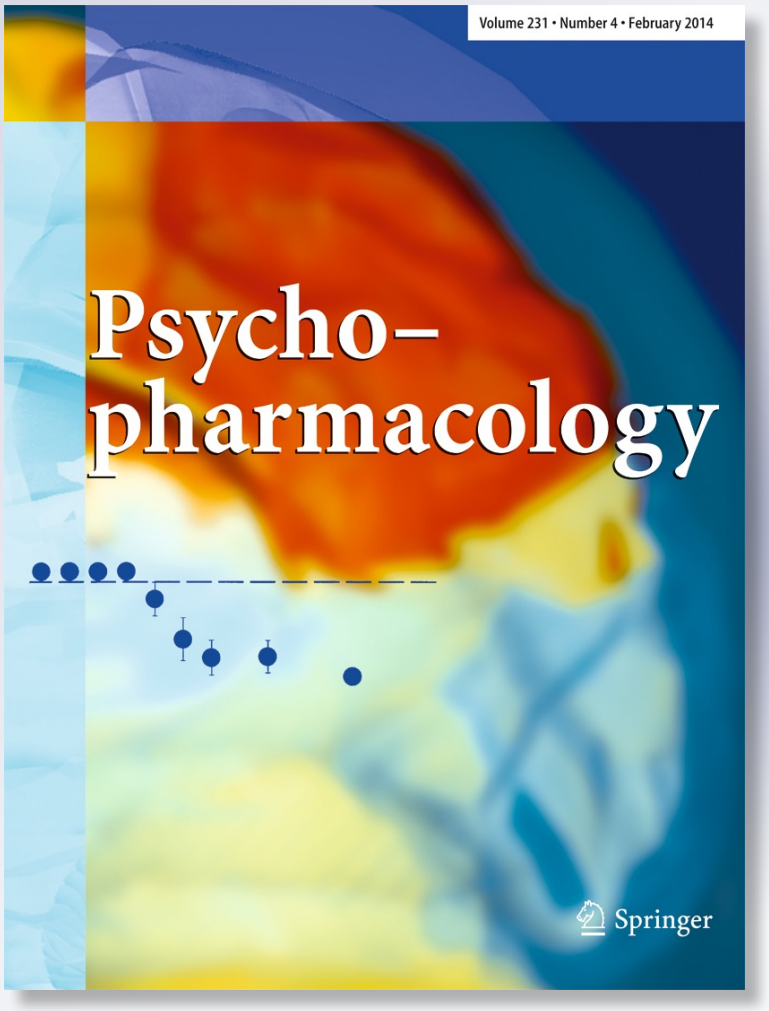

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ORIGINAL INVESTIGATION

Sex-influence of nicotine and nitric oxide on motor coordination and anxiety-related neurophysiological responses

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Abstract
Rationale Nitric oxide (NO) is a messenger synthesized in both the neuronal and glial populations by nitric oxide synthase type 1 (NOS1). Nicotine regulates NO production in a sex-dependent manner, both molecules being involved in motor function.
Objective The present study evaluates sex differences in motor coordination, general movement, and anxiety-related responses resulting from both constant and continuous nicotine treatment and the genetic depletion of NOS1 activity.
Methods Male and female mice were analyzed with the open-field and the rotarod tests. To understand the role of NO, knockout mice for NOS1 (NOS1−/−) were analyzed. Nicotine was administered continuously at a dose of 24 mg/kg/day via osmotic mini-pumps over 14 days because the behavioral effects elicited are similar to those observed with discontinuous administration.
Results Data analyses revealed noteworthy sex differences derived from NOS1 depletion. Control NOS1−/− males exhibited an exacerbated anxiety-related response in relation to control NOS1−/− females and control wild-type (WT) males; these differences disappeared in the nicotine-administered NOS1−/− males. Additionally, nicotine administration differentially affected the horizontal movements of NOS1−/− females with respect to WT animals. NO depletion affected male but not female motor coordination improvement along the test days. However, the drug affected female motor coordination only at the end of the administration period.
Conclusions We show for the first time that NO affects motor and anxiety behaviors in a sex-dependent manner. Moreover, the behavioral effects of constant nicotine administration are dimorphic and dependent on NO production.

Keywords Nicotine · Osmotic mini-pumps · Nitric oxide · Sex differences · Motor behavior · Anxiety

R. Muñoz-Castañeda · D. Díaz · J. R. Alonso · E. Weruaga (✉)
Laboratory of Neural Plasticity and Neurorepair, Institute for Neuroscience of Castilla y León (INCyL), Universidad de Salamanca, C/ Pintor Fernando Gallego 1, 37007 Salamanca, Spain
e-mail: ewp@usal.es

R. Muñoz-Castañeda · D. Díaz · C. A. Ávila-Zarza · J. R. Alonso · E. Weruaga
Institute for Biomedical Research of Salamanca (IBSAL), Salamanca, Spain

C. A. Ávila-Zarza
Department of Statistics, Universidad de Salamanca, Salamanca, Spain

J. R. Alonso
Institute for Higher Research, Universidad de Tarapacá, Arica, Chile

Introduction

Nitric oxide (NO) is a gaseous messenger synthesized by nitric oxide synthases (NOS; Palmer et al. 1987). In the central nervous system (CNS), type 1 nitric oxide synthase (NOS1) is mainly expressed by neuronal but also by astroglial populations (Esplugues 2002; Zhou and Zhu 2009). It is well documented that NOS1 activity is involved in general behavior, affecting motor coordination, locomotion, and aggression (Del-Bel et al. 2011; Gammie and Nelson 1999; Jacoby et al. 2001; Lev-Ram et al. 1997; Tanda et al. 2009; Weitzdoerfer et al. 2004). Long-term changes in synaptic plasticity (especially in long-term depression, LTD) are affected in NOS1 knockout mice (NOS1−/−; Jacoby et al. 2001; Lev-Ram et al. 1997). Although cerebellar LTD is widely accepted as a basis for motor learning and memory (Ito 2001; Koekkoek et al. 2003; Matsuda et al. 2000; Meller and Gebhart 1993; Schweighofer and Ferriol 2000; Siegelbaum and Kandel 1991), previous authors have failed to find impairments in motor coordination in NOS1−/− mice

(Chiavegatto et al. 2001; Kriegsfeld et al. 1999; Weitzdoerfer et al. 2004).

