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Antidystonic efficacy of nitric oxide synthase inhibitors in a rodent model of primary paroxysmal dystonia

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1 In a hamster model (genetic symbol dt^{sz}) of primary paroxysmal non-kinesiogenic dystonic choreoathetosis, recent studies have shown beneficial effects of glutamate and dopamine receptor antagonists. Nitric oxide (NO), synthesized from L-arginine by NO synthase in response to glutamate receptor activation, elicits cyclic GMP and modulates glutamate-mediated processes and striatal dopamine release.

2 Therefore, the effects of NO synthase inhibitors and of L-arginine on severity of dystonia were investigated in dt^{sz} hamsters in which dystonic attacks, characterized by twisting movements and postures, can be induced by stress.

3 The NO synthase inhibitors N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole significantly reduced the severity of dystonia. At antidystonic effective doses neither L-NNA nor L-NAME caused observable side effects, whereas 7-nitroindazole exerted moderate reduction of locomotor activity.

4 The antidystonic effect of L-NAME was reversed by co-administration of the NO precursor L-arginine. However, L-arginine administered alone did not exert any effect on severity of dystonia.

5 Cerebellar cyclic GMP levels in brains of mutant hamsters in comparison to non-dystonic control hamsters did not significantly differ, but the cerebellar cyclic GMP levels tended to be increased in dt^{sz} hamsters during a dystonic attack. L-NAME significantly decreased the cerebellar cyclic GMP levels in both dt^{sz} and control hamsters.

6 Although an overproduction of NO is probably not critically involved in the pathogenesis of paroxysmal dystonia, it may contribute to the manifestation of dystonic attacks, as indicated by the antidystonic effects of NO synthase inhibitors.

7 Peripheral side effects may limit the clinical use of NO synthase inhibitors, but more selective inhibitors of the neuronal NO synthase should be considered as interesting candidates for the treatment of paroxysmal dystonia.

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Abbreviations: L-NAME, N^G-nitro-L-arginine methyl ester; L-NNA, N^G-nitro-L-arginine

Introduction

Nitric oxide (NO) is an important messenger molecule implicated in multiple physiological and pathophysiological events in the central nervous system (Moncada *et al*., 1991; Schuman & Madison, 1994). NO is synthesized from L-arginine by NO synthase in response to Ca^{2+} entry through the activated *N*-methyl-D-aspartate (NMDA) receptor-gated ion channels. NO diffuses rapidly and, as shown particularly in the cerebellum of rodents, exerts its action in surrounding target cells by increasing cyclic GMP levels (Garthwaite *et al*., 1988, Schuman & Madison, 1994). It seems likely that NO acts *via* cyclic GMP dependent mechanisms by modulating the release of various neurotransmitters (Prast *et al*., 1994; Schuman & Madison, 1994), e.g., NO has been shown to enhance the striatal dopamine release (Bowyer *et al*., 1995; Stewart *et al*., 1996). NMDA receptor-mediated neurotransmitter release is regulated, in part, through NO production and can be blocked by NO synthase inhibitors (Dawson & Dawson 1996).

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With regard to the close interaction of NMDA-receptor and NO-mediated processes, NO synthase inhibitors have been investigated in experimental models of neurological diseases associated with abnormal motor behaviour, such as parkinsonism and epilepsy, in which enhanced NMDA-receptor activation is thought to be involved in the pathophysiology (e.g. Eblen *et al*., 1996, Rundfeldt *et al*., 1995). Primary dystonias, a group of a relatively common movement disorder characterized by sustained muscle contractions causing twisting movements or abnormal postures, have been presumed to be caused by biochemical dysfunctions, such as by dopaminergic overactivity or dopaminergic deficits in the striatum, and altered neuronal activity within the basal ganglia. Apart from the Dopa-responsive dystonia, however, no consistent abnormality has been identified as yet and the benefit of medical treatment is often insufficient (Fahn 1995; McGeer & McGeer, 1995). The knowledge about the pathophysiological relevance of excitatory amino acids in dystonias and efficacy of glutamate receptor antagonists is sparse (McGeer & McGeer, 1995, Richter & Löscher, 1998). To our knowledge, there are no data on the effects of NO synthase inhibitors in dystonias.

