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The Effect of Nitric Oxide Synthase Inhibitors Nitro-L-Arginine and 7-Nitroindazole on Spatial Learning and Motor Functions in Lurcher Mutant and Wild Type Mice

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Abstract: Nitric oxide (NO) is an intercellular messenger that, among other things, plays an important role in the nervous system as a gaseous neurotransmitter, modulating long-term potentiation (LTP) induction of synaptic transmission. LTP has been suggested to be the basis of memory formation. On the other hand NO also participates in excitotoxic processes which play an important role in many neuropathological states. The aim of this work was to observe the effect of two NO synthase (NOS) inhibitors (Nω-Nitro-L-arginine, NA; 7-nitroindazole, NI) on spontaneous behaviour, spatial learning and motor functions in Lurcher (+/Lc) and wild type (+/+) mice, derived from the B6CBA strain. Heterozygous Lurcher mutant mice represent a natural model of the olivocerebellar degeneration. They suffer from postnatal, practically total, extinction of cerebellar Purkinje cells (due to the excitotoxic apoptosis) and a partial decrease of granule cells and inferior olive neurons (ION) because of the lost target of their axons. +/+ animals are healthy littermates of +/Lc. NA is a nonselective NOS inhibitor which influences, except neuronal (n), also endothelial (e) NOS with an impact on blood pressure, NI is a selective nNOS inhibitor without any circulatory effect. The adult animals of both types (+/Lc; +/+) were influenced by acute administration of both inhibitors (25 mg/kg i.p. 30 min. before experiments) and newborns only by both acute and long-term administration of NI (1 month, starting from postnatal day 2, P2). Control solutions – saline or solvents of both NA and NI inhibitors – diluted 1M HCl and dimethyl sulfoxide (DMSO) respectively, were given at a relevant volume in the same way. The effect of both inhibitors and control solutions on motor functions was tested using four standard procedures (horizontal wire, slanting ladder, rotating cylinder, foot-bridge); in newborns at the age of 14 days. Spatial learning ability was examined in five-day long procedure in the Morris water maze (MWM) (in newborns started on P21). Spontaneous behaviour was studied only in adult animals (after acutely influencing them) employing the open field method. The results showed, that neither the Lurcher mutant, nor wild type mice derived from the B6CBA strain were significantly affected by NOS inhibitors NA and NI in spatial learning after both the acute and long-term application. Only significant decrease of swimming speed was found in both types of mice after the acute administration of NI and in the wild type animals after the acute administration of NA. Motor functions were significantly negatively affected only in the Lurcher mutants after both the acute and chronic application of NI.

Introduction

Lurcher mutant mice represent a naturally originated model of genetically determined olivocerebellar degeneration [1, 18]. They are in fact heterozygous individuals (+/Lc) suffering from postnatal loss of Purkinje cells, and secondary decrease of cerebellar granule cells and inferior olivary neurons [2, 3]. The

extinction of practically of all Purkinje cells is due to an excitotoxic apoptosis as a consequence of mutation in the γ 2 glutamate receptor gene [4], the partial decrease of the other neurons is because of the disappearance of the Purkinje cells, the targets of their axons [5, 6, 7]. Lurcher mutants are therefore used in the research studying the consequences of a functional cerebellar decortication and the possibility of influencing this degeneration process by various procedures. Because the reduction in Purkinje cell number can be detected since P8 and the degeneration is finalized at P90, the cerebellar ataxia consequently develops in the Lurcher mutants from the end of the second postnatal week. In addition, the mutants reveal deterioration of some cognitive functions, including spatial learning abilities. They also suffer from higher CNS excitability [8]. Homozygous individuals (Lc/Lc) are not viable. Wild type homozygots (+/+) are completely healthy and are employed as ideal controls.

Nitric oxide (NO) is an intercellular messenger that, among other things, plays a role in the nervous system as a gaseous neurotransmitter, modulating LTP induction of synaptic transmission that has been suggested to be the basis of memory formation on the cellular level [9].

NOS is a catalytic enzyme in synthesis of NO from arginin (there arises citrulin and NO). There are 3 isoforms of NOS: endothelial, which is in endothelial vessels cells, in the brain too, inducible, which is induced in activated glia and neuronal NOS – the main isoform in the brain, present in neurons. NO plays a role in the formation of memory trace. Lurcher mutant mice have the decreased cognitive functions and it appeared to be interesting to discover the difference in the action of inhibitors NOS on both the wild type and Lurcher mice.