Nicotine is a drug of abuse whose action is mediated in the CNS by nicotinic acetylcholine receptors (nAChR). Its final effect on motor behavior is well documented and it has also been proposed to play a neuromodulatory role in cerebellar LTD (Kanyt et al. 1999). The different routes of administration of nicotine exert different behavioral effects (Dong et al. 2010; Matta et al. 2007; Salminen et al. 1999), acute and discontinuous administration being the way to produce the most exacerbated behavioral effects (Besson et al. 2007; Salminen et al. 1999), although with contradictory results depending on the dose employed (Huang et al. 2008; Kota et al. 2007). In this sense, the infusion of nicotine through osmotic mini-pumps induces a smaller response of the dopaminergic system than acute administration, both in the *caudate putamen* and the *nucleus accumbens* (Salminen et al. 1999). Thus, to achieve behavioral effects similar to those observed with acute and discontinuous administration, a higher nicotine dose (at least 24 mg/kg/day) is necessary (Damaj et al. 2003; Jonkman et al. 2005; Kota et al. 2007; Matta et al. 2007).

Previous studies have reported changes in NO synthesis derived from nicotine administration, leading to the idea that NO could mediate some of the effects of nicotine (Bauer et al. 1994; Pögün et al. 2000; Shim et al. 2002; Weruaga et al. 2002). Moreover, sex differences in both NO production and nAChR expression have been analyzed extensively (Caldarone et al. 2008; Damaj 2001; Gangitano et al. 2009; Hamilton et al. 2010; Kant et al. 2000; Pögün et al. 2000). Thus, both molecules exert sexually dimorphic behavioral effects (Damaj 2001; Kanyt et al. 1999; López-Figueroa et al. 1998; Mitsushima et al. 2003, 2008; Weruaga et al. 2002; Yilmaz et al. 1997). More precisely, nicotine induces a sexually dimorphic release of NO in brain areas related to motor behavior, such as the *nucleus accumbens* and *dorsal striatum* (Weruaga et al. 2002). Nevertheless, no comparative works studying the interaction of both molecules in NOS1−/− mice attending to possible sex differences have been published.

The aim of this work is to study general motor behavior, motor coordination, and anxiety-related neurophysiological response in NOS1−/− mice under the effect of constant and continuous nicotine administration. Taking into account the previously reported dimorphism in both NO production and the behavioral effects of nicotine, we also considered possible sex differences in the parameters analyzed in both genotypes. Accordingly, we first hypothesized that NOS1−/− mice would be more anxious, hyperactive, and sensitive to nicotine effects because of the lack of NO as a neuromodulatory molecule in the CNS. The second hypothesis was that these parameters would be related to the sex of the animals under the experimental conditions studied.

Material and methods

Animals

Adult mice (P75-110) from the B6/129S4-Nos1tm1Plh strain (The Jackson Laboratory, Bar Harbor, ME, USA) were employed. The animals were housed at constant room temperature and relative humidity with a light/dark photoperiod of 12/12 h and fed ad libitum at the animal facilities of the University of Salamanca. A total of 66 animals were employed for this experiment. They were divided into eight groups based on the following: (1) sex; (2) genotype, the mice being wild type (WT) or knockout for the neuronal nitric oxide synthase (NOS1−/−); and (3) treatment, depending on whether saline solution (control group) or nicotine (experimental group) was administered. Thus, the animals were grouped as follows: control WT males ($n=10$), nicotine-treated WT males ($n=10$), control WT females ($n=10$), nicotine-treated WT females ($n=10$), control NOS1−/− males ($n=8$), nicotine-treated NOS1−/− males ($n=8$), control NOS1−/− females ($n=5$), and nicotine-treated NOS1−/− females ($n=5$).

The animals were housed, handled, and sacrificed according to the guidelines established by European (2010/63/UE) and Spanish (RD53/2013, Law 32/2007) legislation. The Bioethics Committee of the University of Salamanca approved all procedures.

Nicotine administration

Nicotine ((−)-nicotine hydrogen tartrate salt; Sigma-Aldrich) was administered subcutaneously with Alzet osmotic mini-pumps (DURECT Corporation, Cupertino, CA, USA; model 2002) at a dose of 24 mg/kg/day (calculated as the base). Nicotine was dissolved in saline solution (NaCl 0.9 % *w*/*v*) and the pH was adjusted to 7.0. Control animals were treated only with the vehicle, saline solution.

The animals were anesthetized with a mixture of isoflurane and oxygen (2.5–5 %) and the mini-pumps were implanted subcutaneously under sterile conditions. Then, animals were sutured and recovered on a heating pad before being returned to their home cage.

The animals were tested before, during, and after nicotine treatment, as depicted in Fig. 1. More specifically, under the basal condition, time zero (T0), the animals were tested before the implantation of the mini-pumps to analyze their basal motor behavior. Then, at T4, T9, and T14, the animals were also tested to study the effect of continuous nicotine administration. At T14, after the behavioral tests, the mini-pumps were removed. Two days later (T16), the animals were tested again to study the effects of nicotine cessation. The behavioral and neurophysiological tests were carried out early in the light

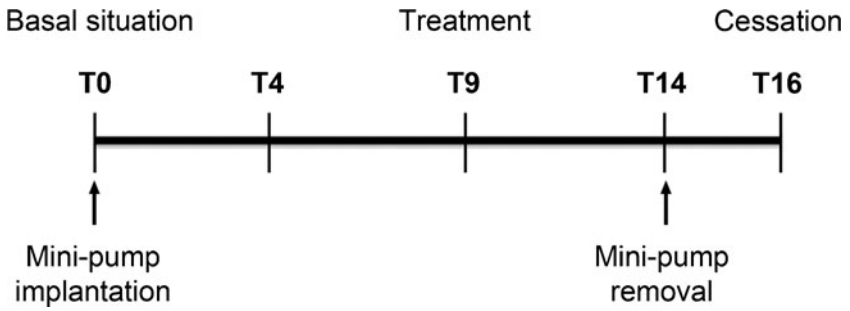

Fig. 1 Timeline representing the implantation (T0) and removal (T14) days of the osmotic mini-pumps and the days of behavioral testing: at the basal situation (T0), during nicotine treatment (T4, T9, T14), and 2 days after nicotine cessation (T16). The mini-pumps were implanted (T0) and removed (T14) after the behavioral tests (open-field test and rotarod test)

cycle, by the same person and always in the same sequence: open-field first and then the rotarod test.

