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Selective inhibition of neuronal nitric oxide synthesis reduces hyperactivity and increases non-selective attention in the Naples High-Excitability rat

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Abstract

The involvement of neuron-derived NO in the process of orienting and scanning times (non-selective attention: NSA) towards environmental stimuli has been investigated in the Naples High-Excitability rat (NHE), a putative animal model of Hyperactivity and Attention Deficit (ADHD). To this aim, orienting and scanning times have been monitored by the frequency and duration of rearing episodes, respectively. Adult male NHE rats were tested in a novelty situation (Làt-maze) for 30 min following single or repeated injections of the non competitive inhibitor 7-Nitroindazole (7-NINA) of the neuronal isoform of the enzyme nitric oxide synthase (n-NOS). In the acute experiments, rats received a single injection of 7-NINA (1 mg/kg) intraperitonealy in a saline vehicle (exp. 1, fast release) or subcutaneously in a lipid carrier, dimethyl sulfoxide (DMSO; exp. 2, slow release) or the vehicles alone as controls 30 min before testing. In the repeated injection experiments, rats received a subcutaneus injection of 1 mg/kg in DMSO or DMSO alone daily for 14 days, and tested 24 h after the last injection (exp. 3, slow release). The results showed a significant differential effect of the drug that was dependent on the release rate, i.p. saline-diluted 7-NINA increased the duration of individual rearing episodes whereas, both single and repeated subcutaneous DMSO-carried 7-NINA exerted an opposite effect. Thus, selective inhibition of n-NOS by an allosteric inhibitor that increases arginine availability without displacing the inhibitor from n-NOS, strengthens the hypothesized role of NO in NSA. These findings may shed light on the mechanism of action of drug treatment of and be useful in the treatment of ADHD in children. (Supported by Telethon-Italy grant E.513).

Keywords: Neuronal nitric oxide synthase; 7-Nitroindazole; Naples high-excitability rats; Rearing frequency; Rearing duration; NSA; ADHD

1. Introduction

An extrasynaptic mode is known to coexist with the synaptic one in the information processing and transfer in the CNS (see e.g. [11]). The former is based on a volume conduction mechanism [11] that implies ions such as K^+, peptides, hormones, prostanoids, and gaseous mediators [nitric oxide (NO)] [18] which exerts a diffuse influence on large neuronal populations.

The duration of individual rearing episodes on the hindlimbs by rats in a spatial novelty situation has been shown to index NSA [5].

Information processing and transfer in different sensory motor domains can be modulated to a great extent by attentive processes. They can be of the selective (SA) or non selective type (NSA). The former has been mostly studied in the visuo-spatial attention that involves the pulvinar and the posterior parietal cortex [23]. In contrast, NSA involves orienting, scanning and detection of stimuli followed by analysis in the SA mode. It pertains to salience of stimuli determined by the motivational state of the animal along with the stimulus properties (reward expectancy [29,33]. The neural substrate of NSA is represented by the so-called

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anterior system [22] localised in the frontal cortex and modulated by multiple subcortical influences, (see [4]).

The major pathology of attentive processes is represented by attention deficit-hyperactivity disorder (ADHD; DSM-IV, [2]; see [32] for a review).

In order to investigate the network operations involved in attentive processes, defective genetic models have been used such as the SHR [17,28], the Wistar–Kyoto hyperactive (WKHA; [15]), and the Naples high-excitability (NHE; see e.g. [8]) rat lines, given the high genetic contribution to ADHD. These rat lines are all hyperactive though to a different degree [12] and in different contexts [27,28] whereas SHR and NHE have been shown to be inattentive [5].

Recently, we have demonstrated in two animal models of ADHD, i.e. the SHR and NHE rats, a pronounced sensitivity to acute inhibition of NO synthesis [5]. In fact, a single intraperitoneal injection of L-NAME (0.1–10 mg/kg) produced a significant increase in the duration of rearing episodes in both rat lines at the highest dose (10 mg). In addition, repeated administration of L-NAME for 2 weeks exerted a paradoxical effect, consisting in a shortening instead of a lengthening of rearing duration. This finding was interpreted as increased availability of NO precursor L-arginine. To determine the site of NO synthesis inhibition in these experiments we have used 7-nitroindazole (7-NINA), a 35-fold selective antagonist for the neuronal isoform of NOS, compared with L-NAME [19]. Furthermore, 7-NINA was chosen for its allosteric mechanism of action, thus by-passing the arginine uptake step that could bias the NO synthesis towards a stimulation rather than an inhibition.