Recent pharmacological and neurochemical studies in mutant dystonic hamsters (gene symbol dt^{sz}), a genetic animal

model of primary paroxysmal non-kinesiogenic dystonic choreoathetosis, indicated that enhanced activation of NMDA receptors and striatal dopaminergic overactivity is involved in this subtype of dystonia in which dystonic attacks can be provoked by stress and anxiety (Nobrega *et al*., 1997; Rehders *et al*., 2000; Richter *et al*., 1991; Richter & Löscher 1997). Although there is evidence that glutamatergic and dopaminergic overactivity probably does not represent the primary defect, these dysfunctions seem to contribute to the manifestation of dystonic episodes in this animal model (for review see Richter & Löscher, 1998). These recent findings prompted us to examine the role of NO in dystonia in the hamster model.

Four distinct isoforms of NO synthase have been identified (Schuman & Madison, 1994). N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole inhibit endothelial and neuronal NO synthase isoforms (Moore *et al*., 1991; Moore & Handy, 1997). Depending on the used compound, dose and pretreatment time, there are conflicting data on effects of NO synthase inhibitors in animal models of epilepsy, parkinsonism or cerebral ischaemia (Eblen *et al*., 1996; Rundfeldt *et al*., 1995; Schuman & Madison, 1994). Therefore, in the present study, we examined the effects of different NO synthase inhibitors, i.e. L-NNA, L-NAME and 7-nitroindazole, as well as the effects of the NO precursor L-arginine on the severity of dystonia. Furthermore, cyclic GMP levels were determined in brains of dt^{sz} hamsters and non-dystonic control hamsters before and after treatment with L-NAME.

Methods

Animals

The present experiments were carried out in groups of dt^{sz} hamsters which were obtained by selective breeding (for detailed descriptions see Löscher *et al*., 1989; Richter & Löscher, 1998). For measurements of cyclic GMP additional groups of age-matched non-dystonic hamsters of an outbred line were used. Dystonic attacks in dt^{sz} mutant hamsters, characterized by generalized twisting movements and abnormal postures of limbs and trunk, can be induced by handling and mild environmental stimuli (Löscher *et al*., 1989; Richter & Löscher, 1998).

As shown by previous clinical, electrophysiological and pharmacological studies, the dt^{sz} hamster shows all characteristics of primary paroxysmal non-kinesiogenic dystonia (for review see Richter & Löscher, 1998). Similar to primary dystonia in humans, dystonia in mutant hamsters occurs in the absence of morphological alterations in the brain or spinal cord (Wahnschaffe *et al*., 1990). Brain regions with abnormal neuronal activity are the basal ganglia, thalamic and deep cerebellar nuclei and the red nucleus (Richter & Löscher, 1998).

In mutant hamsters the severity of dystonia is age-dependent with maximum severity between the 30th and 40th day of life (max-period, suitable to study antidystonic effects of drugs). Thereafter, the severity of dystonia slowly declines (post-max period, suitable to examine prodystonic drug-effects) until complete remission of dystonia appears with the age of about 10 weeks (Richter & Löscher, 1993).

Drug testing

In dt^{sz} hamsters dystonic attacks can be induced by the procedure of triple stimulation (Löscher *et al*., 1989), i.e. (1) taking the animal from its home cage and placing it on a balance, (2) i.p. injection of vehicle (pre- and post-drug control) or of the drug (i.e. in the present study of N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine (L-NNA), 7-nitroindazole or L-arginine), (3) placement of the hamster in a new environment. The severity of dystonia was rated by the following score-system (Löscher *et al*., 1989): stage 1, flattened ears and flattened posture; stage 2, facial contortions, rearing with forelimbs crossing, disturbed gait with retarded setting of the forepaws; stage 3, stiffened hindlimbs so that the animals appear to walk on tiptoes; stage 4, twisting movements and loss of balance; stage 5, hindlimbs hyperextended caudally; stage 6, immobilization in a twisted, hunched posture with hind- and forelimbs tonically extended forward and opisthotonus. The examiner of the severity of dystonia was unaware of the drugs used in the present investigations. The individual maximum stage of dystonia is usually reached within 3 h after the hamsters were placed in the new cage. Therefore the hamsters were observed for 3 h. During this period the severity of dystonia, the latencies to the different stages and, in case of drug trials, the side effects (not quantified) were noticed. After reaching the maximum stage the hamsters usually recover within 2–5 h. The control trials were done 2 days before and 2 days after drug testing.