Behavioral and neurophysiological tests

The *open-field* test was performed in a white Plexiglas® box (50×29 cm) with the floor divided into 15 squares (9×9 cm) to measure general locomotion and neurophysiological responses. The animals' behavior was recorded along 10 min and analyzed only during the last 5 min, when they had become habituated, thus reducing the effect of novelty. The parameters measured were as follows: (1) horizontal movement, defined as the crossing between squares with the four paws, providing information about general locomotion; (2) vertical movement, defined as rearing on two legs, also recording the exploratory behavior of animals; (3) grooming, a parameter related to the behavioral response to anxiety; and (4) defecations, as a neurophysiological response related to stress and anxiety. The open-field device was cleaned with 96 % ethanol and air-dried before each animal was tested.

The *rotarod* test was used to measure both motor coordination and motor learning. This test was performed with an acceleration of 0.6 rpm/s, from 4 to 40 rpm, in 10 min. Seven sessions per animal were conducted with 20 min between the beginning of each session and the next one. For data analysis, the revolution per minute of each animal at the moment of falling off the device was recorded automatically. The device was cleaned with 96 % ethanol before and after each session.

Data analysis

First, the fit of the samples to the assumptions for parametric tests was examined. Samples were found to be unbalanced and asymmetric at some points, a condition that has been demonstrated to be enough to reject the use of parametric analysis (Fagerland 2012). Although both homoscedasticity and normality only fit at some time points, to avoid the use of different statistical procedures depending on each situation, and to achieve the best consistency of the results, we decided to carry out a statistical analysis that would perform best in all situations found. Accordingly, parametric analysis bias becomes extremely exacerbated. Meanwhile, the nonparametric analytical Mann–Whitney *U* test works well in all the cases found, allowing us to perform the same statistical analysis with the minimum effect on its robustness (Fagerland 2012; Kitchen 2009; Vickers 2005). Consequently, comparisons were performed with the nonparametric Mann–Whitney *U* test for two independent samples. Thus, to study our first hypothesis at T0, the basal behavioral differences between the genotypes of each sex were analyzed (WT males vs. NOS1−/− males; WT females vs. NOS1−/− females). For the study of the second hypothesis, the differences in basal behavior between the sexes of each genotype were studied (WT males vs. WT females; NOS1−/− males vs. NOS1−/− females). From T4 onwards, the nicotine variable was added, and hence, finally, four groups for each genotype were studied for data analysis. Thus, possible differences between nicotine-treated mice, related to both genotype and sex, were also studied, depending on the hypothesis. Consequently, in order to understand the influence of each variable individually, pairs of groups were confronted statistically based on our two hypotheses.

Results

Open-field test

Basal evaluation (T0)

Under basal conditions, the open-field test did not reveal differences related to sex. However, regarding genotype, WT and NOS−/− females differed in vertical movement (rearings; $U=91$ and $p=0.021$; Fig. 2c), while no differences were detected in males (Fig. 2).

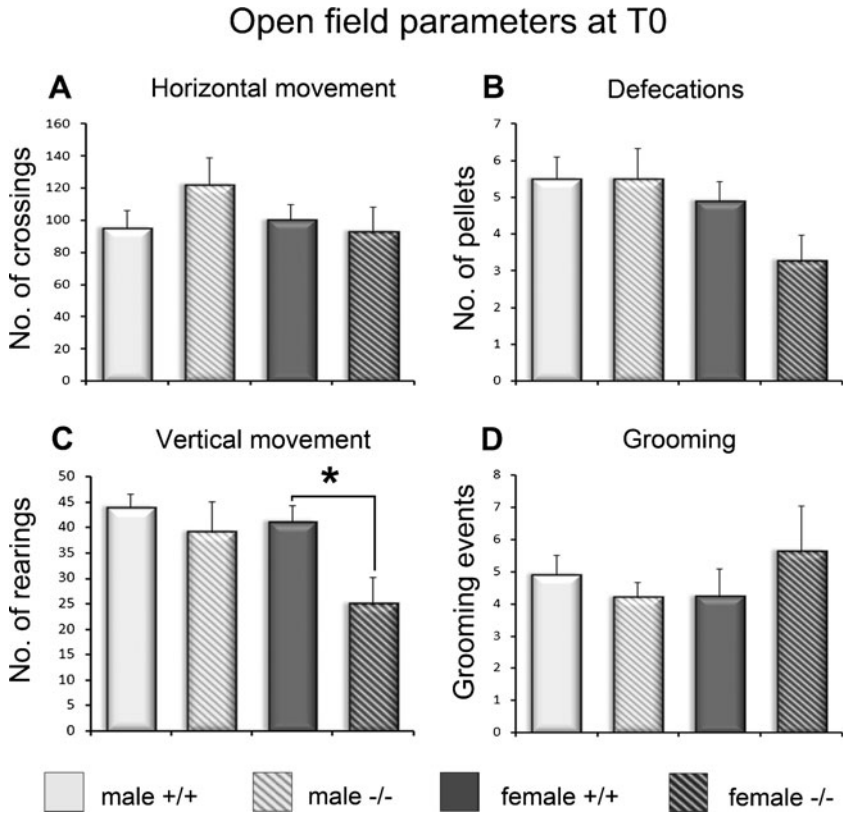

Fig. 2 **a**–**d** Open-field parameters in the basal situation (T0): no differences in motor behaviors or anxiety-related responses were found. However, the NOS1−/− females showed fewer vertical movements (rearings) than the WT females, indicating a diminished exploratory behavior in new environments. *$p<0.05$

Evaluation of nicotine administration and cessation (T4–T16)

The main target of this work was to determine the effect of nicotine administration and its interaction with NO on general motor behavior, taking into account possible influences due to sex. In order to facilitate the data analysis and comprehension, we compared both genotypes for each sex and both sexes for each genotype.