The aim of the studies reported here was to investigate the role of NO in NSA in the NHE rat line.

2. Materials and methods

2.1. Animals

Male NHE rats from our animal colony were used throughout the experiments. They were housed two per group in standard makrolon cages with water and food pellets (Mucedola, Italy) ad libitum. All other parameters fulfilled the requirements of the 'Guide for the Care and Use of Laboratory Animals' by the National Research Council, implemented by EU rules [16].

2.2. Apparatus

The experimental system was a Làt-maze, a $60 \times 60 \times 40$ cm wooden box with a $30 \times 30 \times 40$ cm plastic transparent smaller box inserted in the middle. Rats were allowed to explore the resulting corridor (60 cm long, 15 cm wide and 40 cm high). The experimental box was illuminated by a white, cold 4 W lamp placed 60 cm above the floor in the center of the wooden cover, providing a 0.1–0.2 $\mu W/cm^2$.

2.3. Drugs

The neuronal isoform of nitric oxide synthase (n-NOS) 7-NINA was purchased from TOCRIS (UK). In the fast release experiments the drug was dissolved in a 2-step procedure, in a 40% v/v ethanol solution and then essicated in an oven at 37 °C and newly dissolved in saline solution. Alternatively, the slow release experiments the drug was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich).

2.4. Drug treatment

In the single injection experiments, NHE rats were given intraperitoneally 7-NINA (0.0, 0.1, 1, 10 mg/kg) in saline vehicle (single fast release, S-FR), or subcutaneously 7-NINA (0.0 or 0.1 mg/kg) in DMSO (single slow release, S-SR). In the multiple injection experiment, another group of NHE rats were given subcutaneously 7-NINA (0.0 or 0.1 mg/kg) in DMSO daily for 14 days (multiple slow release, M-SR). The volume of saline and DMSO vehicle was 2 ml/kg.

2.5. Experimental procedure

In the S-FR experiments NHE rats were exposed to a Làt-maze during three 10 min tests at 24 h interval or during a single 30 min test in the S-SR experiment 0.5 h after injection. In the M-SR experiment, NHE rats were exposed to a Làt-maze during a single 30 min test 24 h after the last drug or vehicle injection. Behavioural testing was carried out at the beginning of the light phase of the circadian cycle on both members of the same cage to minimise the interference with the arousal state and with the post-trial period. The behaviour was monitored by a CCD camera and videotaped to be analysed off-line. The behavioural variables, i.e. frequency and duration of rearings on hindlimbs and leanings against the walls with one or both forepaws were visually monitored in 3 min blocks. At the end of the test, the number of fecal boluses was counted and the floor was carefully cleaned with a wet sponge. The reliability index was quite high ($r = 0.914$; $df = 198$; $P < 0.001$).

2.6. Data analysis and statistics

Frequency and duration of rearings are presented as mean $\pm$ S.E.M. of 7-NINA treated NHE rats, expressed as percent of vehicle treated controls with zero indicating the vehicle level. Data were submitted separately two-way factorial analysis of variance (ANOVA) treat-

ment × time blocks, as dependent variable. Planned comparisons between group means were carried out by two-tailed t-test for non-paired data [9]. The rejection level was set at $P > 0.05$, two-tailed.

3. Results

3.1. Inhibition of neuronal oxide synthesis by 7-NINA

3.1.1. Single injection: fast and slow release

3.1.1.1. Rearing frequency. As shown in Fig. 1, the frequency of rearing episodes was significantly lower in NHE rats receiving 1 mg/kg of 7-NINA in saline (fast release) than in DMSO (slow release). The effect of 7-NINA in saline vehicle pertained to the entire testing period, though it was more pronounced in the first part (-58.6%; $t = 7.111$, $P < 0.001$) than in the second part (-21.9%) of the testing period though borderline. In contrast, there was a time dependent differential drug effect in DMSO vehicle with an increase in the second part ($+31.4\%$) at borderline level and no effect in the first part of the testing period ($+8.1\%$).

