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ORIGINAL ARTICLE

Prepuberal intranasal dopamine treatment in an animal model of ADHD ameliorates deficient spatial attention, working memory, amino acid transmitters and synaptic markers in prefrontal cortex, ventral and dorsal striatum

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Abstract Intranasal application of dopamine (IN-DA) has been shown to increase motor activity and to release DA in the ventral (VS) and dorsal striatum (DS) of rats. The aim of the present study was to assess the effects of IN-DA treatment on parameters of DA and excitatory amino acid (EAA) function in prepuberal rats of the Naples high-excitability (NHE) line, an animal model for attention-deficit hyperactivity disorder (ADHD) and normal random bred (NRB) controls. NHE and NRB rats were daily administered IN-DA (0.075, 0.15, 0.30 mg/kg) or vehicle for 15 days from postnatal days 28–42 and subsequently tested in the Làt maze and in the Eight-arm radial Olton maze. Soluble and membrane-trapped L-glutamate (L-Glu) and L-aspartate (L-Asp) levels as well as NMDAR1 subunit protein levels were determined after sacrifice in IN-DA- and vehicle-treated NHE and NRB rats in prefrontal cortex (PFc), DS and VS. Moreover, DA transporter (DAT) protein and tyrosine hydroxylase (TH) levels were assessed in PFc, DS, VS and mesencephalon (MES) and in ventral tegmental area (VTA) and substantia nigra, respectively. In NHE rats, IN-DA (0.30 mg/kg) decreased horizontal activity and increased nonselective attention relative to vehicle, whereas the lower dose (0.15 mg/kg) increased selective spatial attention. In NHE rats, basal levels of soluble EAAs were reduced in PFc and DS relative to NRB controls, while membrane-trapped EAAs were elevated in VS. Moreover, basal NMDAR1 subunit protein levels were increased in PFc, DS and VS relative to NRB controls. In addition, DAT protein levels were elevated in PFc and VS relative to NRB controls. IN-DA led to a number of changes of EAA, NMDAR1 subunit protein, TH and DAT protein levels in PFc, DS, VS, MES and VTA, in both NHE and NRB rats with significant differences between lines. Our findings indicate that the NHE rat model of ADHD may be characterized by (1) prefrontal and striatal DAT hyperfunction, indicative of DA hyperactivty, and (2)

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prefrontal and striatal NMDA receptor hyperfunction indicative of net EAA hyperactivty. IN-DA had ameliorative effects on activity level, attention, and working memory, which are likely to be associated with DA action at inhibitory D2 autoreceptors, leading to a reduction in striatal DA hyperactivity and, possibly, DA action on striatal EAA levels, resulting in a decrease of striatal EAA hyperfunction (with persistence of prefrontal EAA hyperfunction). Previous studies on IN-DA treatment in rodents have indicated antidepressant, anxiolytic and anti-parkinsonian effects in relation to enhanced central DAergic activity. Our present results strengthen the prospects of potential therapeutic applications of intranasal DA by indicating an enhancement of selective attention and working memory in a deficit model.

Keywords ADHD · Intranasal dopamine · L-Glutamate · L-Aspartate · NMDA receptor · Dopamine transporter · Tyrosine hydroxylase · Working memory · Attention

Introduction

Attention-deficit hyperactivity disorder (ADHD) is a chronic, behavioral disorder characterized by inattention, impulsivity, and hyperactivity (for review see Steinhoff 2008). Pathophysiologically, ADHD is mainly ascribed to dopamin(DA)ergic dysfunctions in mesocorticolimbic regions (for review see Del Campo et al. 2011). In vivo imaging studies have shown that ADHD patients display an increased availability of striatal DA transporters (DAT), while D2 receptor binding as well as DA synthesis and release in the majority of investigations have been unaltered (for reviews see Krause 2008; Nikolaus et al. 2009). The pivotal role of DAT binding sites is supported by the therapeutic efficacy of DAT inhibitors, such as methylphenidate (for review see Volkow et al. 2005). Recently, also disturbance of glutamate(Glu)ergic function has been implied (for review see Lesch et al. 2013), which is based on the effectiveness of the *N*-methyl-D-aspartate (NMDA) antagonists amantadine (for review see Hosenbocus and Chahal 2013a) and memantine (for review see Hosenbocus and Chahal 2013b) in treating pediatric as well as adult ADHD.

Animal models for studying ADHD can be of the genetic and nongenetic type (for review see Russell 2011). Examples of genetic models are (1) the spontaneously hypertensive rat (SHR) (for review see Sagvolden 2000) and (2) the Naples high-excitability (NHE) line (for review see Viggiano et al. 2002a, 2003). These models reproduce two specific subtypes of the syndrome (for review see Purper-Ouakil et al. 2004), which are characterized by the key symptoms of delay aversion and hyperactivity, respectively. Delay aversion is considered to be due to a dysfunctional mesolimbic branch of the DA system and is modeled by the SHR (for review see Sonuga-Barke 2003), while hyperactivity, along with alterations of executive functions, are linked to a dysfunctional mesocortical branch of the central DA system and is modeled by the NHE rats (for review see Viggiano et al. 2002a, b, 2003). D-Amphetamine and methylphenidate reduce hyperactivity and ameliorate attention deficit in both animal models, as well as in ADHD patients (for review see Solanto 1998).

Because DA does not cross the blood brain barrier, its intranasal application (IN-DA) has emerged as a promising alternative for targeting the central nervous system (Graff and Pollack 2005; Illum 2007; Thorne and Frey 2001; Tayebati et al. 2013). There is evidence that IN-DA can be transported directly from the nasal mucosa into the brain, bypassing the blood brain barrier (Chemuturi et al. 2006; Dahlin et al. 2000, 2001). IN-DA application in rats has been shown to increase DA release in the neostriatum and nucleus accumbens, to reduce anxiety, increase activity and have antidepressant-like effects (Buddenberg et al. 2008; De Souza Silva et al. 2008), as well as to ameliorate parkinsonian-related behavior in the hemiparkinsonian rat (Pum et al. 2009).

We have demonstrated that subchronic IN-DA, administered during the prepuberal period in NHE rats, reduced behavioral hyperactivity and improved both nonselective and selective attention/working memory in this animal model of ADHD (Ruocco et al. 2009a). These results suggested the potential of employing IN-DA for therapeutic purposes. The aim of the present study was to enhance our understanding of the mechanisms responsible for the behavioral effects of IN-DA in this model. For this purpose, prepuberal NHE rats again received repeated applications of IN-DA during the fifth and sixth week of postnatal life, i.e., the prepuberal period that corresponds to adolescence in children. Male rats were employed, based on the high male-to-female ratio (4:1) of ADHD (Sergeant et al. 2003). We employed the Làt maze to measure nonselective attention towards environmental stimuli and the Eight-arm radial Olton maze to assess selective spatial attention/working memory (Aspide et al. 1998).

L-Glutamate (L-Glu) and L-aspartate (L-Asp) neurotransmission between prefrontal cortex (PFc) and both dorsal (DS) and ventral striatum (VS) plays a major role in information processing (Errico et al. 2008; D'Aniello 2007). Previous studies have demonstrated higher levels of L-Glu and L-Asp in the PFc of NHE rats (Ruocco et al. 2009b). Moreover, as tyrosine hydroxylase (TH) is the rate limiting enzyme for DA synthesis (for review see, Tolleson and Claassen 2012), also TH levels might play a role in the pathophysiology of ADHD. This is supported by previous findings showing an increase in TH in PFc and ventral tegmental area (VTA) of young adult NHE rats (Viggianao and Sadile 2000; Viggiano et al. 2002a, b, 2003). DAT

function is crucial to determine the duration of DA action and to maintain DA homeostasis in the central nervous system (for review see, Chen and Reith 2000). Previous studies have revealed increased DAT levels in the PFc of NHE rats, which can be related to the hyperactive state of the mesocortical DA system (Viggiano et al. 2002a, 2003; Ruocco et al. 2009c). This finding is consistent with the elevation of DAT binding observed in ADHD patients (for review see, Krause 2008). Furthermore, an elevation of both NMDA- and quisqualate-sensitive [3H]glutamate binding was detected in the neostriatum, thalamus, frontal cortex, occipital cortex, dentate gyrus and hippocampus of NHE rats (Sadile et al. 1996).

The present study aimed to assess the influence of prepuberal long-term treatment with IN-DA on activity and selective attention/working memory and nonselective attention in the NHE rat model of ADHD using both the Làt maze and the Eight-arm radial Olton maze. Moreover, we aimed to determine the effects of IN-DA on the levels of the excitatory amino acids (EAAs) L-Glu and L-Asp, and of the NMDAR1 subunit protein in prefrontal cortex (PFc), DS and VS, as well as on the levels of the DAT protein in PFc, DS, VS and mesencephalon (MES) and of TH in VTA and substantia nigra (SN). The expected results were considered as important to further our understanding of the plasticity of the mesocortical system and long-term changes in its functional state as well as to assess the potential of intranasal delivery of dopamine for therapeutic purposes.

Materials and methods

Animals

The subjects were 28 day old male rats of the Sprague–Dawley-derived Naples High-Excitability line (NHE; $n = 32$) and the random bred line (NRB; $n = 24$) from which the selective inbreeding started in 1976. The experiments were carried out at the end of the sixth postnatal week. Rats were housed in groups of four in standard Makrolon cages and maintained in a reversed 12:12 light–dark cycle (lights on from 7 PM to 7 AM) with food and water freely available. All experiments were authorized by the Ministero della Salute. Ethical advice was issued from the Istituto Superiore di Sanità.