Analysis between genotypes: males No differences were found in the horizontal (Fig. 3) or vertical (data not shown) movements of males. However, along the experimental days, our data revealed a higher number of defecations in the control (nontreated) NOS1−/− males as compared to the control WT ones (Fig. 4; T4, $U=12$ and $p=0.012$; T9, $U=6.5$ and $p=0.001$; T14, $U=15.5$ and $p=0.027$), indicating a higher anxiety-related neurophysiological response during the saline treatment. In addition, differences in the grooming behavior at T9 were also observed ($U=12$ and $p=0.012$) between the control WT and control NOS1−/− males (data not shown). Surprisingly, within the males, nicotine administration completely eliminated all these differences in defecations and grooming (Fig. 4).

Thus, basal levels of neural NO seemed to have an anxiolytic effect in males, the neurophysiological response related to stress and anxiety being exacerbated with the genetic depletion of NOS1. Furthermore, nicotine administration completely eliminated these anxiety-related neurophysiological responses in the NOS1−/− mice, compensating NO depletion.

Analysis between genotypes: females Data analysis of the genotypes of the control females did not reveal differences in any parameter studied in the open-field test. However, the nicotine-treated females differed in horizontal movement on the last 2 days of nicotine administration (T9, $U=8$ and $p=0.026$ and T14, $U=5$ and $p=0.008$; Fig. 3), NOS1−/− females being less active than the WT females.

In this sense, NOS1 depletion seemed to have a stronger effect on males than females. In addition, nicotine administration also had a sexual dimorphic effect, appearing early and being maintained in males, whereas it was more delayed in females.

Analysis between sexes: wild-type mice No sex differences were observed in WT mice in the motor parameters of the open-field test (horizontal and vertical movements and grooming; Fig. 3). However, the control WT males showed a higher number of defecations than the females at T4 ($U=23.5$ and $p=0.043$; Fig. 4).

Analysis between sexes: NOS1−/− mice NOS1−/− mice did not show differences in their movement parameters (horizontal and vertical movements and grooming). However, at T4 ($U=0.5$ and $p=0.002$), T9 ($U=2.5$ and $p=0.016$), and T14

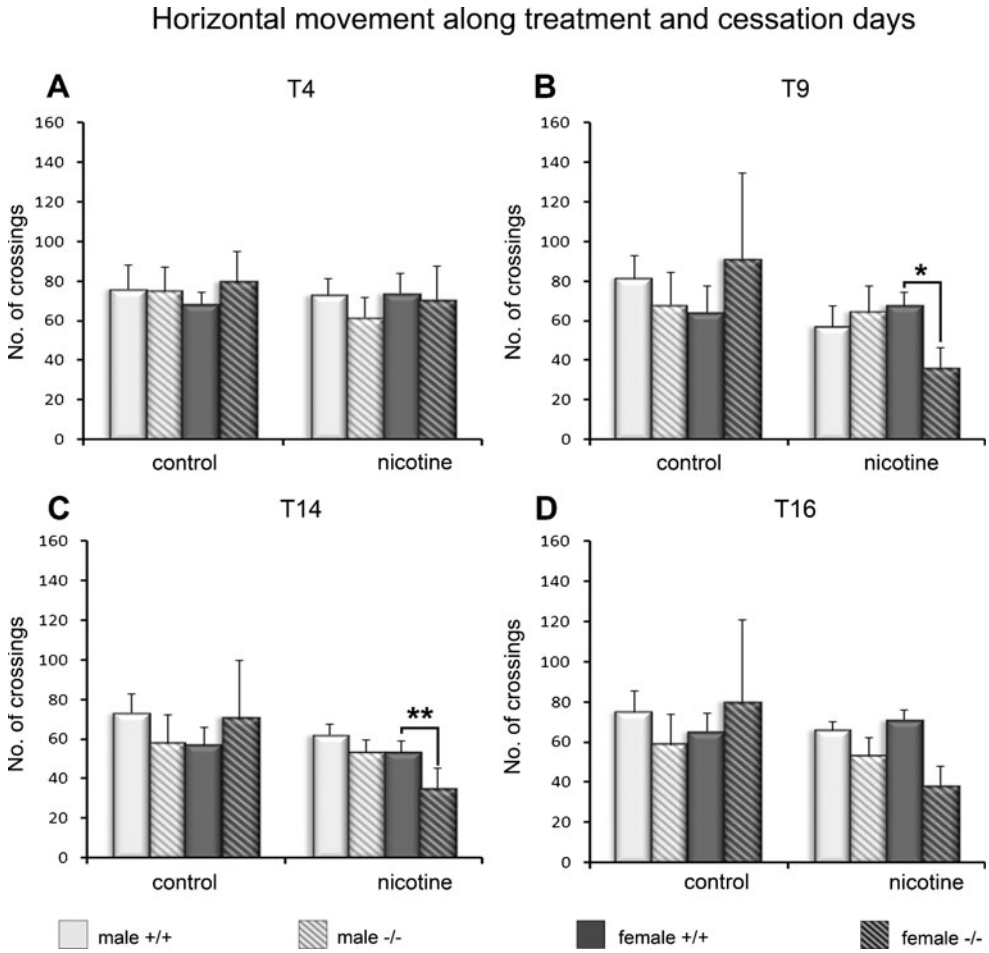

Fig. 3 Horizontal movement along nicotine administration (T4 to T14, **a**–**c**) and cessation (T16, **d**). The data only show differences between the WT and NOS1−/− nicotine-treated females at the end of drug treatment but not at the beginning or after drug cessation. *$p<0.05$; **$p<0.01$

($U=3$ and $p=0.011$), the control NOS1−/− males exhibited a higher number of defecations than the females, an effect that also disappeared with nicotine treatment (Fig. 4). According to the results reported above, it seems that NOS1 activity could play an important role in male habituation to new environments, modulating their anxiety-related neurophysiological responses, although it is irrelevant in females.

In sum, NO seemed to modulate anxiety-related neurophysiological responses, especially in males, which showed the highest number of defecations. In this sense, nicotine had an earlier and persistent anxiolytic-related effect in NOS1−/− males, and a time-delayed anxiogenic-related response in females. Thus, nicotine seems to compensate the behavioral effects derived from NOS1 depletion in an early and persistent manner in males.

Rotarod test

Basal evaluation (T0)

Under basal conditions, motor coordination in the rotarod test did not reveal differences related to genotype. However, differences between male and female NOS1−/− mice were found ($U=45$ and $p=0.034$), but not between the sexes of the WT animals (Fig. 5).