3.1.1.2. Rearing duration. As shown in Fig. 2, the duration of rearing episodes was significantly higher in NHE rats receiving 1 mg/kg of 7-NINA in saline (fast release) than in DMSO (slow release). The effect of 7-NINA in saline vehicle pertained to the entire testing period, though it was more pronounced in the second part ($+75.7\%$; $t = 2.954$, $P < 0.02$) than in the first part ($+41.7\%$; $t = 7.798$, $P < 0.001$) of the testing period. In contrast, the drug effect in DMSO vehicle was in the opposite direction towards a shortening of rearing duration and pertained equally to both parts of the testing period (-22.1% and -23.0%; $t = 3.695$ and 3.186, $P < 0.01$ for both).

3.1.2. Multiple injections of 7-NINA

3.1.2.1. Rearing frequency. As shown in Fig. 1, the frequency of rearing episodes in NHE rats receiving multiple injections of 7-NINA (1 mg/kg, 14 days) in DMSO vehicle (slow release) and tested 24 h following the last injection demonstrated a small but significant effect in the first and the second part of the testing period ($+16.2\%$; $t = 2.659$, $P < 0.02$ and $+9.3\%$; $t = 2.171$, $P < 0.05$, respectively).

3.1.2.2. Rearing duration. As shown in Fig. 2, the duration of rearing episodes in NHE rats receiving multiple injections of 7-NINA (1 mg/kg, 14 days) in DMSO vehicle (slow release) and tested 24 h following the last injection showed a small decrease that was more pronounced in the second part (-23.8%; $t = 1.897$, $0.05 < P < 0.10$) than in the first part (-13.6%; $t = 1.935$, $0.05 < P < 0.10$) of the testing period.

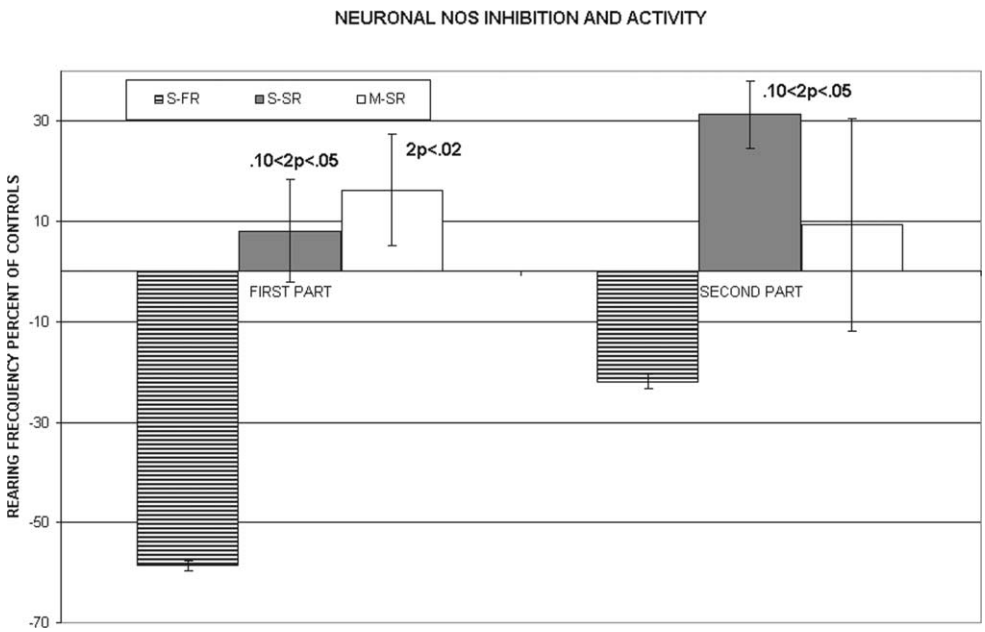

Fig. 1. Inhibition of neuronal n-NOS and activity in a spatial novelty. Rearing frequency (Mean ± S.E.M.) by 7-NINA treated NHE rat (1 mg/kg). Data are expressed as percent of vehicle treated controls with zero indicating the vehicle level for groups given a S-FR, a S-SR or M-SR injections. Significance values refer to comparison of drug vs. vehicle treated groups by two-tailed t-test for non-paired data, following two-way ANOVA.