L-NAME (used as HCL), L-NNA and L-arginine, purchased from Sigma (Munich, Germany), were freshly dissolved in water (L-NNA with the aid of dilute HCL). 7-nitroindazole (RBI Biotrend, Cologne, Germany) was freshly suspended in 1% Tween 80. All drugs were administered intraperitoneally. Injection volume was 5 ml kg^{-1}. For control trials (pre- and post-drug recordings) the hamsters received the same volume of vehicle.

The significance of differences in severity of dystonia and latencies to onset of the first unequivocal signs of dystonia (stage 2) and to maximum severity (stage 6) were calculated by the Wilcoxon signed test for paired replicates.

Measurements of cyclic GMP

Determinations of cyclic GMP concentrations were undertaken to examine if antidystonic effects of 50 mg kg^{-1} L-NAME are accompanied by decreases of cyclic GMP. For the determinations of brain cyclic GMP levels two groups of dt^{sz} mutant hamsters and two groups of control hamsters were decapitated (at the age of 34 days) 3 h after triple stimulation procedure. One group of dt^{sz} and control hamsters was decapitated 3 h after administration of vehicle (basal) and a second group 3 h after administration of L-NAME (50 mg kg^{-1} i.p.). When the animals were decapitated, dt^{sz} hamsters exhibited severe (basal) or moderate (after L-NAME) dystonia, while no motor disturbances occurred in both groups of control hamsters.

The brains were quickly dissected (frontal cortex, striatum, cerebellum) and homogenized in an ice-cold 6% TCA. The homogenates were centrifuged at $2500 \times g$ for 15 min and the supernatants were extracted three times with ether. The remaining homogenates were used for protein determinations. The extracts were vacuum-dried overnight. Dried samples were kept at $-80^{\circ}C$ until analysis. For cyclic GMP detection, a commercial enzymimmunoassay kit (Biotrak, Amersham) was used. Samples were redissolved in 1 ml assay buffer and 50 μl aliquots were used in the assay. cyclic GMP values were expressed as pmol/mg protein. Protein determinations were done using the method of Lowry *et al*. (1951) using a microplate reader.

The significance of differences in cyclic GMP levels were calculated with ANOVA and *post hoc* by the Tukey test.

Results

As shown in Figure 1, L-NAME significantly reduced the severity of dystonia in mutant hamsters at the dose of 50 mg kg^{-1} during the 2nd and 3rd hour of observation. L-NAME did not exert significant effects on the latency to onset of dystonic symptoms. At 5 or 10 mg kg^{-1}, no significant effects on severity or latency to onset of dystonia were recorded. At all doses tested, L-NAME did not cause any observable adverse effects.

L-NNA retarded the progression of dystonia in mutant hamsters (Figure 2). The severity of dystonia was decreased during the 1st (75 mg) or 2nd (50 mg) hour of observation. At both doses tested, L-NNA did not exert significant effects on latency to onset of dystonia, but the latency to the maximum severity (stage 6) was significantly increased ($P<0.05$) from 89.4 ± 22.7 (pre-drug) or 74.0 ± 23.8 (post-drug) to 163.8 ± 4.7 min (50 mg) and from 69.4 ± 6.5 (pre-drug) or 87.0 ± 16.9 (post-drug) to 129.9 ± 16.4 min (75 mg). At both doses no behavioural side effects were observed.