Evaluation of nicotine administration and cessation (T4–T16)

The second objective of this work was to evaluate the effect of nicotine administration and NO on motor coordination along the test days, attending to possible sex differences.

Analysis between genotypes: males Along the test days, the data analysis revealed significant differences in rotarod performance between the control (saline-treated) NOS1−/− and control WT males at T9 ($U=6.5$ and $p=0.001$), T14 ($U=10$ and $p=0.006$), and T16 ($U=10$ and $p=0.006$; Fig. 6): the knockout males had a lower rotational speed performance (i.e., a lower latency time) than the WT males. Similarly, the nicotine-treated NOS1−/− males showed the same differences (T9, $U=1$ and $p=0.000$; T14, $U=4$ and $p=0.001$; T16, $U=13$ and $p=0.016$). Thus, there would be a strong influence of NOS1 depletion on motor coordination in males and an absence of the effect of nicotine administration (Fig. 6).

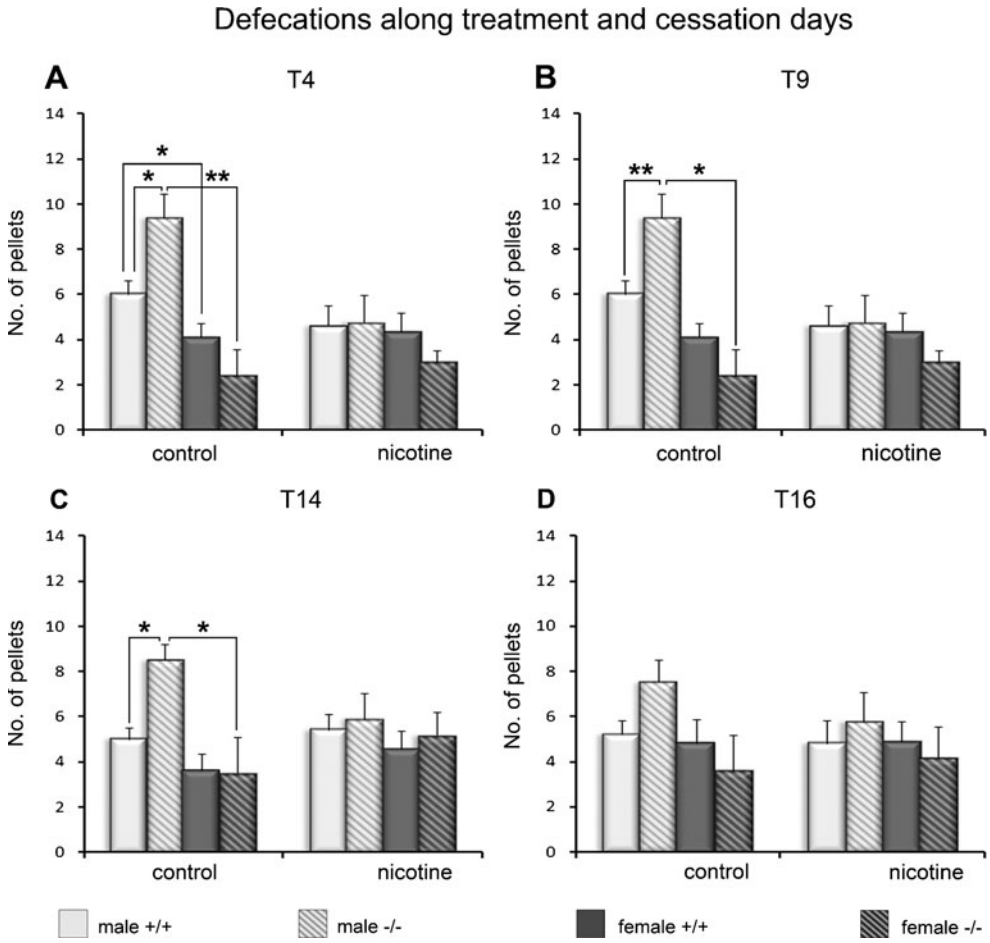

Fig. 4 Number of defecations during nicotine administration (T4 to T14, **a**–**c**) and nicotine cessation (T16, **d**). The control NOS1−/− males showed a higher number of defecations than the control WT males and control NOS1−/− females from T4 to T14. However, the nicotine-treated NOS1−/− males did not show differences with respect to the nicotine-treated WT males and nicotine-treated NOS1−/− females. In addition, the control WT males defecated more than the control WT females only at T4. *$p<0.05$; **$p<0.01$

Analysis between genotypes: females Surprisingly, control NOS1−/− and WT females had similar scores on the rotarod test along all days tested (T4–T16; Fig. 6). However, differences were found between nicotine-treated WT and NOS1−/− females on the last 2 days of administration of the drug, at T9

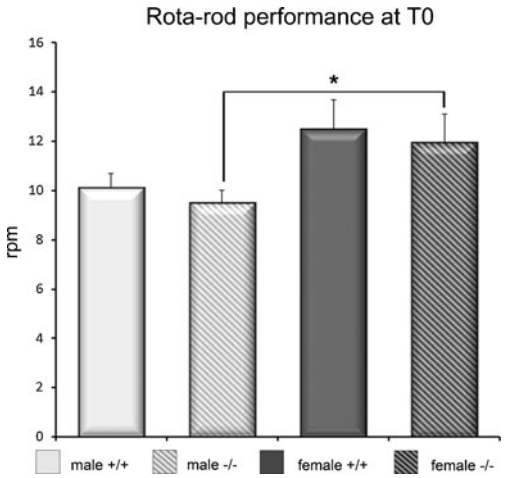

Fig. 5 Rotarod performance in the basal situation (T0). No differences between genotypes were found, except between theNOS1−/− males and females. *$p<0.05$

($U=8$ and $p=0.026$) and T14 ($U=10.5$ and $p=0.05$; Fig. 6). Hence, as seen in the open-field test, nicotine administration in NOS1−/− females seemed to have a delayed effect, being significant only after several days of administration.