On the other hand NO also participates in the excitotoxic processes which play an important role in many neuropathological states.

Nω-Nitro-L-arginine (NA) is an unselective nitric oxide synthase (NOS) inhibitor which except neuronal, also influences endothelial NOS with an impact on blood pressure [10]. 7-nitroindazole (NI) is a selective neuronal NOS inhibitor without this circulatory effect [11, 12].

The aim of our work was to observe the acute effect of both these NOS inhibitors (NA and NI) on spatial learning, spontaneous behaviour and motor functions in adult +/Lc and +/+ derived from the B6CBA strain and to compare selective and nonselective types of inhibition. In young animals we studied the effect of acute NI administration only on motor functions while the effect of long-term NI administration we observed in both testing of their motor activities and spatial learning.

Materials and Methods

All experiments were performed in full compliance with the EU Guidelines for scientific experiments on animals and with permission of the Ethical Commission of the Faculty of Medicine in Pilsen.

In acute experiments 90 adult animals of both sexes (approximately half and half) were used, 45 wild type and 45 Lurcher mutants. The animals of both types were divided into five groups with the application of:

- NT-Nitro-L-arginine dissolved in diluted 1M HCl (NA),
- Diluted 1M HCl (control solution- CS),
- 7-nitroindazole dissolved in DMSO (NI),
- Dimethyl sulfoxide (DMSO)
- Saline (S).

NA and NI were administrated in the dose 25 mg/kg i.p. in the solvent, altogether 4 μl/g, control solution and DMSO in relevant volumes, 30 min. before the experiment in the same way.

Spatial learning was examined using the Morris water maze (MWM) [13] with a platform in the south-west quadrant hidden under the water surface. Four starts daily from different cardinal points (N, S, E, W) were performed for 5 consecutive days (D1–D5). The parameters measured were: the latency of animals reaching the platform, the trajectory length and the swimming speed.

Motor functions were examined employing four standard tests that have been already described [14]. We measured the latencies of staying animals on individual tools (horizontal wire, rotating cylinder, foot-bridge, slanting ladder). Behavioural characteristics were observed using the open field method (area 40 × 40 cm) so that the length of trajectory and place preferences was measured.

In chronic experiments 54 animals were used, 27 +/+ and 27 +/Lc. The animals of both types were divided into three groups with application of:

- NI,
- DMSO,
- S (saline).

NI in the dose 25 mg/kg as well as control solutions in relevant volumes were administered subcutaneously from P2. Motor functions were tested in the same way as in the acute experiments in animals aged 14 days. Tests were repeated on the next day once after the acute application of these substances.

Spatial learning was examined using MWM according to the same protocol as in the acute experiments in mice aged 21–25 days.

Statistical evaluation of all results was performed using paired t-test.

Results

Acute experiments

In +/+ only one small insignificant difference was found between the influenced and control animals in motor functions. It was lower latency of staying on individual tools in mice after administration of NI versus animals that were given saline (NI/ S – $p<0{,}134$), (Figure 1A).

In MWM there were significantly longer latencies of reaching the platform in animals that were given NI and NA (NA/CS D5 – $p<0.021$, NA/S D5 – $p<0.049$, NI/DMSO D5 – $p<0.018$, NI/S D5 – $p<0.002$, NI/S D4 – $p< 0.0001$, NI/S D3 – $p<5.5 \times 10^{-6}$, NI/S D2 – $p<0.007$) (Figure 1B). Both these inhibitors also highly significantly decreased the swimming speed – measured in D5 (NA/CS – $p<5.1 \times 10^{-6}$, NA/S – $p<5.37 \times 10^{-6}$, NI/DMSO – $p<0.0006$, NI/S – $p<1.17 \times 10^{-6}$) (Figure 1C). In addition, it was typical to observe that mice affected by NI were lying on the water surface for a few seconds when placed into the MWM and they began to swim only after audio-stimulus. Nevertheless, the mean length of the swimming trajectories, as a marker of spatial learning, was not significantly changed in the influenced animals compared to controls on D5 (NA/CS – $p<0.075$, NA/S – $p<0.122$, NI/DMSO – $p<0.751$), though the comparison of NI administered mice to individuals of saline given group (NI/S) was – $p<0.056$. (Figure 1D).