Drug treatment

The animals were randomly assigned to treatment and control groups receiving vehicle or dopamine hydrochloride (DA-HCl, Sigma, USA) in doses of 0.075, 0.15, or 0.3 mg/kg. DA-HCl was suspended in a volume of 10 µl of gel composed of a viscous castor oil mixture (M & P Pharma, Emmetten, Switzerland), and applied into both nostrils (5 µl each) by the use of a micropipette for viscous media (Transferpettor, Brand GMBH +CO KG, Wertheim, Germany). Treatments were given daily at the beginning of the dark phase for 14 days from postnatal day 29 onward. One hour after the last application of drug or vehicle, rats were tested for activity and nonselective attention in the Làt maze and 24 h later, in the Eight-arm radial Olton maze. This interval was chosen on the basis of neurochemical and behavioral effects of IN-DA reported in previous studies (Buddenberg et al. 2008; De Souza Silva et al. 2008).

Behavior

Làt maze

The Làt maze consisted of a 60 × 60 × 40 cm box made of PVC material (KÖMACEL^R), closed by a cover. A smaller plastic transparent 30 × 30 × 40 cm box was inserted in the middle of the latter, thus providing a 60 cm long, 15 cm wide and 40 cm high corridor, which the animals could traverse (for details see Sadile et al. 1988). The box was illuminated by a white, cold 4 W lamp placed 60 cm above the floor in the centre of the cover, providing 0.1–0.2 µW/cm^2. Two such boxes were located in a sound-attenuated room.

Procedure

At the beginning of the dark phase of the inverted light/dark cycle (between 9 AM and 4 PM) the rats were individually exposed to the Làt maze and allowed to explore the corridor for 10 min. Pairs of rats from the same cage were tested at the same time. The behaviors were registered with a high-resolution charge-coupled device (CCD) camera and stored on a video tape recorder to be analyzed offline. Measures of activity were: horizontal activity (number of corner crossings) and vertical activity (frequency of rearing on hind limbs or leaning against the walls with one or both forepaws). Nonselective attention was operationalized as the duration of rearing and leaning episodes (Aspide et al. 1998). Behaviors were measured in 1-min blocks. The reliability index was reasonably high ($r = 0.914$; $df = 198$; $p < 0.001$). At the end of the behavioral test, the fecal boluses were counted and the floor was cleaned with a wet sponge.

Eight-arm radial (Olton) maze

The apparatus consisted of eight arms (8 × 60 cm) extending from an octagonal centre platform (diameter 18.5 cm). The distance from the centre platform to the end

of each arm was 69.25 cm. The apparatus was constructed of gray poly-vinylchloride with a smooth surface and 14 cm high side walls of transparent Plexiglas. The maze was placed on the floor in a dimly lit room, surrounded by a circular higher wall without visual cues. Behavior was monitored by a high-resolution CCD camera and stored on DVD to be analyzed offline.

Procedure

In the Eight-arm radial Olton maze each rat was placed on the centre platform into a cardboard cylinder to avoid immediate escape into an arm. The trial commenced with removal of the cylinder, allowing the animal to explore. The parameters assessed in the Olton maze were horizontal activity (frequency of alley visits) and frequency of rearings on the hind limbs as well as duration of rearing as measures of nonselective attention. The number of arms visited before occurrence of the first repetition occurred (first error [FE]) and the number of arms visited before completion of visits to all eight arms (NVTC) were also recorded. The latter two indices were considered as measures of selective spatial attention as well as of working memory.

Dissection of brain areas

The animals were killed and the brain was removed and put in ice-cold saline. After removal of the olfactory tubercles, the first coronal cut was made at 4.20 AP from Bregma, using the stereotaxic coordinates of a brain atlas as a reference (Paxinos and Watson 2007). Thus, small remnants of the PFc and cingulate cortex area1 were included. The entire upper portion of the DS was removed by a sagittal pinch extending between 2.20 and −3.8 AP, up to 7 mm DV in depth, which included putamen and globus pallidus. The VS was removed by a sagittal pinch between 2.70 and 0.48 AP, at 1 mm from the midline and at about 1 mm in depth, thus incorporating the nucleus accumbens. After removing the cerebellum and the lamina quadrigemina at the level of the fourth ventricle, the MES was dissected out including both medial and lateral portions. The rectangular strip of tissue extended from −5.20 to −6.80 AP.

Excitatory amino acids

Extraction procedure

Groups treated with IN-DA (0.150 and 0.300 mg/kg) and vehicle were analyzed; the 0.075 mg/kg IN-DA dose was excluded because it did not show any significant effect in the behavioral analysis. Brain samples were homogenized in 1 ml ice-cold saline in Eppendorf vessels and centrifuged at 7,500*g* at +4 °C for 20 min. The supernatant was filtered and used for the analysis of the soluble fraction (SF). The precipitate was shocked in bidistilled water and centrifuged at 7,500*g* at 4 °C for 20 min to obtain the membrane-trapped form (MTF). For both SF and MTF forms of the free aminoacids L-Glu, L-Asp and L-Leu were measured employing the LC/MS/MS technique.

Table 1 Liquid chromatography-tandem mass spectrometry parameters

Analyte	Retention time (min)	Selected reaction monitoring transitions (m/z)	Collision energy (eV)
Aspartate	1.36	134.0 → 134.0	4.0
		134.0 → 116.0	4.0
Glutammate	1.38	148.0 → 148.0	4.0
		148.0 → 130.0	7.5
Leucine	1.42	132.2 → 132.2	4.0
		132.2 → 86.0	10.0

Liquid chromatography/tandem mass spectrometry

The analysis was performed using an Varian 310-MS triple quadrupole mass spectrometer (Varian, Palo Alto, CA, USA) in positive ionization mode and selected reaction monitoring (SRM) mode (Table 1). The settings of the electrospray ionization (ESI) source were as follows: spray voltage, 5,000 V; capillary temperature 300 °C; sheath gas pressure (spraying), 20 arbitrary units; auxiliary gas pressure (desolvating), 10 arbitrary units; ion sweep gas pressure (curtain), 5 arbitrary units. The collision cell (Q2) pressure was 2.2 m Torr of argon. The collision energies were optimized for a maximum detection of each product ion (Table 1). Chromatographic separation was performed with a ProStar™ 300 HPLC system (Varian, Palo Alto, CA, USA) on an Varian Polaris® C_{18}column (5 µm, 2.1 mm × 100 mm) at a flow rate of 0.3 mL/min. The mobile phase consisted of aqueous 0.1 % formic acid (A) and acetonitrile (B). Samples were eluted with a linear gradient from 10 to 90 % B in 5 min. At 5:01 min, solvent B was decreased from 90 to 10 % and remained constant for 5 min. The total run time was 10 min.

Morphological analysis

Tissue preparation

Animals ($n = 6$ per group) were deeply anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with saline (NaCl 0.9 %) for 2 min followed by 4 % paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) for 5 min. The brain was quickly removed and

post-fixed in the same fixative for 2 h. After washing in PBS for 30 min, the brain was placed in a sucrose solution (18 % sucrose in PBS) at 4 °C. After equilibration in sucrose, forebrain and MES were divided from brainstem and cerebellum and sagittally cut in half along the midline. Each half was frozen on dry ice and stored at −80 °C. For this experiment, only one half of the prosencephalon was used. Frozen prosencephali were sagittally cut (slice thickness, 50 μm) using a cryostat. Random sampling took place every four sections. Before washing and staining, cryostat sections were left floating in cold PBS. All the sections were stained in the same run, to reduce experimental variability.

Tyrosine hydroxylase immunohistochemistry

Sections were washed in Tris buffered saline (TBS) and incubated with mouse monoclonal antibody against TH (Diasorin, Stillwater, USA) at a 1:5,000 dilution in 10 % normal bovine serum and 0.2 % triton X-100 in PBS at 4 °C. After overnight incubation, sections were washed three times in PBS and then incubated for 1 h with anti-mouse-biotin conjugated antibody (Vector, USA) at a 1:200 dilution in 10 % normal bovine serum and 0.2 % Triton X-100 in PBS at room temperature. Subsequently, sections were washed three times in TBS and incubated in ABC (Vector lab, USA) for 1 h. After three further washings in TBS, the reaction was visualized with 0.1 % diaminobenzidine and 0.02 % hydrogen peroxide in TB (0.05 M; pH 7.4) for 10 min in the dark. The reaction was terminated with cold TBS and the sections were flattened on to nontreated glass slides, air-dried and coverslipped with Permount. Slides were analyzed with a Zeiss Axioscope equipped with a CCD high-resolution camera (Hamamatsu Photonic Italy, C5405). The images were converted by a microcomputer-assisted analysis system (MCID-M2; Imaging Res. Inc. Canada) to a 640 × 512 pixel file and quantitatively analyzed according to the guidelines by Capowski (Capowski 1989). The following areas were considered [according to the atlas by Paxinos and Watson (2007)] for TH immunocytochemistry: DS (caudateputamen), VS (nucleus accumbens and olfactory tubercle), VTA and SN pars compacta. The sections from different animals corresponding to the same level were aligned using neuroanatomical markers. Measurements were taken at low magnification (objective 2.5× Plan-Neofluar, Zeiss). The number of sections was 8–10 for each animal per staining technique. The region of interest was outlined in each section and the relative optical density (ROD % log10 (256/observed gray levels) of the TH signal was measured. To obtain digital pictures of brain preparations from different groups, slices were photographed with a CCD digital camera (C-5985, Hamamatsu Photonics, Milan, Italy) and Image Pro-Plus software (Media Cybernetics, Silver Springs, MD, USA).