Analysis between sexes: wild-type mice No differences in motor coordination between the sexes were apparent in the control WT mice. However, when the WT animals were treated with nicotine, the females had a better rotarod performance than the males at T4 ($U=20.5$ and $p=0.043$) and T9 ($U=18$ and $p=0.028$), but not thereafter (Fig. 7). Therefore, nicotine treatment would lead to sex differences in the motor coordination of WT mice at the beginning of the treatment (i.e., in a short-term manner).

Analysis between sexes: NOS1−/− mice Along the test days (T4–T16), an unexpected decrease in the performance of the motor coordination test was found in the control NOS1−/− males in relation to the females ($U=5.5$ and $p=0.03$; $U=6$ and $p=0.045$; $U=3$ and $p=0.011$; $U=5$ and $p=0.03$, respectively; Fig. 7). Moreover, in the nicotine-treated NOS1−/− mice, this difference was not detectable until after 9 days of drug treatment, including the nicotine cessation time point

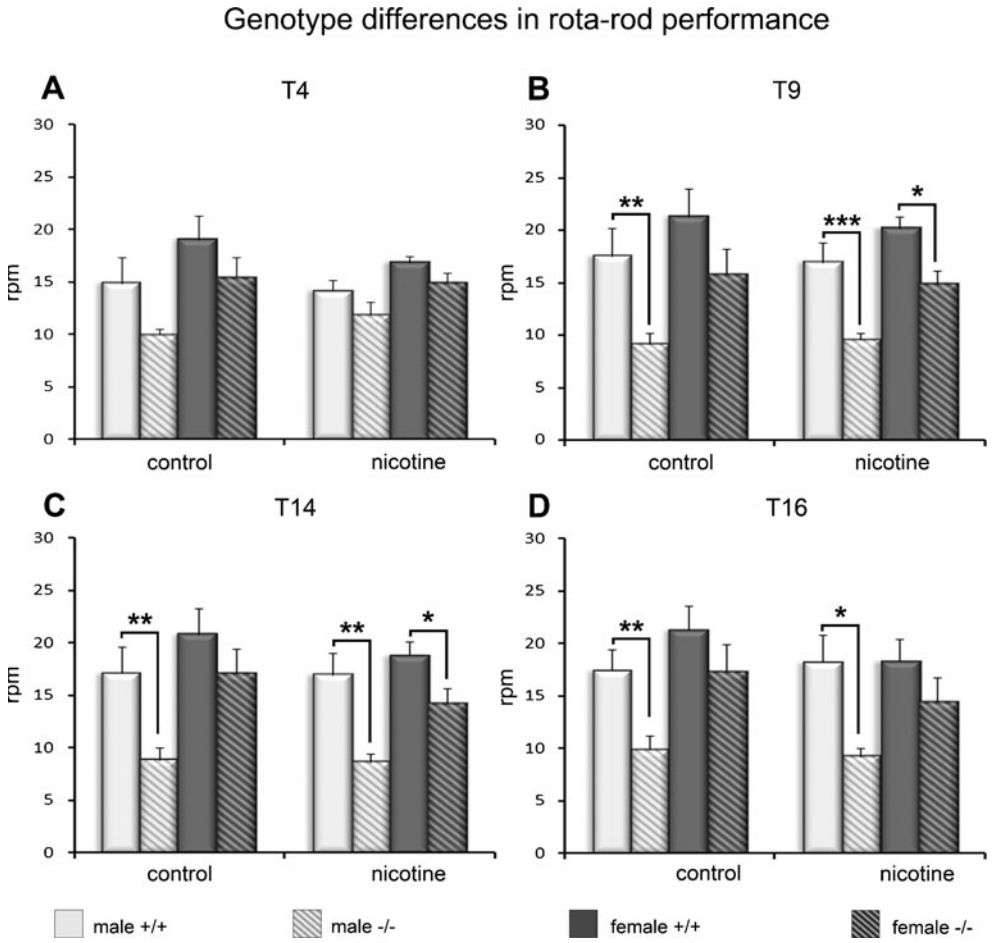

Fig. 6 Genotype differences in rotarod performance (revolutions per minute; mean ± SEM) during nicotine administration (T4–T14, **a**–**c**) and cessation (T16, **d**). From T9 to T16, the control NOS1−/− males had a diminished performance in relation to the control WT males. Moreover, the nicotine-treated NOS1−/− males also showed these differences in relation to the nicotine-treated WT males (T9–T16). The control NOS1−/− females did not show differences with the control WT females. However, the nicotine-treated NOS1−/− females showed differences with the nicotine-treated WT females on the last 2 days of administration (T9 and T14), but not after nicotine cessation (T16). *$p<0.05$; **$p<0.01$; ***$p<0.001$

(T9, $U=2$ and $p=0.003$; T14, $U=2$ and $p=0.003$; T16, $U=8$ and $p=0.043$). Similar to the situation occurring in WT animals, nicotine-treated NOS1−/− males had diminished motor coordination as compared to the corresponding females (Fig. 7). Accordingly, NOS1−/− males showed a diminished motor coordination compared to NOS1−/− females, which emphasizes the highly sexual dimorphic effect of NO in motor coordination improvement along the test days.

In summary, our data from the rotarod experiments strongly support a sexual dimorphic effect of NOS1 activity in motor coordination. In this sense, NO seems to be essential for the motor coordination improvement of males but not of females.

Discussion

Our results show different patterns of environmental adaptation in the NOS1−/− mice. The NOS1−/− males showed a specific, increased anxiety-related neurophysiological response; nicotine infusion completely eliminated these differences, acting as an anxiolytic in the NOS1−/− males. Further, motor coordination improvement along test days was strongly affected in the NOS1−/− males but not in the females.

General motor behavior

WT males and females show different anxiety-related neurophysiological responses in the open-field test

Summarizing our results, the control WT males showed a higher anxiety-related neurophysiological reactivity than the females on the second day (T4; Fig. 4). This parameter has been associated, among other factors, with reactivity to new environments (Bindra and Thompson 1953; Hall 1943). Therefore, in agreement with previous results, WT females show a faster adaptation to the open-field arena than males (Frick and Gresack 2003; Palanza 2001).