In +/Lc only the NI influenced animals revealed significantly shorter latencies of staying on individual tools in acute experiments of motor tests compared to mice

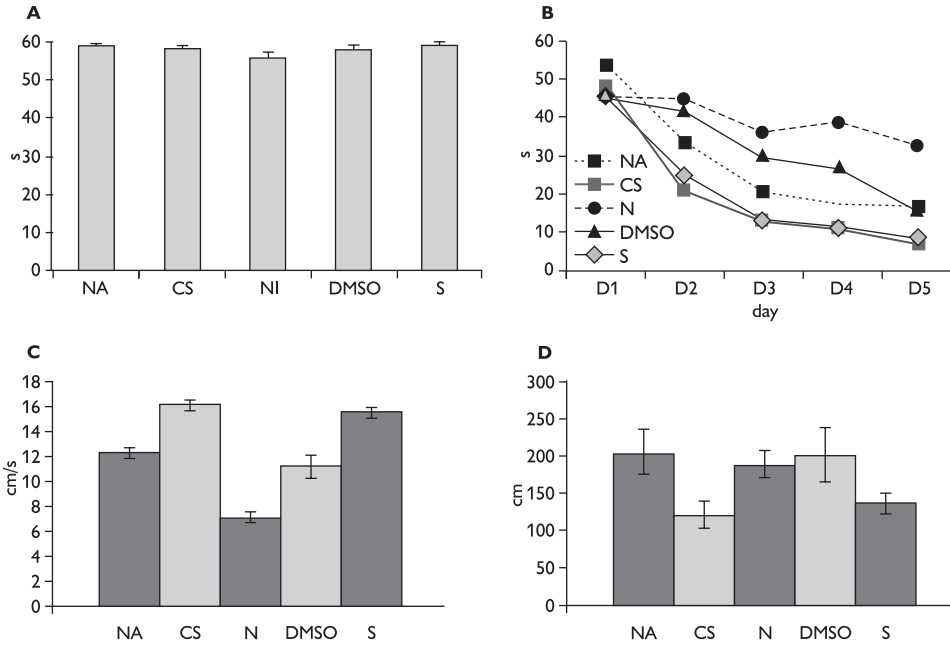

Figure 1 – a) Mean latencies in four various motor tests ± SEM, $p > 0.05$; b) Mean latencies of reaching the platform in the Morris water maze in individual experimental days (D1–D5), significant differences ($p < 0.05$): NA/CS – D5, NA/S – D5, NI/DMSO – D5, NI/S – D5, NI/S – D4, NI/S – D3, NI/S – D2; c) Mean of the swimming speed on the 5th experimental day ± SEM, NA/CS, NA/S, NI/S – $p < a.10^{-6}$, NI/DMSO – $p<0.0006$, DMSO/S – $p<0.002$; d) Mean length of the swimming trajectory on the 5th experimental day ± SEM, $p > 0.05$.

of the saline group (NI/S – $p<0.047$). All the other differences were insignificant (Figure 2A).

In MWM there were significant differences in latencies of reaching the platform only in NI versus DMSO and S groups of animals (NI/DMSO D3 – $p<0.026$,