NMDAR1 subunit and DAT protein

Preparation of protein extracts

Membrane and cytosolic protein fractions were prepared as previously described (Fumagalli et al. 2008) with minor modifications. Tissues were homogenized in a glass-Teflon potter in cold 0.32 M sucrose buffer (pH 7.4) containing 1 mM HEPES, 0.1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride and commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich) inhibitors. The homogenate was clarified at 1,000*g* for 10 min obtaining a pellet (P1) corresponding to the nuclear fraction. The supernatant (S1) was then centrifuged at 13,000*g* for 15 min to obtain a clarified fraction of cytosolic proteins (S2) and a pellet (P2) corresponding to the crude membrane fraction that was homogenized in a glass potter in 1 % Triton X-100 buffer containing 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA and 0.02 % sodium azide. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as calibration standard.

Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transfer of proteins to nitrocellulose membranes were performed using conventional methods with minor modifications. The proteins were solubilized in loading buffer at 54 °C for 45 min, separated on 10 % polyacrylamide gels and transferred to PVDF membranes (GE Healthcare, Munich, Germany) in transfer buffer (25 mM Tris [pH 8.3], 192 mM glycine, 20 % methanol). The membranes were incubated with blocking buffer (5 % ECL blocking agent [GE Healthcare] in PBST [0.1 M phosphate buffered saline {pH 7.0}, 0.1 % Tween-20]) for 1 h at room temperature. The conditions for the primary antibodies were as follows: overnight incubation at 4 °C with the antibodies of either NMDAR1 (1:300 anti-NMDAR1 Chemicon-Millipore AB9864), DAT (1:500 anti Dopamine Transporter Millipore AB2231), Actin (1:500 anti-Actin Sigma A2066) or Actin (1:3000 anti-Actin clone C4 Chemicon-Millipore MAB1501). After three 10-min washings in PBST, the blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (antirabbit or antimouse ECL antirabbit IgG HRP-linked whole Ab; GE Healthcare NA934, NA931). After washing, immunocomplexes were visualized with chemiluminescence using the ECL Western

blotting kit (GE Healthcare). All protein bands were within linear range of standard curves, and were normalized for actin level in the same membrane. Quantity One software (BioRad Laboratories, Hercules, CA) was used for standardization and quantification of protein bands obtained with Western blot analysis.

Statistics

All data were tested for fulfillment of the requirements for parametric analysis (Levene's test for univariate analysis and Box test of equality of covariance matrices for repeated measures analysis) using SPSS software (Version 11.0). For body weight and each behavioral parameter, a separate two-way repeated measures analysis of variance (ANOVA) was carried out, with treatment as between-group factor and time as repeated measures factor. Duration of leaning and rearing as measures of nonselective attention were evaluated by two-way ANOVAs with treatment as between-group factor and time as covariate. For selective attention, emotionality index and regional levels of soluble and membrane-trapped L-Glu/L-Leu and L-Asp/L-Leu ratios one-way ANOVAs were calculated with treatment as between-groups factor. The level of significance was set at $p_{\text{two-sided}} < 0.05$. Post hoc analysis was performed by Tukey's or LSD tests. Regional DAT protein levels and TH levels (expressed as percentage of baseline) after In-DA were compared between rat lines using the independent t test (two-tailed, $p_{\text{two-sided}} < 0.05$). NMDAR1 subunit levels could not be statistically analyzed due to the small sample sizes.

Results

Body weight

During the entire treatment period, the body weight of animals receiving IN-DA in different doses did not differ from vehicle-treated controls. Two-way repeated measures ANOVA (treatment × time) showed a significant main effect only for time ($F = 618.0$, $df = 2/50$, $p < 0.000$) with no interaction. Thus, all groups exhibited a similar increase in body weight (data not shown).

Behavior

Làt maze

Horizontal activity In the NHE rats, two-way ANOVA revealed significant main effects for treatment ($F = 3.01$, df 3/28, $p < 0.046$) and for time ($F = 16.27$, df 5/140, $p < 0.000$), with no interaction effects. Post hoc analysis with Tukey's test showed that the treatment effect—the reduction in horizontal activity—was due to the high dose of 0.3 mg/kg DA ($p < 0.028$). In contrast, in the NRB rats no significant differences were obtained for either treatment or time. As shown in Fig. 1a, only the dose of 0.3 mg/kg significantly reduced horizontal activity in the NHE rats. Horizontal activity over time of testing was similar across groups, as demonstrated by a significant main effect for time (see above) in the absence of a significant treatment per time interaction.

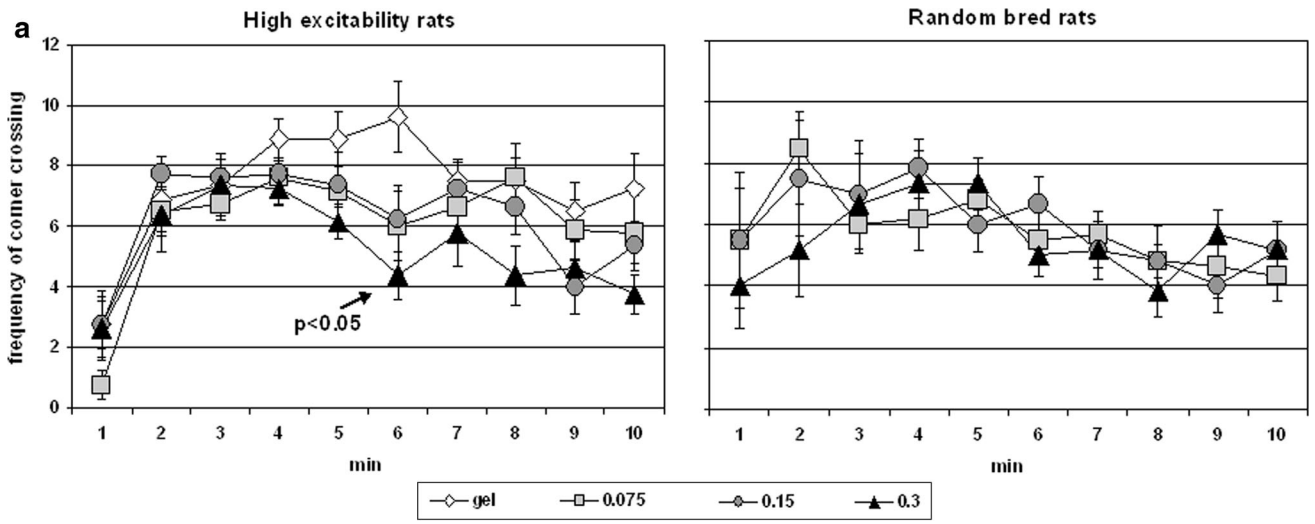
Frequency of leaning and rearing IN-DA treatments did not affect the orienting frequency, as measured by the frequency of leaning on the hind limbs against the wall in either NHE or NRB rats. Two-way ANOVAs yielded significant main effects only for time in leaning frequency in the NHE rats ($F = 8.11$, df 9/252, $p < 0.0001$) with a treatment per time interaction effect ($F = 1.66$, df 27/250, $p < 0.024$) and for time in rearing frequency in NRB controls ($F = 1.98$, df 9/180, $p < 0.043$) with no interaction.

Duration of leaning and rearing (measure of non-selective attention) IN-DA treatment with 0.3 mg/kg increased the duration of leaning against the wall in the NHE but not NRB rats, as demonstrated by one-way ANOVA ($F = 3{,}175$, $df = 3/28$, $p < 0.039$) (Fig. 1b). Two-way ANOVAs for treatment × time yielded significant main effects in both NHE ($F = 2.48$, df 5/139, $p < 0.034$) and NRB ($F = 3{,}011$, $df = 9/144$, $p < 0.003$) rats only for time without interaction.

Eight-arm radial Olton maze

Horizontal activity A two-way ANOVA for treatment and for rat line was carried out for frequency of horizontal activity in the radial maze. This analysis revealed significant main effects only for rat line in horizontal activity ($F = 12.41$, df 1/45, $p < 0.001$) (Fig. 2a), but not for treatment without interaction. This result confirms the higher horizontal activity typically exhibited by NHE rats, which has been interpreted to be of allocentric origin in the Olton maze (Berke et al. 2009).

FE and NVTC (measures of selective spatial attention/working memory) Perfect performance on this working memory task would entail exploration of each of the eight arms only once. The analysis of FE revealed a significant effect only for treatment ($F = 3.88$, df 3/45, $p < 0.015$). Separate one-way ANOVAs for each rat line revealed a significant treatment effect only in the NHE rats ($F = 5.73$, df 3/28, $p < 0.004$). Tukey's test showed that the treatment with 0.15 mg/kg significantly increased the FE value. In



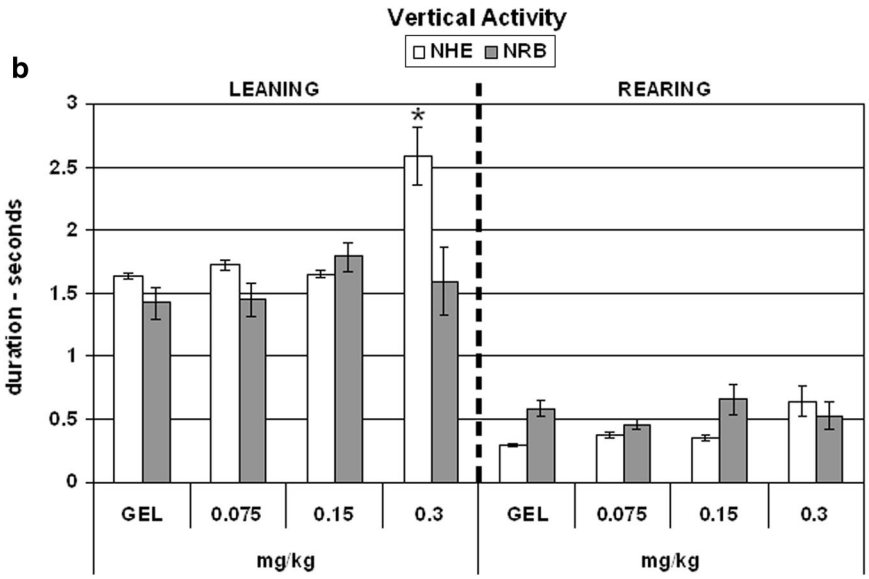


Fig. 1 Làt maze: behavioral effects of IN-DA on activity and nonselective attention in prepuberal NHE and NRB rats. Frequency of corner crossings plotted over the 10-min exposure per-min blocks. **a** Mean duration of rearing and leaning, **b** data are given as mean ± SEM (*$p < 0.05$)

fact, NHE rats typically re-entered a previously visited alley, whereas the control rats did this after having visited eight alleys in a row ($p < 0.045$) (Fig. 2b). This result indicates that the intermediate dose of IN-DA improved spatial attention/working memory in the NHE rats.