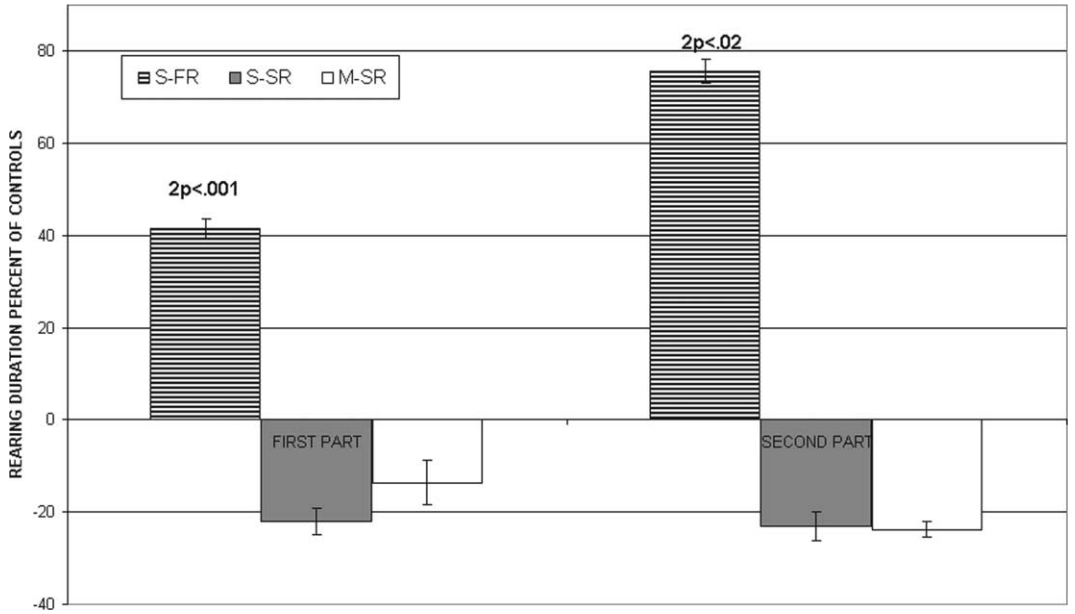

Fig. 2. Inhibition of neuronal n-NOS and NSA in the NHE rat. Rearing duration (mean ± S.E.M.) by 7-NINA treated NHE rats (1 mg/kg). Data are expressed as percent of vehicle treated controls with zero indicating the vehicle level for groups given a S-FR, a S-SR or M-SR injections. Significance values refer to comparison of drug vs. vehicle treated groups by two-tailed t-test for non-paired data, following two-way ANOVA.

4. Discussion

Within the domain of genetic models of ADHD, both SHR and NHE rats show in a special novelty situation higher frequency of rearing episodes of shorter duration than controls, along with higher horizontal activity in a spatial novelty situation (locomotion; [5]).

Since L-NAME blocks all isoforms of NO synthase, we used 7-NINA that is highly selective in blocking (see Section 1) the neuronal isoform of NOS. The first major finding is that a single injection of 1 mg/kg of 7-NINA differentially affected a measure of activity and NSA in the NHE rats in a time-dependent manner. In fact, when carried in a vehicle such as saline and given intraperitonealy allowing a fast release, the drug significantly decreased activity in the first more than in the second part of the testing period. This was paralleled by an increase in NSA more in the second part. The second major finding was that the drug in a vehicle such as DMSO and by the subcutaneous root allowing a slow release, produced a quite different effect. As a matter of fact, a single injection of 1 mg/kg of 7-NINA in DMSO produced a reduction in orienting frequency only in the second part of the testing period, whereas it produced shorter scanning times in the entire testing period.

Orienting and scanning phases of NSA in a spatial novelty are known to be modulated by multiple subcortical influences of different nature exerted by five systems (see for a review [5]).