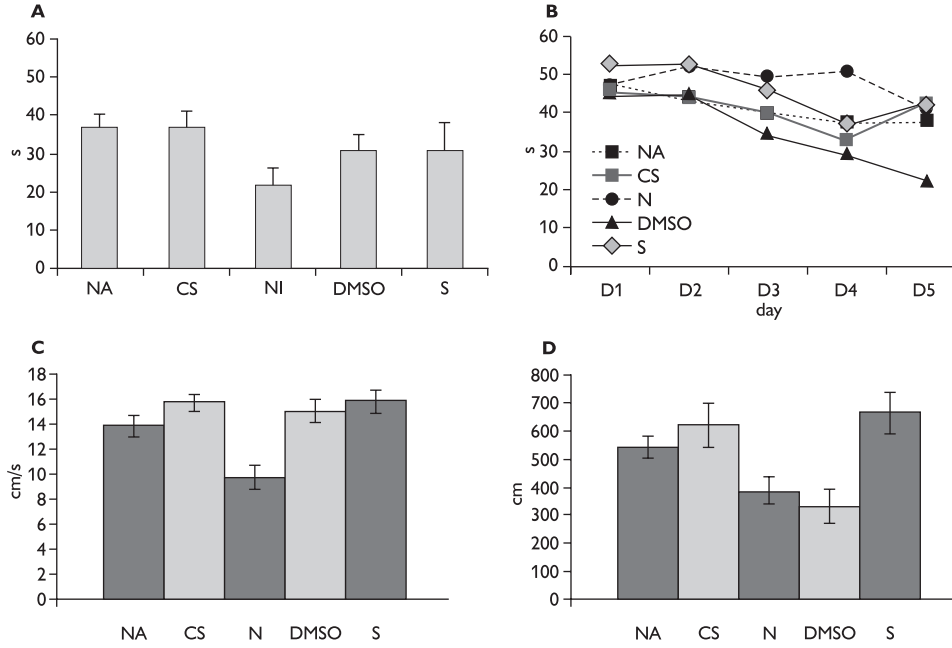

Figure 2 – a) Mean latencies in four various motor tests ± SEM NI/S, $p < 0.05$; b) Mean latencies of reaching the platform in the Morris water maze in individual experimental days (D1–D5), significant differences ($p < 0.05$): NI/DMSO – D3, D4, D5, NI/S – D4; c) Mean of the swimming speed on the 5^{th} experimental day ± SEM, NI/DMSO – $p < 0.04$, NI/S – $p < a.10^{-5}$; d) Mean length of the swimming trajectory on the 5^{th} experimental day ± SEM, NI/S – $p < 0.01$.

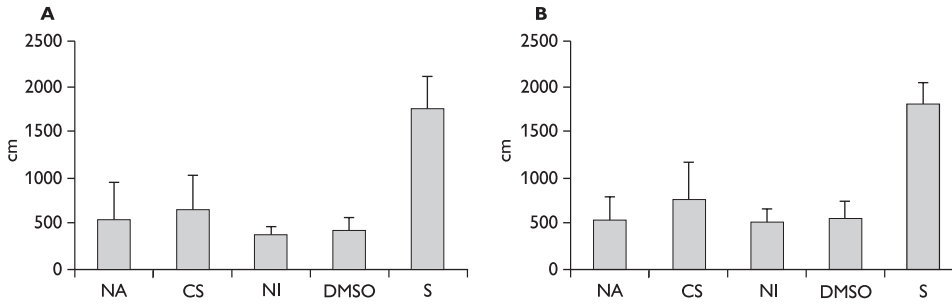

Figure 3 – a) Mean length of trajectory in the open field ± SEM, NA/S – $p < 0.0007$, NI/S – $p < 0.0004$; b) Mean length of trajectory in the open field ± SEM, NA/S, NI/S – $p < a.10^{-6}$.

D4 – $p<0.0008$, D5 – $p<0.0082$, NI/S D4 – $p<0.036$) (Figure 2B). The swimming speed /measured in D5/ was significantly different only between NI mice compared to DMSO and S groups (NI/DMSO – $p<0.04$, NI/S – $p<8.2 \times 10^{-5}$) (Figure 2C). The mean length of the swimming trajectory /D5/ was significantly shorter in both NI and DMSO influenced mice compared to S group. (NI/S – $p<0.0095$, NI/DMSO – $p<0.513$ (Figure 2D). No other differences were significant.

The observation of behavior in open field showed significant differences in animals of all groups (NA, CS, NI, DMSO) of both types (+/Lc, +/+) that revealed lower activity as compared to mice that were given saline (NA/S – $p<0.00074$, NI/S – $p<0.0004$ in, +/+), (Figure 3A) and **(**NA/S – $p<7.6 \times 10^{-6}$, NI/DMSO – $p<0.6$, NI/S – $p<3.5 \times 10^{-6}$, +/L), (Figure 3B).