Emotionality index

The defecation score, indexed by the number of fecal boli laid down during testing in the Làt maze and Eight-arm radial Olton maze, yielded no significant differences across treatment groups. Separate one-way ANOVAs showed no treatment effect in the two mazes (data not shown).

Basal levels of glutamate and aspartate

Prefrontal cortex

The values of Glu and Asp corrected for Leu (which does not participate in neurotransmission) for NHE and NRB rats for the soluble and membrane-trapped form under basal conditions are shown in Fig. 3a. A one-way ANOVA demonstrated a lower level of soluble L-Glu/L-Leu in NHE rats relative to NRB controls ($F = 26.19$; $df = 1/4$, $p < 0.007$). Moreover, the level of membrane-trapped L-Glu/L-Leu was lower in NHE rats relative to NRB controls ($F = 88.47$; $df = 1/4$, $p < 0.001$). Soluble L-Asp/L-Leu

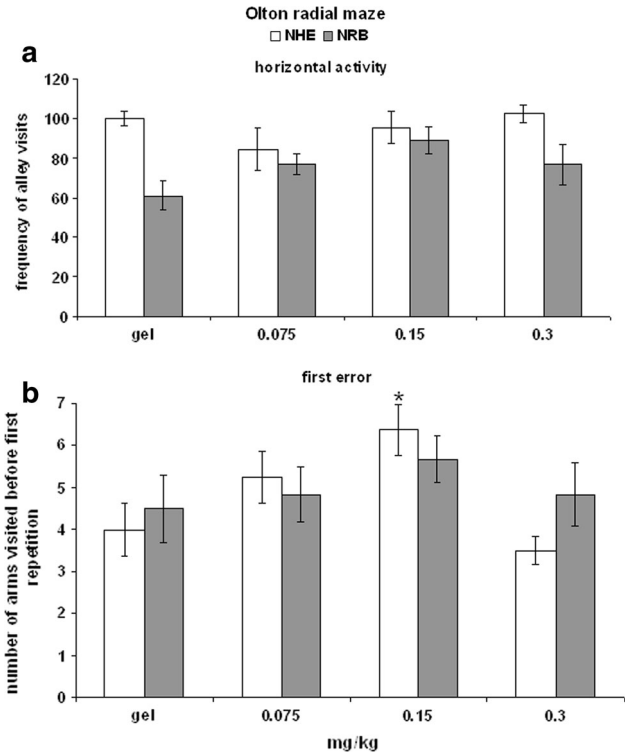


Fig. 2 Eight-arm radial Olton maze: effects of prepuberal IN-DA on indices of horizontal activity (**a**) and selective spatial attention/working memory (FE values, i.e., number of arms visited before repetition error) (**b**) in adult NHE and NRB rats. An efficient rat would explore the eight arms only one time; thus, the best possible score would be 9 for first error. Data given as mean ± SEM (*$p < 0.05$)

was also lower in NHE than in NRB rats ($F = 219.81$; $df = 1/4$, $p < 0.001$), whereas no significant difference of the membrane-trapped form of L-Asp/L-Leu was observed between rat strains.

Dorsal striatum

Levels of soluble and membrane-trapped L-Glu/L-Leu were lower in the DS of NHE rats relative to NRB controls ($F = 186.80$; $df = 1/4$, $p < 0.001$ and $F = 171.68$; $df = 1/4$, $p < 0.001$, respectively) as shown in Fig. 3b. Soluble L-Asp/L-Leu was lower in the DS of NHE as compared to NRB rats ($F = 3.352$; $df = 1/4$; $p < 0.027$), whereas membrane-trapped L-Asp/L-Leu was higher in the DS of NHE rats relative to NRB controls ($F = 250.50$; $df = 1/4$; $p < 0.001$).

Ventral striatum

Soluble L-Glu/L-Leu was lower in the VS of NHE rats compared to NRB controls ($F = 20.72$; $df = 1/4$, $p < 0.010$). No significant difference was evident for soluble L-Asp/L-Leu, as shown in Fig. 3c. Membrane-trapped L-Glu/

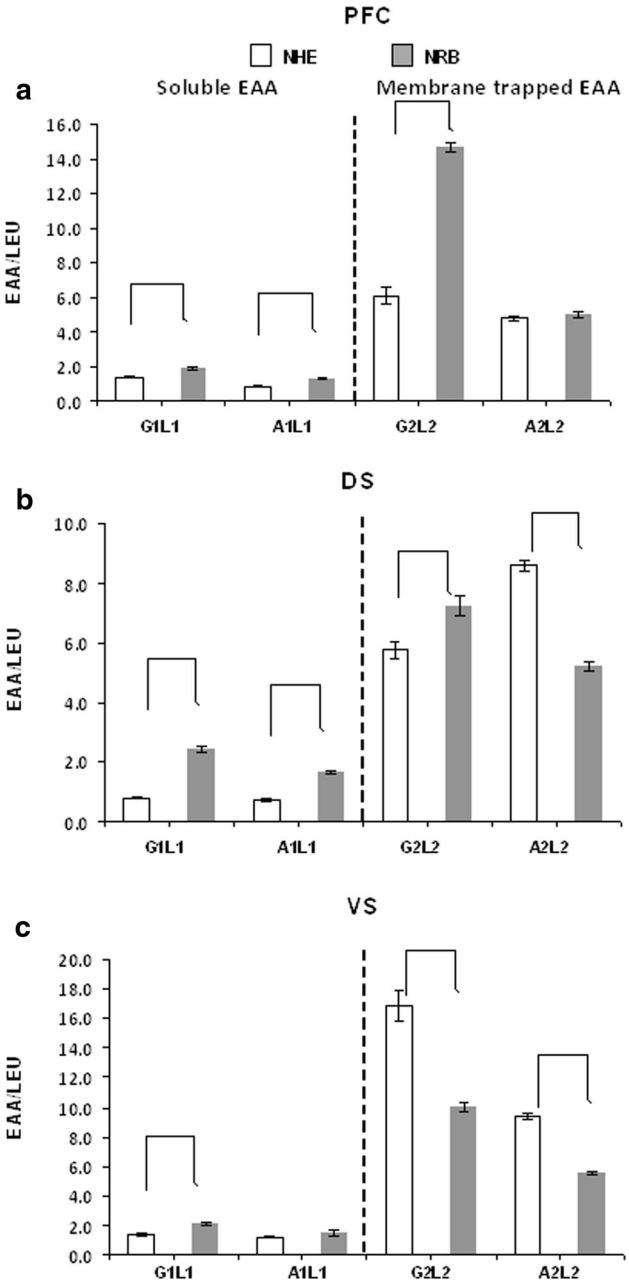


Fig. 3 L-Glu/L-Leu and L-Asp/L-Leu ratios for soluble and membrane-trapped forms in **a** prefrontal cortex (PFC), **b** dorsal striatum (DS) and **c** ventral striatum (VS) of NHE and NRB rats under basal conditions. Data given as mean ± SEM (*$p < 0.05$)

L-Leu and L-Asp/L-Leu were higher in the VS of NHE rats relative to NRB controls ($F = 38.53$; $df = 1/4$, $p < 0.003$ and $F = 268.588$; $df = 1/4$, $p < 0.001$, respectively).

Glutamate and aspartate after IN-DA

Prefrontal cortex

An one-way ANOVA on NHE rats showed an increase of soluble L-Glu/L-Leu ($F = 7.90$; $df = 2/6$, $p < 0.021$).