7-Nitroindazole significantly decreased the severity of dystonia during the 1st and 2nd hour of observation at a dose of 75 mg kg^{-1}, while no effects on severity or latencies were found after administration of 50 mg kg^{-1} (see Figure 3). At a dose of 75 mg kg^{-1}, 7-nitroindazole significantly ($P<0.05$) increased the latency to onset of dystonia and the latency to the maximum severity (stage 6) from 74.6 ± 12.5 (pre-drug) or 74.4 ± 4.8 (post-drug) to 121.8 ± 19.6 min. At the antidystonic effective dose, 7-nitroindazole caused a moderate decrease of spontaneous locomotion and in four animals moderate ataxia during the first hour, i.e. 20–60 min after administration, while no side effects were observed at the lower dose of 50 mg. The disappearance of side effects caused by 75 mg kg^{-1} 7-nitroindazole already within the 1st hour after administration and the lack of antidystonic effects during the 3rd hour of observations suggested a short duration of action of 7-

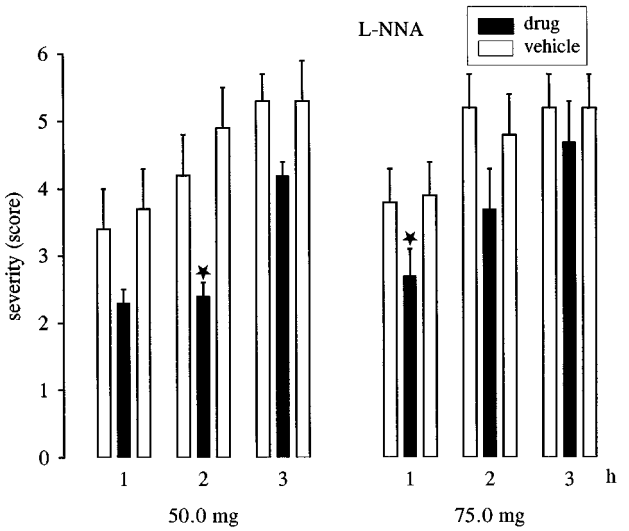

Figure 2 Effect of L-NNA on severity of dystonia in mutant hamsters at the age of maximum severity (max period). The figure shows the average of the maximum individual severity scores of dystonia reached within the 1st, 2nd and 3rd hour after i.p. administration of 50.0 or 75.0 mg kg^{-1}. Control recordings were taken 2 days before (pre-drug control) and 2 days after (post-drug control) the drug trial. Asterisks indicate significant reduction of severity in comparison to the pre-drug and post-drug control (*$P<0.05$; **$P<0.01$). Data are shown as mean$\pm$s.e.mean of nine (50.0 mg kg^{-1}) or 10 (75.0 mg kg^{-1}) dystonic hamsters.