There is a dual regulation of such modulatory inputs, at the site of origin of these neurons at the base of the brain and at the target sites. In addition, NO is known to affect synaptic neurotransmission and efficacy at both sites. Although the different aminergic influences have been postulated to affect different aspects of behaviour [24] and the integration among the different systems is yet to be ascertained, there is a wealth of data indicating that the mesocorticolimbic (MCL) dopamine system plays a crucial role in the modulation of these early attentive phases [24]. Conversely, several lines of evidence both in vivo and in vitro lend support to the hypothesis that ADHD are associated to a hyperfunctioning MCL dopamine system in genetic models and ADHD children [25]. In fact, a mouse model of ADHD bearing a knock-out of DA D3 autoreceptors that normally regulate DA release at terminal sites shows pronounced hyperactivity [1,7]. In addition, in the juvenile SHR [26], we have recently shown evidence for a hyperfunctioning MCL system that differentially affects the dorsal and ventral striatum in the rostro-caudal plane [20]. The anterior portions of the striatum becomes 'hyporesponsive' to DA release due to the presence of an increased density of DA D-1 binding sites which are probably non functional and derive from altered pruning during development [3]. Further, this 'hypofunctioning segment' is due to uncoupling of DA D-1 receptor binding sites from transduction mechanisms such as Ca^{++}-Calmodulin dependent protein kinase (CaMKII) and transcription factors onto medium spiny GABA neurons projecting to ventral pallidum and the ventral tegmental area

(VTA). As a consequence, there is a weak feedback inhibition on these target neurons that eventually leads for instance to increased firing probability by VTA DA neurons. On the other hand, in the posterior portions of the shell of nucleus accumbens the increased DA release is associated to a lower density of D-2/D-3 autoreceptors [10,20]. Support to this hypothesis comes from the observation that repeated injections of a low dose of methylphenidate (3 mg/kg, 14 days) in the SHR block firing probability of VTA DA neurons. This is likely to be related to activation of D-2 hyperpolarizing autoreceptors. This, in turn, leads to increase the density of D-2/D-3 binding sites in the caudal shell of the n. accumbens to the level of control rats. Moreover, inhibition of DA release by blockade of endogenous cannabinoid transport has been recently shown to decrease activity and increase NSA to novelty in the SHR [6] and NHE [13] (rat line). Consonant with these findings, psychostimulants have been reportedly shown to ameliorate ADHD symptoms in ADHD children for five decades which have been attributed to reduced DA functioning [30,31].

Accordingly, it is tempting to speculate that selective inhibition of NO release at neuronal level by 7-NINA might decrease DA neurotransmission in the midbrain and at target forebrain sites. Interestingly, the differential effect of fast versus slow release of the drug lend further support to the hypothesized hyperfunctioning MCL DA system. This is probably due to the 'phasic' nature of inhibition of tonically hyperfiring DA neurons under basal conditions (low motivational level).

The third major finding comes from repeated subcutaneous injection of 7-NINA (1 mg/kg) in the slow release mode (DMSO). The rational for such experiments derives from previous findings that multiple subcutaneous injections of the non-selective NOS inhibitor of L-NAME (1 mg/kg) during 14 days produced a paradoxical shortening of rearing duration (see Section 1) [4]. As a matter of fact, repeated 7-NINA injections differentially affected orienting, frequency and scanning times (NSA). The former was increased in the first part while the latter was decreased in the second part of the testing period. First of all, rats where tested 24 h after the last injection and different evidences suggest a high reversibility of the modulatory mechanism influenced by NO. In fact, repeated injections of methylphenidate have been shown to modify DA receptor binding, CaMKII and transcription factors in the dorsal and ventral striatum of the SHR in a reversible manner with effects fading out within 72 h [21]. Therefore, repeated injections of 7-NINA should be followed in further experiments by behavioural testing within 0.5–2 h from drug withdrawal. Secondly, the root of administration of 7-NINA should be a fast rather than a slow release as the latter appears to be unable to 'phasically' switch the hyperfiring DA neurons. Thirdly, a role of NO on other non-dopaminergic modulatory influences cannot be excluded, as NO has been shown to modify neurotransmission for instance at cholinergic and glutamatergic synapses [14], thus modifying complex network-related operations in the attentional and motivational domains.

In perspective, the data lend support to a role of NO in the modulation of the orienting and scanning phases of attentive processes and to a hyperfunctioning DA system in ADHD in genetic models of ADHD in children. Finally, they might shed light to the understanding of the mechanism of action of drug treatment of and new strategies in the treatment of this syndrome.

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