The neurophysiological response related to anxiety derived from the depletion of NOS1, which is sexually specific, is abolished by nicotine administration

When compared with WT animals, the general motor behavior of the knockout mice was more heterogeneous. The control (saline-treated) NOS1−/− males showed a higher number of defecations than the control WT males and the control NOS1−/− females (Fig. 4), indicating a strong sex-

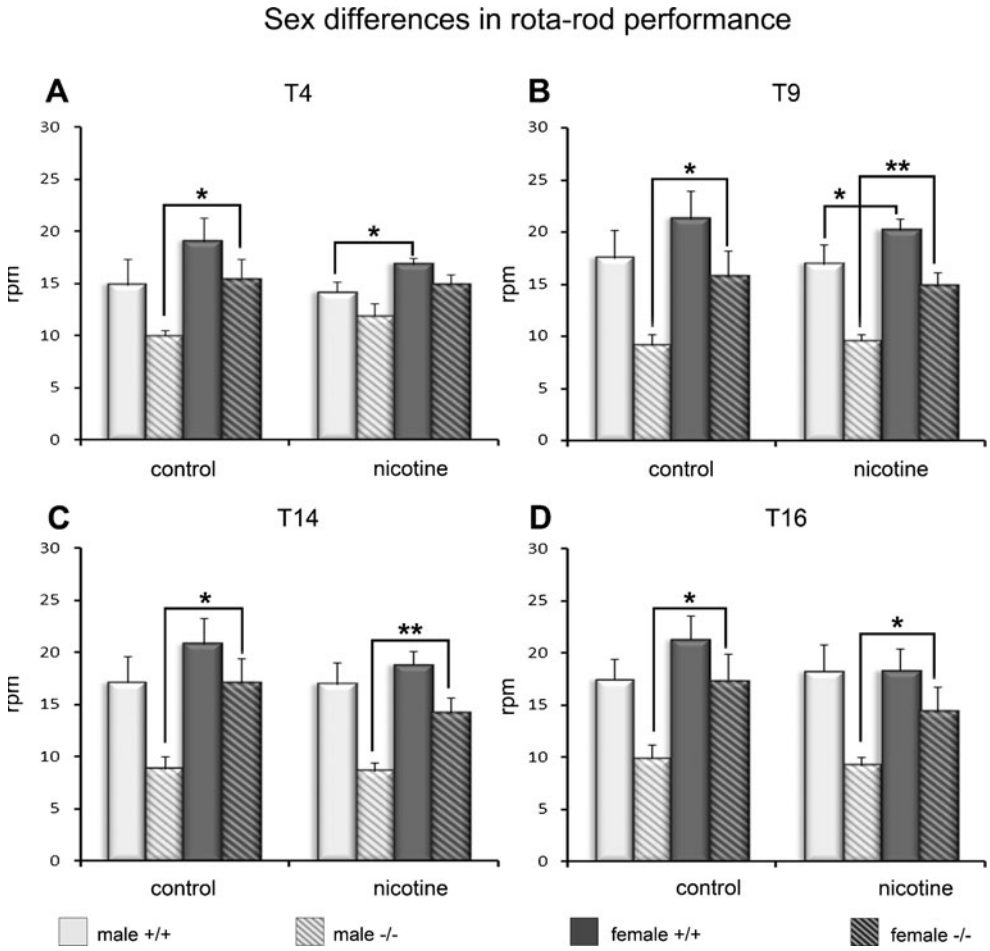

Fig. 7 Sex differences in rotarod performance (revolutions per minute; mean ± SEM) during nicotine administration (T4–T14, **a**–**c**) and cessation (T16, **d**). Along the treatment and cessation days (T4–T16), the control NOS1−/− males had a diminished performance with respect to the control NOS1−/− females. However, the nicotine-treated NOS1−/− males showed differences with respect to the nicotine-treated NOS1−/− females from T9 to T16, but not at T4. The control WT mice did not show sex differences in rotarod performance along the test days. Nevertheless, the nicotine-treated WT males showed a poorer rotarod performance than the nicotine-treated WT females at T4 and at T9. *$p<0.05$; **$p<0.01$

specific effect of NOS1 depletion in the anxiety-related neurophysiological response. Taking into account that NO and sexual hormones, especially estrogens, interact and influence neurotransmitter release (Castel and Vaudry 2001; Chanrion et al. 2007; Fink et al. 1996; Fossier et al. 1999; Garthwaite 2007; Joffe and Cohen 1998; Rubinow et al. 1998), NOS1 depletion probably influences anxiety-related behavior in a sex-dependent manner. This idea is supported by previous results indicating that NO depletion affects serotonin clearance in males (Chiavegatto et al. 2001), serotonin being the most widely studied neurotransmitter related to anxiety and mood disorders (Blanchard et al. 1991, 1995; Hale et al. 2012; Lowry et al. 2008; Maier and Watkins 2005; Zohar and Westenberg 2000). However, to our knowledge, no comparative studies have been carried out between NOS1−/− males and females as regards the serotonergic or other neurotransmission systems.

Interestingly, nicotine infusion completely eliminated this sex difference in the anxiety-related neurophysiological response (Fig. 4). Thus, constant and continuous nicotine administration seems to act as a potent anxiolytic in NOS1−/− males. This is in accordance with previous results indicating a modulatory role of nicotine on the serotonergic system, hence influencing anxiety-related behavior (Benwell and Balfour 1982a, b; File et al. 2000; Seth et al. 2002; Suemaru et al. 2006).

Taken together, our data support the idea of a modulatory action of NO in anxiety-related neurophysiological responses (López-Figueroa et al. 1998; Weruaga et al. 2002). Moreover, we show for the first time that this NO release from NOS1 sources has a stress-related neurophysiological response that is sexually dimorphic. Additionally, constant and continuous nicotine administration seems to produce a highly anxiolytic response in NOS1−/− males but not in females. Taking our own results and those reported in previous works together, the anxiety-related neurophysiological sex differences could be related to a sexual dimorphism of the neurotransmission systems in NOS1−/− mice.

Nicotine affects the motor parameters of NOS1−/− mice in a dimorphic manner

We only detected changes in vertical movements (rearing) in the open-field test of the control NOS1−/− females in comparison with the control WT females in the basal condition (T0; Fig. 2). This behavior has been related to the reactivity of mice to new environments and novelty seeking (Adriani et al. 2003;

Denenberg and Grota 1964). Accordingly, our data reveal a sex-specific effect of NO release from NOS1 on the initial reaction of females to new environments, since no differences were observed between the WT and NOS1−/− males.