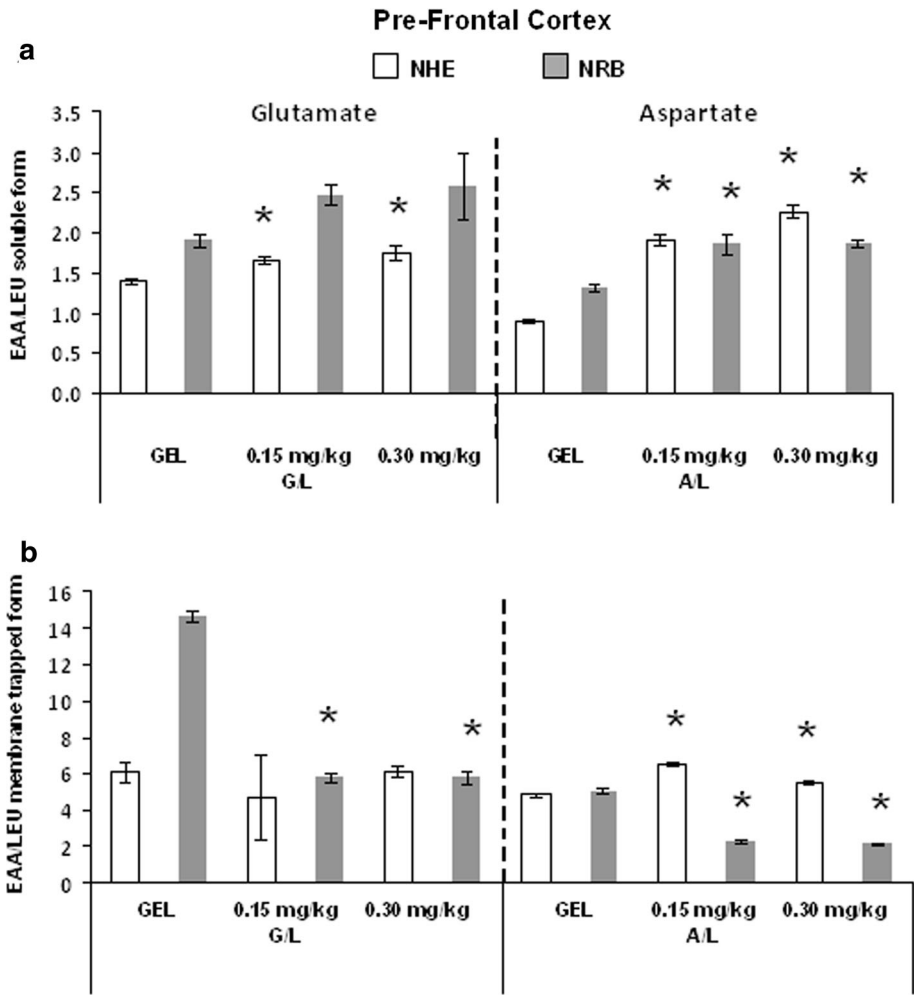


Fig. 4 L-Glu/L-Leu (G/L) and L-Asp/L-Leu (A/L) ratios in the soluble form (**a**) and in the membrane-trapped form (**b**) in the prefrontal cortex of NHE and NRB rats for each dose of IN-DA treatment. Data given as mean ± SEM (*$p < 0.05$)

Moreover, increases of both soluble ($F = 143.85$; $df = 2/6$, $p < 0.000$) and membrane-trapped L-Asp/L-Leu ($F = 39.85$; $df = 2/6$, $p < 0.000$) were observed. The LSD test revealed that this was due to both the 0.15 and the 0.3 mg/kg doses (soluble L-Glu/L-Leu, 0.15 mg/kg, $p < 0.028$; 0.3 mg/kg, $p < 0.009$; soluble L-Asp/L-Leu, $p < 0.0001$ both; membrane-trapped L-Asp/L-Leu, $p < 0.0001$ both).

A reduced level of L-Glu/L-Leu only in the membrane-trapped form was observed in the NRB rat line ($F = 302.90$; $df = 2/6$, $p < 0.000$), whereas increased and decreased levels of L-Asp/L-Leu were observed in soluble ($F = 14.31$; $df = 2/6$, $p < 0.005$) and membrane-trapped ($F = 177.40$; $df = 2/6$, $p < 0.000$) form, respectively. The LSD test showed that both 0.15 and 0.3 mg/kg doses affected the EEA levels (soluble L-Asp/L-Leu, $p < 0.004$ both; membrane-trapped L-Glu/L-Leu, $p < 0.0001$ both; membrane-trapped L-Asp/L-Leu, $p < 0.0001$ both) as shown in Fig. 4a, b.

Dorsal striatum

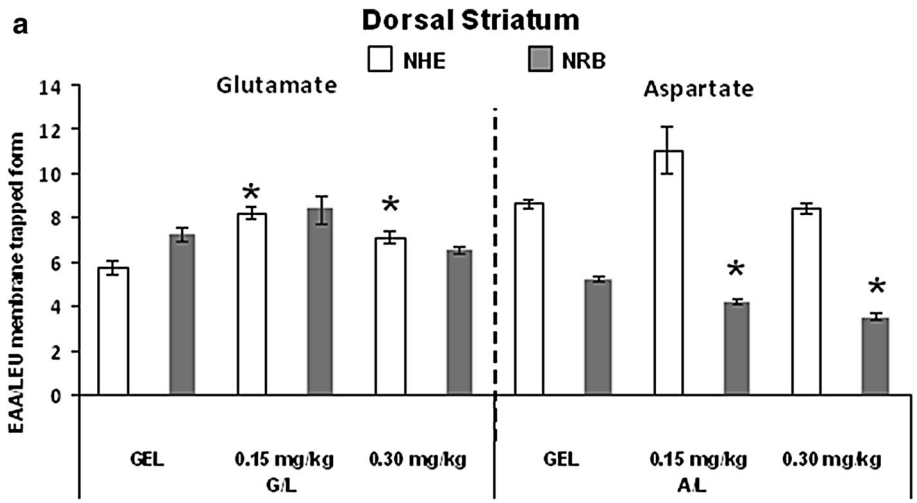
One-way ANOVA on NHE rats showed an increased ratio of L-Glu/L-Leu only for the membrane-trapped form ($F = 17.83$; $df = 2/6$, $p < 0.03$; Fig. 5a). LSD tests evidenced that this was due to either the 0.15 mg/kg dose (L-Glu/L-Leu, $p < 0.01$) or the 0.300 mg/kg dose (L-Glu/L-Leu, $p < 0.016$). A reduced ratio of L-Asp/L-Leu only in the membrane-trapped form ($F = 4.70$; $df = 2/6$, $p < 0.001$) was observed in the NRB rat line. The LSD test showed that both 0.15 and 0.3 mg/kg dose affected EEA levels (L-Glu/L-Leu, $p < 0.003$ and L-Asp/L-Leu, $p < 0.0001$).

Ventral striatum

One-way ANOVA on NHE rats evidenced no differences of L-Glu/L-Leu levels between either dose of IN-DA and vehicle. An increase of soluble L-Glu/L-Leu ($F = 6.72$; $df = 2/6$, $p < 0.029$) was obtained for NRB controls with the LSD test revealing that this was due to the 0.150 mg/kg dose ($p < 0.026$; Fig. 5b).

Levels of tyrosine hydroxylase

The ratio of TH levels obtained after IN-DA (0.015 and 0.3 mg/kg) and vehicle in the VTA is shown in Fig. 6



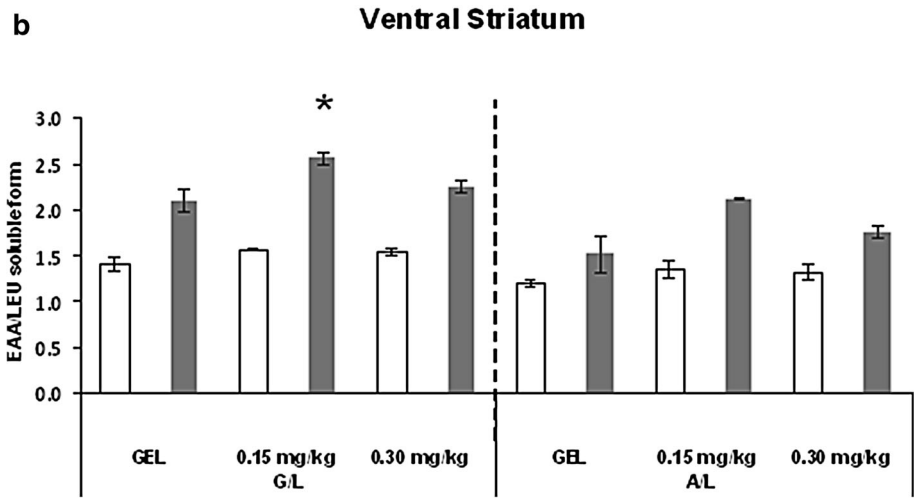


Fig. 5 L-Glu/L-Leu a (G/L) and L-Asp/L-Leu (A/L) ratios in the membrane-trapped form in the dorsal striatum (**a**) and in the soluble form in the ventral striatum (**b**) of NHE and NRB rats for each dose of IN-DA treatment. Data given as mean ± SEM ($*p < 0.05$)

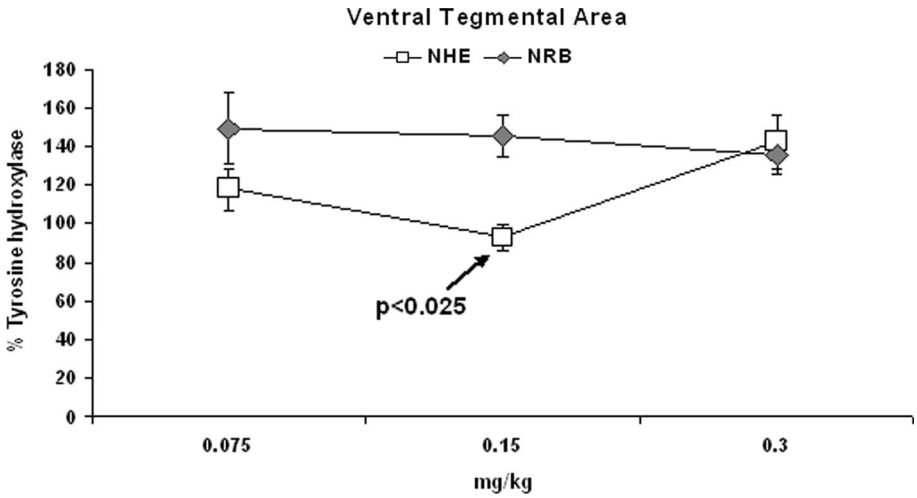


Fig. 6 Tyrosine hydroxylase levels after IN-DA (expressed as percentage of gel) for NHE and NRB rat lines in the ventral tegmental area for each dose of IN-DA. Data are given as mean ± SEM ($*p < 0.05$)

for both NHE and NRB rats. In NHE rats, TH was decreased after 0.15 mg/kg IN-DA, but increased after 0.3 mg/kg IN-DA ($t = 3.19$; *df* 8/10; $p < 0.025$). On the contrary, in the NRB rats treated with IN-DA no significant changes of TH levels were observed for either dose. Moreover, no significant changes were found in the SN of either NHE or NRB rats after any dose of IN-DA (Table 2).