Chronic experiments

The results of motor testing in +/+ showed no significant differences between animals of NI, DMSO and S groups (Figure 4A). A similar situation was observed in the spatial learning examination in MWM when no significant differences in latencies, the swimming speed and distance among animals of the three groups

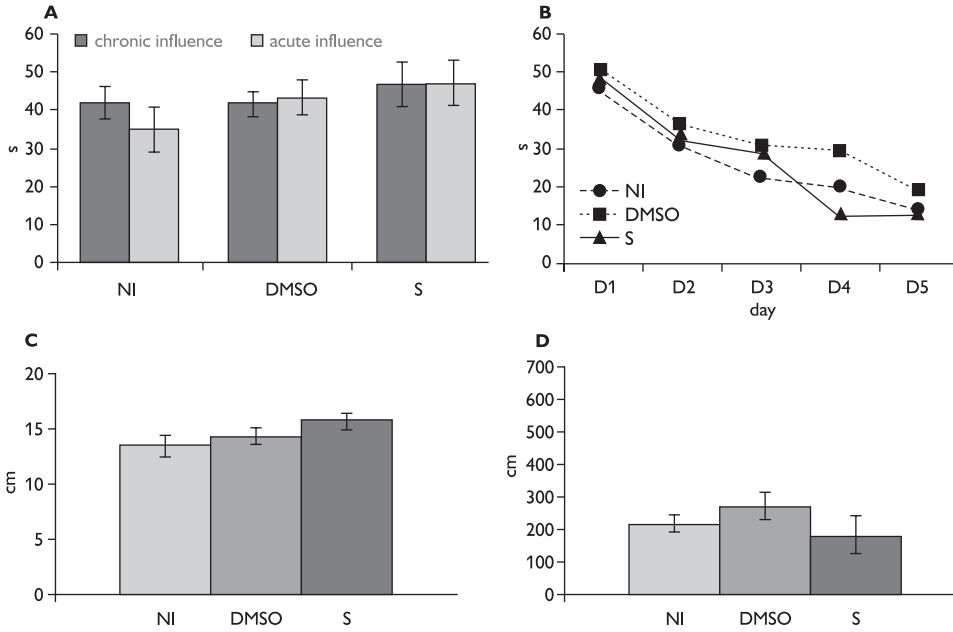

Figure 4 – a) Mean latencies in four various motor tests ± SEM, $p > 0.05$; b) Mean latencies of reaching the platform in the Morris water maze in individual experimental days (D1–D5), NI/DMSO, NI/S – $p > 0.05$; c) Mean of the swimming speed on the 5th experimental day ± SEM, $p > 0.05$; d) Mean length of the swimming trajectory on the 5th experimental day ± SEM, $p > 0.05$.

were found. The following acute administration of all substances did not significantly change the results (Figure 4B, C, D).

On the contrary, in +/Lc we found significantly decreased motor ability in both NI and DMSO influenced animals (NI/S chr – $p<0.016$, DMSO/S chr – $p< 0.027$), which revealed lower latencies of staying on individual tools compared to those ones that were given saline. The acute administration of the drugs did not lead to any other significant changes (NI, DMSO chr, ac /S – $p<0.05$), (Figure 5A).

In MWM no significant differences were found among animals of individual groups in latencies, trajectories and swimming speed, (Figure 5B, C, D).

Discussion

The non-specific NOS inhibitor NA in the doses used significantly decreased the swimming speed in +/+ tested in spatial learning. However, this test also showed that cognitive functions as well as motor functions were not significantly affected by this drug. In +/Lc NA did not have any significant effect.

The specific n NOS inhibitor NI after acute application in +/+ significantly decreased the mean swimming speed and even the willingness to swim, which could be a consequence of catalepsy [15] during swimming in MWM. It was