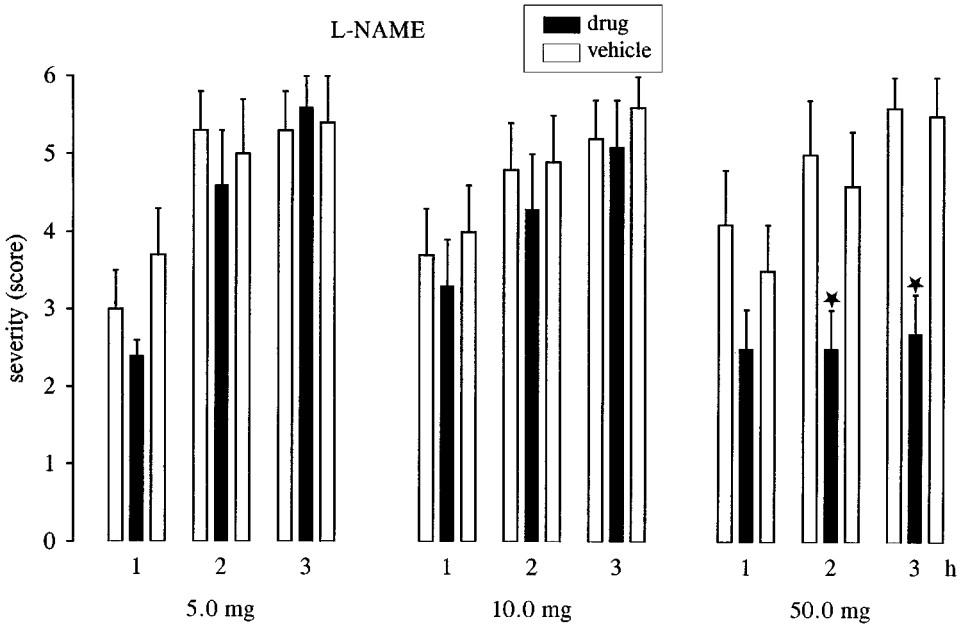

Figure 1 Effect of L-NAME on severity of dystonia in mutant hamsters at the age of maximum severity (max period). Usually, the individual maximum severity of dystonia is reached within 3 h after induction of dystonia by triple stimulation including the i.p. injection of drugs (black bars) or vehicle for pre- and post-drug controls (open bars). The figure shows the average of the maximum individual severity scores of dystonia reached within the 1st, 2nd and 3rd hour after i.p. administration, reflecting the progression of dystonia in mutant hamsters after treatment with the compounds and without drug-treatment (vehicle controls). Control recordings were undertaken 2 days before (pre-drug control) and 2 days after (post-drug control) the drug trial. Asterisks indicate significant reduction of severity in comparison to the pre- and post-drug control (*$P<0.05$). Data are shown as mean$\pm$s.e.mean of seven (5.0 mg kg^{-1}), nine (10.0 mg kg^{-1}) or eight (50.0 mg kg^{-1}) dystonic hamsters.

nitroindazole. Therefore, the NO synthase inhibitor L-NAME was used for co-administrations with L-arginine and for examinations of the effects on cyclic GMP levels.

The NO precursor L-arginine did not exert any effects on the severity of dystonia and on latencies to onset or to maximum severity (Figure 4). Even high doses of L-arginine did not cause observable behavioural side effects. As shown in Figure 4, the antidystonic efficacy of L-NAME was reversed by co-administration of L-arginine.

As shown in Figure 5, no significant differences of cerebellar cyclic GMP levels could be detected between groups of mutant hamsters and non-dystonic control animals ($P=0.092$) which were decapitated 3 h after triple stimulation procedure including the injection of vehicle (basal). The cyclic GMP concentration only tended to be increased in mutant hamsters which exhibited severe dystonia (mean 5.1 ± 0.6) after vehicle injections. Antidystonic effective doses of L-NAME (50 mg kg^{-1}) significantly decreased cyclic GMP concentrations in the cerebellum in both mutant hamsters and control animals. The dt^{sz} hamsters showed only moderate dystonia (mean 2.6 ± 0.5) before decapitation, i.e. 3 h after administration of L-NAME, supporting the marked antidystonic efficacy of this NO synthase inhibitor. The cyclic GMP levels in the striatum and frontal cortex of dt^{sz} and control hamsters (not illustrated) were too low to allow any comparisons between the groups, possibly because higher volumes of aliquots of the samples were necessary for analyses of cyclic GMP levels in these regions of hamster brains than those used in the present study, which have been shown to be suitable for determinations in rat brains (Eblen *et al*., 1996).