Table 2 Tyrosine hydroxylase levels as percentage of vehicle with standard error of mean (SEM) in NHE and NRB rats after IN-DA treatment

Tyrosine hydroxylase in ventral tegmentum (VTA)				
Dose IN-DA	NRB		NHE	
	% Vehicle	Sem %	% Vehicle	Sem %
0.075	149.36	18.24	117.92	11.10
0.150	145.48	10.86	92.65	7.01
0.300	135.37	9.48	142.42	13.88
SN				
Dose IN-DA	NRB		NHE	
	% Vehicle	Sem %	% Vehicle	Sem %
0.075	137.40	26.09	117.97	12.29
0.150	122.47	13.62	92.04	6.58
0.300	162.64	15.48	144.35	14.90

Basal DAT protein levels

Prefrontal cortex

The levels of DAT protein in the PFc of NHE and NRB rats are shown in Fig. 7a. The present preliminary analysis indicated that in the NHE rat line the DAT protein levels were higher relative to NRB controls e ($\approx$143 %; $p < 0.0001$).

Dorsal striatum

In the DS, no differences between strains were observed (Fig. 7b).

Ventral striatum

In the VS of NHE rats, DAT protein levels were higher relative to NRB controls ($\approx$156 %; $p < 0.057$; Fig. 7c.

Mesencephalon

DAT protein levels were lower in the MES of NHE compared to NRB rats (about 41 %; $p < 0.013$; Fig. 7d).

DAT protein levels after IN-DA

Prefrontal cortex

An increased DAT protein level was observed in NHE rats (Fig. 8a) treated both with IN-DA 0.15 mg/kg (+79 %, *t* test, $p < 0.037$) and 0.300 mg/kg (+120 %, $p < 0.004$) compared to vehicle. In NRB controls (Fig. 8b) the 0.15 mg/kg dose increased l DAT protein levels (+62 %; $p < 0.029$), whereas no significant differences were observed after 0.3 mg/kg IN-DA.

Dorsal striatum

DAT protein levels of NHE rats (Fig. 8a) treated with IN-DA at 0.150 and 0.3 mg/kg did not differ relative to vehicle. In NRB rats (Fig. 8b) treatment with 0.15 mg/kg yielded increased DAT protein levels (+48 %, *t* test, $p < 0.0194$) whereas no significant differences were observed after the 0.3 mg/kg dose.

Ventral striatum

In neither NHE nor NRB rats IN-DA treatment produced a significant effect on DAT protein levels (Fig. 8a, b).

Mesencephalon

In neither NHE nor NRB rats IN-DA treatment produced a significant effect on DAT protein levels (Fig. 8a, b).

NMDAR1 subunit protein levels

In the present study, IN-DA was only injected in a small sample ($N = 3$/group) of prepuberal NHE rats. Although the group size did not allow for statistical analysis, the results may be indicative of action (Fig. 9).

In the PFc of NHE rats the NMDAR1 subunit protein level increased from 0.12 to 0.15, whereas in NRB controls it decreased from 0.06 to 0.03 starting, however, from a lower baseline level. In the DS of NHE rats the NMDAR1 subunit protein level decreased from 0.15 to 0.07, whereas in the NRB rats, it increased from 0.04 to 0.08 after the 0.15 mg/kg dose (starting from a lower baseline level) and remained at 0.04 after the 0.30 mg/kg dose. In the VS of NHE rats the NMDAR1 subunit protein level increased from 0.12 to 0.20, and in the VS of NRB rats from 0.03 to 0.09.

Discussion

NHE vs NRB—basal conditions

Findings showed reduced levels of soluble L-Glu in the PFc, DS and VS of NHE rats relative to NRB controls, while membrane-trapped L-Glu levels were decreased in PFc and DS, but elevated in VS. Levels of soluble L-Asp were lowered in PFc and DS of NHE rats relative to NRB controls, but unaltered in the VS. In contrast, membrane-trapped Asp levels were unaltered in the PFc, but elevated

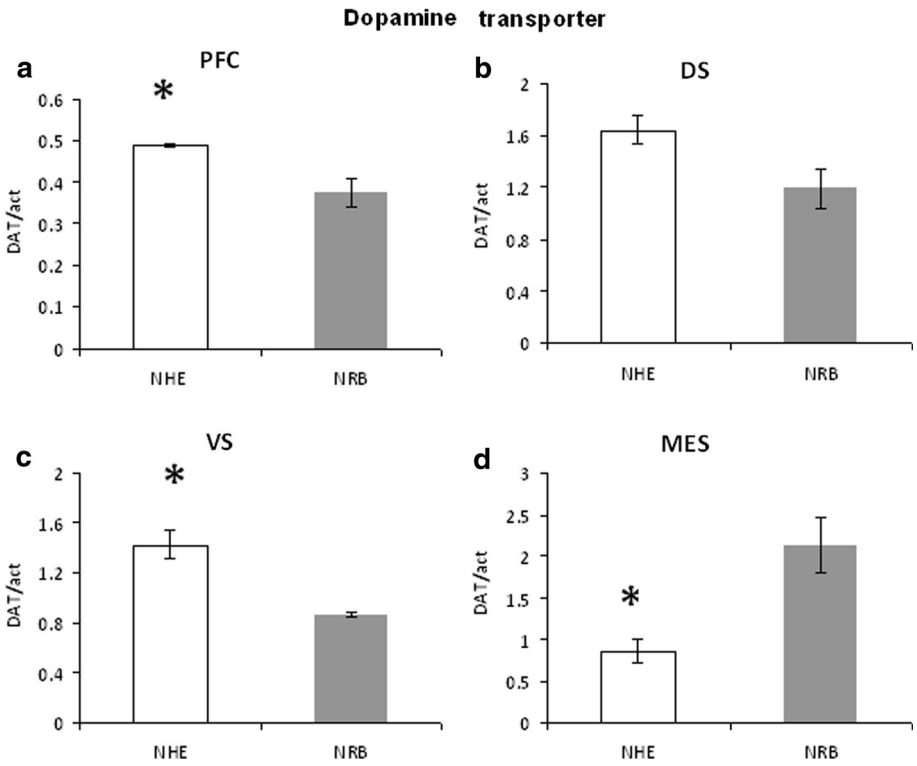


Fig. 7 Basal dopamine transporter (DAT) protein levels in **a** prefrontal cortex (PFC), **b** dorsal striatum (DS), **c** ventral striatum (VS) and **d** mesencephalon (MES) of NHE and NRB rats. Data given as mean ± SEM of DAT over actine (DAT/act). Data are given as mean ± SEM (*$p < 0.05$)

in the DS and VS of NHE rats relative to controls. Taken together, findings evidence a reduction of soluble EAAs in the PFc and DS and an elevation of membrane-trapped EAAs in the VS of NHE rats. Moreover, NMDAR1 subunit proteins were elevated in PFc, DS and VS of NHE rats, and DAT protein levels were increased in PFc and VS, decreased in MES and unaltered in DS.

The results obtained on EAA levels in PFc correspond to previous findings of higher EAA levels in the PFc of NHE rats (Ruocco et al. 2009b; however, see below). Likewise, findings obtained on NMDAR1 subunit protein levels in PFc and DS are consistent with previous findings of increased NMDA-sensitive [3H]glutamate binding in these regions (Sadile et al. 1996). Moreover, the relevance of EAA hyperactivity in neuropsychiatric disorders is underlined by the recent findings of increased Glu levels in the anterior cingulate of ADHD and borderline patients (Hoerst et al. 2010; Rüsch et al. 2010). The finding of increased prefrontal DAT protein levels corroborates previous findings on NHE rats (Viggiano et al. 2002a, 2003; Ruocco et al. 2009c). Moreover, results obtained on DAT protein levels in DS and MES of NHE rats relative to NRB controls are consistent with findings of unaltered neostriatal DAT (van Dyck et al. 2002; Jucaite et al. 2005) and decreased midbrain DAT binding in ADHD patients (Jucaite et al. 2005). They do not correspond, however, to the increases of neostriatal DAT binding observed in the majority of in vivo investigations (Dougherty et al. 1999; Krause et al. 2000; Dresel et al. 2000; la Fougere et al. 2006; Larisch et al. 2006; Spencer et al. 2007).

If we proceed from the assumption that regulatory mechanisms aim to maintain functional homeostasis throughout the central nervous system, the decrements of soluble L-Glu in PFc, DS and VS and of soluble L-Asp in PFc and DS are in agreement with the observed increases of the NMDAR1 subunit protein in these regions, which may be interpreted in terms of a compensatory up-regulation of available binding sites. This also holds for the decrease in membrane-trapped L-Glu in PFc and DS, but, interestingly, not for membrane-trapped L-Glu in VS, as well as membrane-trapped L-Asp in PFc, DS and VS. Therefore, it may rather be inferred that the concentrations of membrane-trapped EAAs have increased in response to the increased availability of NMDA binding sites. If this be the case, then NHE rats may be hypothesized to have more NMDA receptors relative to NRB controls, leading to, first, an increase in L-Glu binding in the VS as well as of L-Asp binding in both DS and VS, and, secondly, to corresponding decreases of soluble L-Glu and L-Asp in these regions. It remains to be seen, however, in as much NMDAR1 subunit protein levels correspond to the final expression of NMDA receptor molecules. Yet, for the time being, the role of NMDA dysfunction in human ADHD is underlined by the association of ADHD with variations of the NMDA-type Glu receptor subunit genes (GRIN1, 2A-D; Dorval et al. 2007).