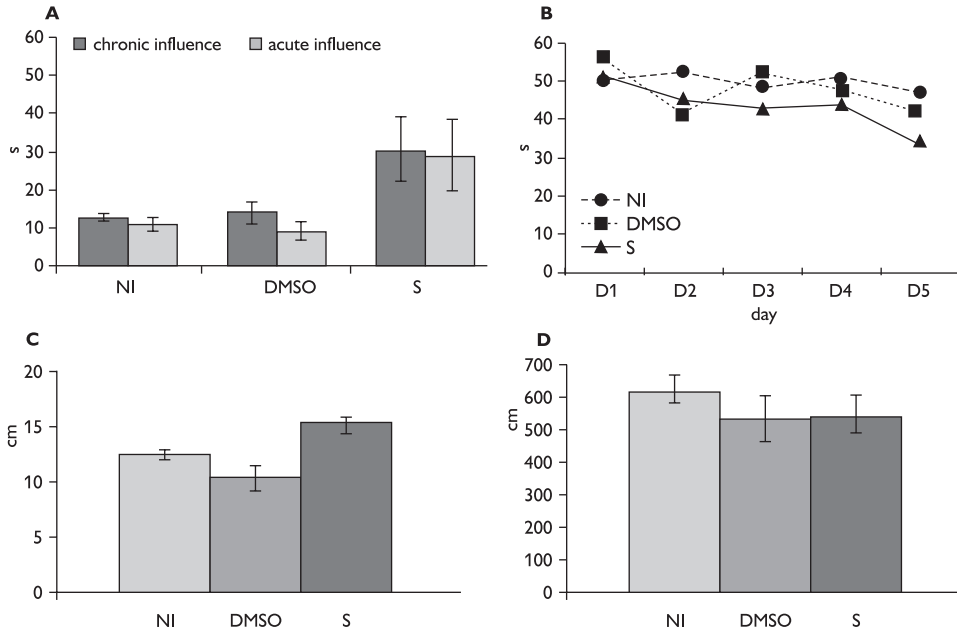

Figure 5 – a) Mean latencies in four various motor tests ± SEM, NI/S chr, NI/S ac – $p < 0.03$; b) Mean latencies of reaching the platform in the Morris water maze in individual experimental days (D1–D5), NI/DMSO, NI/S – $p > 0.05$; c) Mean of the swimming speed on the 5th experimental day ± SEM, $p > 0.05$; d) Mean length of the swimming trajectory on the 5th experimental day ± SEM, $p > 0.05$.

manifested by significantly longer latencies of reaching the platform. Nevertheless, the swimming speed was also significantly (yet not so highly) decreased in the DMSO treated mice. Because DMSO as a solvent of NI was given also to mice of NI group, there is a question to what extent the results are influenced by one and/or other substance. Concerning the length of swimming trajectory no significant differences between the experimental and control animals were found. After the chronic application of these substances +/+ were not significantly affected in any experiments performed.

+/Lc in acute experiments revealed significantly decreased motor ability after the application of NI in both motor tests and the swimming speed in MWM. In these experiments DMSO did not influence either motor functions or swimming speed of the examined animals. However, the swimming trajectories of animals from both groups (NI, DMSO) were significantly shorter than in mice of the S group. It means that in this case the spatial learning process was not negatively affected, but rather opposite. The reason can be that the lower swimming speed was a consequence of a certain damping or inhibition of these animals which made them to be able to climb the platform more easily compared to those with saline. These animals were hyperactive and they often only stuck to the platform instead of climbing it. By chronic administration NI significantly decreased the motor ability of influenced animals, but DMSO mice alike. Nevertheless, the age of animals also may play a role here. In the acute experiments only adult mice were used and no negative effect of DMSO on their performance was observed. On the contrary, the chronic experiments were performed on 2–3 weeks old mice and the effect of this solvent on their motor ability was evident. The results which showed the decreased spontaneous activity in the open field test were likely a consequence of inhibitory effect of i. p. administered drugs we used on motor activity of experimental animals. In spite of these non uniform results, it is necessary to state, that DMSO as a solvent has some effect and could influence the results.

Conclusion

Taken together, our results suggest, that both specific and non-specific inhibitors of NOS negatively influenced some motor functions and behaviour in the Lurcher mutant and wild type mice derived from B6CBA strain in a different way. Concerning the effect of these drugs on spatial learning, it is possible to conclude, that the learning and memory were not negatively influenced despite longer latencies of animals to find and climb the platform in MWM. Paradoxically, in Lurchers we found the positive effect of acutely applied NI on swimming trajectory (the marker of learning) which was shorter in NI acutely influenced mice than in S controls. Because not only the acute or chronic administration of these drugs but undoubtedly also the means of application, the types of animals, their age and last but not least also solvents used played a role here, this interesting problem will be the subject of further study.

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