On the last two testing days (T9 and T14), nicotine administration reduced horizontal movements in NOS1−/− females as compared to WT females, although the NOS1−/− males were unaffected by nicotine infusion (Fig. 3). Previous authors have related hypothalamic neurotransmission to motor control (Jessop 1999; Lee et al. 2009; Patel et al. 2012; Ter Horst et al. 1984; Wayner 1970) and nicotine-induced motor responses (Semba et al. 2004; Yu et al. 2008). Moreover, gonadal hormones, especially estrogens, influence hypothalamic neurotransmission and NO release in WT females (Gingerich and Krukoff 2005; Shih 2009). These data support a sex-specific effect of NOS1 depletion in nicotine-induced motor responses.

Thus, the open-field data strongly support the idea of a dimorphic effect derived from both the NO release from NOS1 and the nicotine administration. In this sense, NOS1 deletion seemed to produce a persistent anxiogenic response in males but a short-term effect in females, only seen at the baseline (T0). Moreover, the NOS1−/− males were affected by nicotine administration in a short-term and persistent manner, eliminating the anxiogenic effect of NOS1 deletion, while in terms of motor parameters, the effect on females was delayed in time.

Motor coordination

Only nicotine-treated WT mice showed sex differences in rotarod performance

The results from the rotarod test did not point to sex differences in the motor coordination of the control WT mice (Fig. 5). Nevertheless, the nicotine-administered WT mice showed sex differences in motor coordination on the first 2 days of nicotine administration (T4 and T9; Fig. 7), which could be related to the learning of motor coordination.

NOS1−/− mice have a strong deficit in motor coordination learning/memory

Control NOS1−/− male mice did not show initial differences in motor coordination compared to control WT males (Fig. 6). However, the motor coordination of the NOS1−/− male mice was decreased as from the third test day (T9) onwards (Fig. 6), which allowed us to infer a possible effect on motor coordination learning/improvement. Surprisingly, the control female NOS1−/− mice also showed differences with respect to the control NOS1−/− male mice, but not with the control WT females (Figs. 6 and 7). This variability in motor coordination can be related to cerebellar function. In this sense, it is widely accepted that long-term cerebellar changes in synaptic transmission, LTD, and long-term potentiation (LTP) are the main electrophysiological processes underlying cerebellar motor learning and memory (Ito 2002; Kakegawa et al. 2011; Lev-Ram et al. 1997; Roberts et al. 2013; Swinny et al. 2005; Thach 1998). Moreover, cerebellar NO is considered to be crucial for long-term cerebellar changes to be generated in synaptic transmission (Crepel and Jaillard 1990; Lev-Ram et al. 1995, 1997; Shibuki and Okada 1991). Although it has been extensively shown that NOS1−/− mice have strong deficiencies in long-term cerebellar changes in synaptic transmission (Crepel and Jaillard 1990; Lev-Ram et al. 1997; Shibuki and Okada 1991), nothing is known about possible sex differences in the cerebellum. However, in the cerebral cortex, it has recently been shown that long-term plasticity is absent in NOS1−/− males but not in females (Dachtler et al. 2012). Thus, our results support the notion of sexual dimorphism in the action of NOS1 during motor coordination learning and memory, possibly due to differences in the molecular bases of cerebellar synaptic plasticity. In this sense, the noradrenergic (NA) system enhances an essential cGMP pathway in the induction of NO-dependent long-term plasticity (Ito 2002). However, estrogens activate the cerebellar NA system, thus influencing cerebellar plasticity in a sexually dimorphic manner (Andreescu et al. 2007; Etgen and Petitti 1987; Freedman et al. 1977; Moises et al. 1979; Morton and Bredt 1998). Consequently, it is plausible that the lack of NOS1 in the cerebellum could be compensated by an enhancement in the NA system through the action of estrogens. This idea is also supported by the fact that cGMP is enhanced in the cerebellar cells of NOS1−/− mice (Morton and Bredt 1998). Finally, testosterone does not affect the cerebellar NA system (Siddiqui and Gilmore 1988; Simpkins et al. 1980), in accordance with the proposed hypothesis. Thus, NO release from NOS1 sources seems to be necessary for motor coordination learning and memory in males but not in females.

Nicotine administration affects the motor coordination of NOS1−/− females

Although the NOS1−/− control females did not show differences in the rotarod test in comparison with control WT females, nicotine administration diminished the motor performance of the drug-treated NOS1−/− females on the last 2 days of its administration as compared with the treated WT females (T9 and T14; Fig. 6). This poorer motor coordination on the last 2 days of drug treatment combined with the diminished horizontal movement seen in the open-field could be related to a delayed effect on general motor behavior in NOS1−/− females derived from nicotine administration.

Finally, although to our knowledge there is no work addressing possible sex differences in long-term cerebellar changes in synaptic transmission, recent data on the cerebral cortex in NOS1−/− mice have unveiled a sexual dimorphism

in LTD (Dachtler et al. 2012). In this sense, here we also report for the first time sex differences in the motor coordination learning and memory of NOS1−/− mice. Accordingly, the molecular basis of cerebellar-dependent learning and memory seems to be sexually dimorphic since its behavioral expression is also dimorphic.

Conclusions

In male but not in female rats, NO depletion produces a remarkable impact on anxiety-related neurophysiological responses and on motor coordination learning. Regarding nicotine, the constant and continuous administration of this drug completely abolished this higher anxiety-related neurophysiological response of NOS1−/− male mice, although it exerted a reduction in NOS1−/− female horizontal movement and motor coordination only at the end of the treatment. This specific effect leads us to hypothesize a general effect on motor behavior due to the combined effect of nicotine administration and the lack of NOS1.

This sexual dimorphism in NOS1−/− mice supports the idea of a sex-specific effect of NOS1 deletion in neurotransmission, especially in the serotonergic system, which has previously been related to both motor behavior (Jessop 1999; Ter Horst et al. 1984; Wayner 1970) and anxiety (Julio-Pieper et al. 2012; Miyata et al. 1992; Sanger et al. 2000). Moreover, our results, together with recent data reporting sex differences in cortical LTD (Dachtler et al. 2012), support the idea of a sex-specific effect of NOS1 deletion in neurotransmission, including motor coordination learning and memory basis.

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