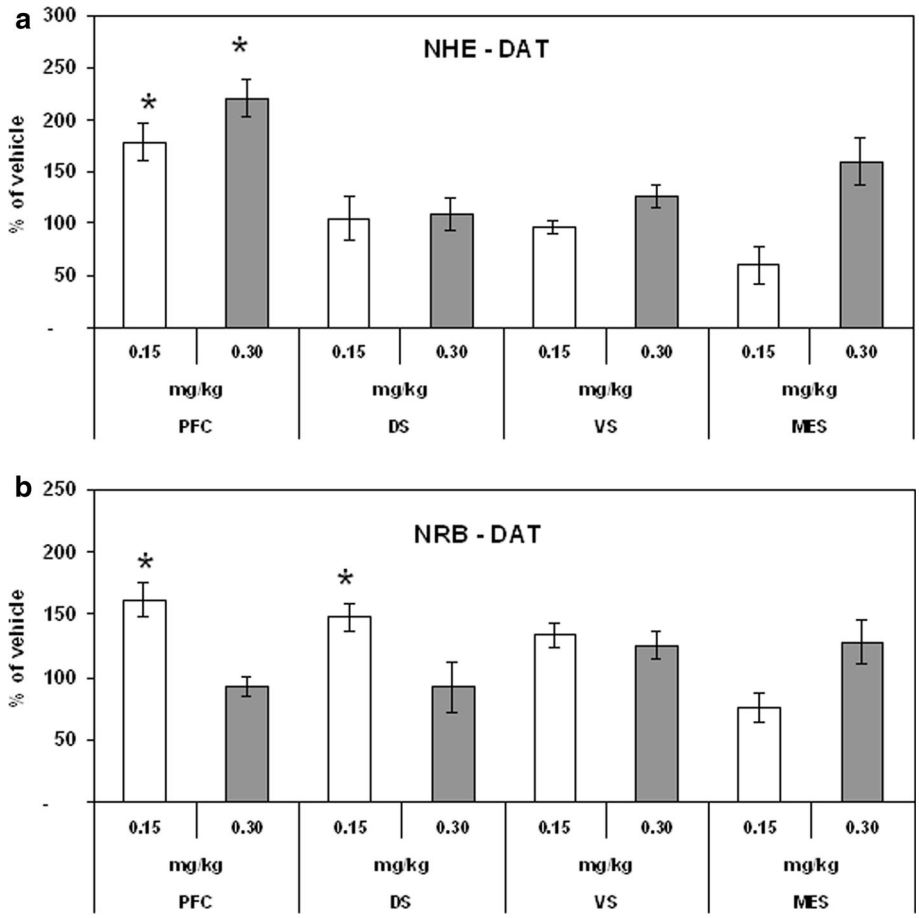


Fig. 8 DAT protein levels (as percent of vehicle) in prefrontal cortex (PFC), dorsal striatum (DS), ventral striatum (VS) and mesencephalon (MES) of NHE (**a**) and NRB (**b**) rats after IN-DA (0.15 and 0.30 mg/kg). Data are given mean ± SEM (*$p < 0.05$)

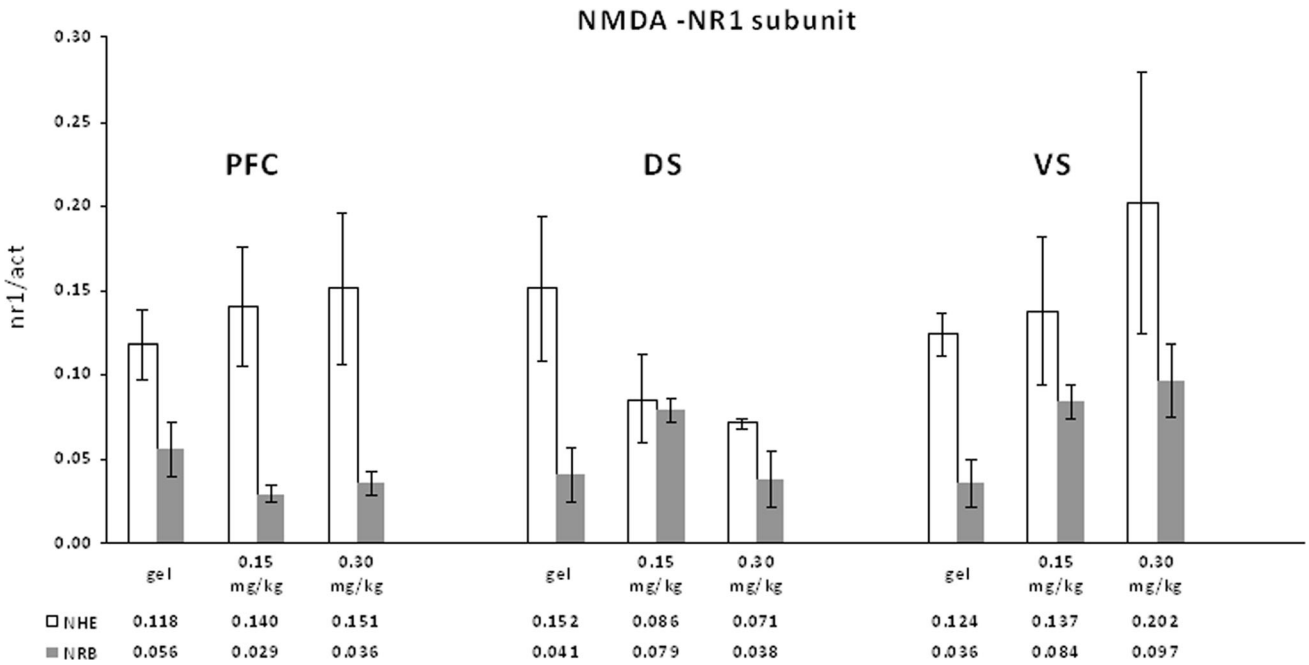


Fig. 9 NMDAR1 subunit protein levels in prefrontal cortex (PFC), dorsal striatum (DS) and ventral striatum (VS) in NHE and NRB rats after IN-DA (0.15 and 0.30 mg/kg) and vehicle. Data given as mean ± SEM of NMDA-R1/nr1 over actine (nr1/act)

Taken together, NHE rats may be characterized by both elevated prefrontal NMDAR1 subunit proteins and elevated prefrontal and ventral striatal DAT protein levels. Glu is known to stimulate DA release (Clow and Jhamandas 1989), while Glu release is inhibited by the D2 receptor subtype (Yamamoto and Davy 1992). Thus, it may be hypothesized that the elevation of excitatory input to the prefrontal target region of mesostriatal efferents in NHE rats induces an increase of DA efflux, which, in turn, diminishes EAA concentrations. This conjecture is consistent with the observed increases of DAT protein in both the VS and the prefrontal target region of mesolimbic DAergic projections. It remains to be elucidated, however, in as much the level of transporter protein corresponds to the final expression of DAT molecules on the presynaptic terminal, and whether human ADHD and the rat NHE model of this disease basically differ with respect to the impact of DAT function in DS and VS (the latter of which in NHE rats displayed a significant increase of DAT protein levels relative to NRB controls).

NHE versus NRB—IN-DA treatment

Firstly, in the PFc of NHE rats, IN-DA elicited an increase of both soluble L-Glu and L-Asp, whereas in NRB controls prefrontal soluble L-Asp was increased, but prefrontal soluble Glu remained unaltered. Moreover, both membrane-trapped L-Glu and L-Asp were decreased in the PFc of NRB rats, whereas they were unaltered and augmented, respectively, in prefrontal tissues of NHE controls. Secondly, in the DS of NHE rats, IN-DA elicited an increase in membrane-trapped L-Glu with no alterations of soluble L-Glu, soluble L-Asp and membrane-trapped L-Asp in this region, whereas in the DS of NRB controls membrane-trapped L-Asp was elevated and soluble L-Asp, soluble L-Glu and membrane-trapped L-Glu remained unaltered. Thirdly, in the VS of NHE rats, no effect was exerted on EAA concentrations by IN-DA, whereas in the VS of NRB controls concentrations of soluble L-Glu were elevated with no alterations of soluble L-Asp and both membrane-trapped L-Glu and L-Asp. From this, it follows that NHE and NRB lines differ as to the regional effects of IN-DA on soluble and membrane-trapped EAAs. It may be hypothesized that the varying effects on soluble and membrane-trapped EAA levels in NHE rats and NRB controls are associated with between-strain differences of regional DA and EAA function.

The application of IN-DA can be assumed to increase DA levels in the mesocorticolimbic system (de Souza Silva et al. 2008). If DA is applied to NRB controls, DAT protein levels, therefore, may be expected to rise in both striatum and prefrontal target regions of mesostriatal efferents, as was the case in the present investigation. In the NHE rat model, basal striatal DAT binding sites may be expected to be higher relative to normal animals; thus, an increase of available DA is not likely to lead to a further elevation of striatal DAT protein levels. Accordingly, in the present investigation, both dorsal and ventral striatal DAT protein levels were unaltered after IN-DA relative to vehicle. Findings indicate, however, that prefrontal DAT protein levels had risen, presumably as an adaptive response to the increased availability of DA in the prefrontal target regions of striatal efferents.

DA synthesis and release are modulated by a negative feedback loop, which is established by DA acting upon presynaptic terminal autoreceptors of the inhibitory D2/3 receptor subtype (for review see Langer 1997). In NRB controls, the elevation of DA levels by 0.15 and 3 mg/kg IN-DA had no effect on ventral tegmental TH levels. Apparently, these doses did not augment mesostriatocortical DA levels to an extent requiring the onset of regulatory feedback mechanisms in SN or VTA. In contrast, in NHE rats a biphasic action was observed in the VTA with a decrease of TH after 0.15 mg/kg and an increase of TH after 0.3 mg/kg IN-DA. From this it may be concluded that DA concentrations after 0.3 mg/kg IN-DA at the time of sacrifice and brain dissection were lower compared to the 0.15 mg/kg dose. Evidently, the application of the higher dose had resulted in DA concentrations sufficient to activate feedback inhibition at the presynaptic terminal resulting in reduced levels of TH, which catalyzes the conversion of L-tyrosine to the DA precursor L-3,4-dihydroxyphenylalanine. The subsequent reduction of DA levels, then, is likely to have disinhibited presynaptic D2 autoreceptors leading again to an enhancement of DA synthesis (indicated by the increase of TH levels) and release.

The application of IN-DA can be expected to enhance both inhibitory and excitatory DAergic neurotransmission in the mesocorticolimbic system. As Glu release is inhibited by DA (Yamamoto and Davy 1992), an elevation of available DA is likely to lead to an inhibition of EAA efflux in both DS and VS. This conjecture is in agreement with the increases of NMDAR1 subunit protein levels observed in both NHE and NRB rats in these regions, which may be interpreted in terms of a compensatory up-regulation of available NMDA receptor binding sites. As a matter of fact, the inhibition of striatal EAA efflux by the increased availability of striatal DA offers a more extensive explanation for the efficacy of methylphenidate treatment of human ADHD beyond the elevation of DA levels via blockade of re-uptake sites (for review see Volkow et al. 2005). In NHE rats also prefrontal NMDAR1 subunit protein levels were found to be increased, while they were decreased in NRB controls. From the increase of striatal NMDAR1 subunit protein levels a net elevation of

EAAergic input to prefrontal projection areas may be inferred, which is likely to be compensated by the decrease of NMDAR1 subunit protein levels in this area. This adaptive mechanism of prefrontal NMDA receptor function could not be detected in NHE rats, supporting our hypothesis of NMDA hyperfunction in this ADHD animal model. Moreover, in NHE rats prefrontal soluble L-GLu and L-Asp as well as membrane-trapped L-Asp levels were elevated, which may also be interpreted in terms of prefrontal EAA hyperactivity relative to NRB controls.

The regional differences between both soluble and membrane-trapped EAAs in both baseline and after IN-DA imply that EAA function is severely disturbed in the NHE rat model of ADHD also after long-term IN-DA treatment. Basal prefrontal and dorsal striatal levels of both soluble and membrane-trapped EAA levels appear to be lowered relative to normal rats, which may be causally related to an increased availability of NMDA binding sites (as indicated by the elevation of baseline prefrontal and both dorsal and ventral striatal NMDAR1 subunit protein levels relative to NRB controls) and, possibly, a compensatory net EAA hyperfunction. A further decrease of EAA levels as induced by IN-DA, then may be surmised to result in a further increase of prefrontal and (ventral) striatal NMDAR1 subunit protein levels (and probably also NMDA binding sites). However, also the levels of soluble and membrane-trapped EAAs appear to rise adding to the effect of net EAA and NMDA hyperfunction.

Behavioral results

Horizontal activity assessed with the Làt maze was significantly reduced in NHE rats after 0.3 mg/kg IN-DA, while nonselective attention (vertical activity in terms of duration of leaning against the wall with one or both forepaws) was significantly increased after the same dose. Moreover, selective spatial attention (in terms of FE values), as obtained with the Eight-arm radial Olton maze, were significantly elevated after 0.15 mg/kg IN-DA. Interestingly, IN-DA produced no effect in NRB controls.

Findings show that IN-DA significantly influenced activity and attention in NHE rats relative to vehicle. Thereby, results on horizontal activity and FE values were consistent with previous findings on NHE rats after the same doses of IN-DA (Ruocco et al. 2009a). In contrast, however, to the present investigation, IN-DA exerted no effect on vertical activity. This might be due to the fact that the previous study did not differentiate between rearing on hind limbs and leaning against the wall with one or both forepaws, which may have led to disparate findings.

As stated above, DA synthesis and release are modulated by a negative feedback loop, which is established by DA acting upon presynaptic terminal autoreceptors of the inhibitory D2/3 receptor subtype (for review see Langer 1997). In the present study, a biphasic action was observed after IN-DA with a decrease of TH in the VTA after 0.15 mg/kg and an increase after 0.3 mg/kg IN-DA. From this may be concluded that DA concentrations after 0.3 mg/kg IN-DA at the time of killing and brain dissection were lower compared with the 0.15 mg/kg dose, as the application of the higher dose, in contrast to the lower one, had resulted, firstly, in DA concentrations sufficiently high to activate feedback inhibition at the presynaptic terminal leading to a reduction of DA synthesis and release, and then, secondly, in DA concentrations sufficiently low to disinhibit presynaptic D2 autoreceptors, leading again to an enhancement of DA synthesis (indicated by the increase of TH levels) and release. The reduction in horizontal activity, as well as the increase in nonselective attention at 1 h after IN-DA administration are, thus, likely to be related to the action of DA occurring first in order at the inhibitory D2 autoreceptor binding sites and leading to an activation of feedback inhibition and subsequent reduction in DA synthesis and release.

With the improvement of selective spatial attention (or working memory), matters are different, as here the lower IN-DA dose of 0.15 mg/kg proved to be effective. Moreover, in the Eight-arm radial Olton maze, data were acquired 24 h instead of 1 h after the last administration of IN-DA or vehicle. It follows that 0.15 mg/kg IN-DA exerted long-term effects on selective attention/working memory which was not the case for the 0.3 mg/kg dose. The fact that 0.3 mg/kg, but not 0.15 mg/kg was effective in reducing horizontal activity and increasing nonselective attention in the Làt maze suggests that the lower dose was not sufficient to induce feedback inhibition at D2 autoreceptor binding sites and reduce DA levels. The increased availability of DA in both DS and VS (elicited by the disinhibition of presynaptic D2 autoreceptors occurring second in order), however, may have induced a lasting decline of EAA levels, which may have acted back on striatal and mesostriatocortical DA neurons. Thereby, it may not be dismissed, that also the reduction of horizontal activity as well as the improvement of nonselective attention may be additionally related to the inhibition of EAA release.

Our findings may have been confounded by the fact the basal level of both EAAs were strikingly lower in NHE rats across all brain areas compared to a previous study (Ruocco et al. 2009b). This could be explained in several ways. First, in the present study, EAAs were expressed as ratio over L-Leu; as L-Leu levels were higher in NHE rats, this may have decreased the levels of soluble EAAs relative to NRB controls. Secondly, in the present study, we attempted to probe the functional compartmentalization of L-Glu and L-Asp, whereas in the previous study we considered whole

brain levels. Thirdly, discrepancies might be due to methodological differences, such as extraction from brain samples, sensitivity and selectivity of detection system (LC/MS/MS vs. HPLC). Fourthly, the higher sensitivity of LC/MS/MS allowed detection of EAAs in individual samples versus average content from pooled areas. Lastly, animal handling before the beginning of the experiment and the intranasal route of administration are likely to have resulted in milder stress (Ruocco et al. 2009d).

In addition, it must be considered that DA and EAAs not only interact with each other, but also with a variety of other neurotransmitters, including noradrenaline (NA), serotonin (5-HT), μ-aminobutyric acid (GABA) and histamine (Flint et al. 1985; Peris and Dunwiddie 1985; Barbeito et al. 1989; Rodriguez et al. 1997). The far-reaching impact of neurotransmitter interactions has been demonstrated for a variety of neuropsychiatric conditions including anxiety disorder, major depression, bipolar disorder and schizophrenia (Nikolaus et al. 2010, 2012, 2014). Also in ADHD and the NHE rat model of this disorder, the disturbances of DA and EAA function are likely to induce alterations of NAergic, 5-HTergic, GABAergic and/or histaminergic neurotransmission, which may be expected to positively or negatively influence human as well as animal behaviors. Therefore, investigations are needed, which specifically assess NA, 5-HT, GABA and/or histamine function in NHE rats. Moreover, in addition to NMDA receptor dysfunction, also disturbances of Glu signaling via metabotropic receptor subtypes is implied in the pathophysiology of ADHD (for review see Lesch et al. 2013). Thus, future efforts should be also directed towards assessing the contribution of other EAA receptor subtypes to the behavioral abnormalities of the NHE rat.

Taken together, the findings obtained in the present investigation indicate that the NHE rat model of ADHD may be characterized by (1) prefrontal and striatal DAT hyperfunction, indicative of DA hyperactivty, and (2) prefrontal and striatal NMDA receptor hyperfunction, indicative of net EAA hyperactivty. IN-DA has ameliorative effects on activity, attention and working memory, which are likely to be associated (1) with DA action at inhibitory D2 autoreceptor binding sites, leading to a reduction in striatal DA hyperactivity and, possibly, (2) with DA action on striatal EAA levels leading to a reduction in striatal EAA hyperfunction (with persistence of prefrontal EAA hyperfunction). Previous studies on IN-DA treatment in rodents have indicated antidepressant, anxiolytic and anti-parkinsonian effects in relation to enhanced central dopaminergic activity (Buddenberg et al. 2008; De Souza Silva et al. 2008; Pum et al. 2009). Our present results strengthen the prospects of potential therapeutic application of IN-DA by indicating an enhancement of selective attention and working memory in a deficit model.

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Conflict of interest None of the authors declare any conflict of interest, except for C. Mattern, who is employed by M & P Pharma AG, Emmetten, Switzerland.

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