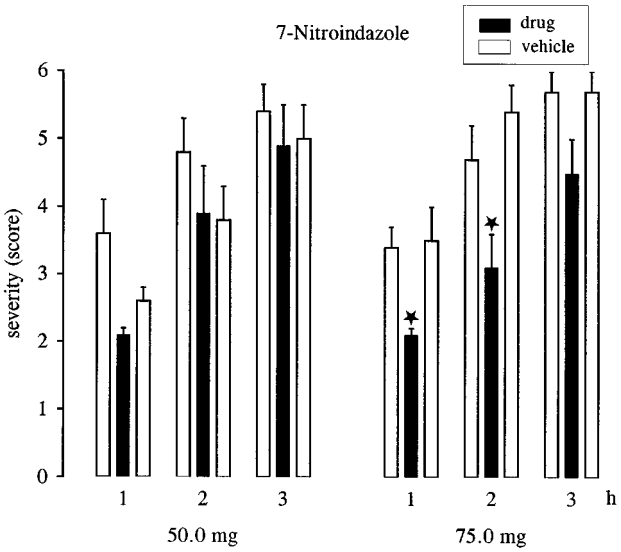

Figure 3 Effect of 7-nitro indazole on severity of dystonia in mutant hamsters at the age of maximum severity of dystonia (max period). The figure shows the average of the maximum individual severity scores of dystonia reached within the 1st, 2nd and 3rd h after i.p. administration of 50.0 or 75.0 mg kg^{-1}. Control recordings were taken 2 days before (pre-drug control) and 2 days after (post-drug control) the drug trial. Data are shown as mean ± s.e.mean of nine (50.0 mg kg^{-1}) or 11 (75.0 mg kg^{-1}) dystonic hamsters. Asterisks indicate significant reduction of severity in comparison to the pre- and post-drug control (*$P<0.05$).

Discussion

The present data demonstrate for the first time antidystonic activity of NO synthase inhibitors at doses which did not cause marked central side effects in dt^{sz} mutant hamsters. As reported for various other effects of NO synthase inhibitors in rats (Connop *et al*., 1995; Rundfeldt *et al*., 1995), the different NO synthase inhibitors showed a different time-course of antidystonic efficacy in mutant hamsters. In contrast to L-NNA and 7-nitroindazole, L-NAME did not only retard the progression of dystonia, but significantly reduced the severity of dystonia during the whole period of observation. In

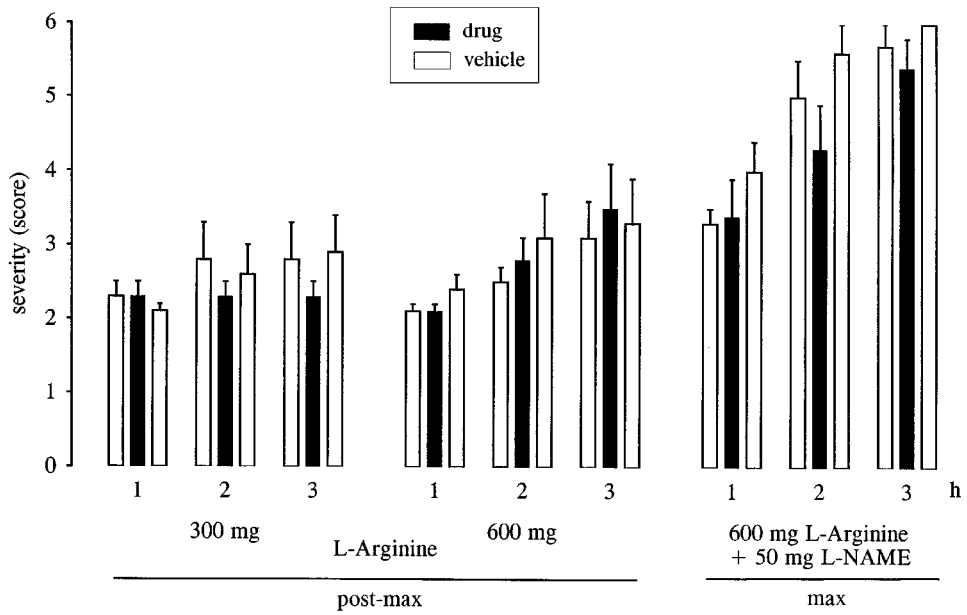

Figure 4 Effect of the NO precursor L-arginine on severity of dystonia in mutant hamsters administered alone (300 or 600 mg kg^{-1}) at an age of 50–55 days (at which the severity of dystonia is decreased, post-max period) or in co-administration with L-NAME (600 mg L-arginine + 50 mg L-NAME) at the age of maximum severity. The figure shows the average of the maximum individual severity scores of dystonia reached within the 1st, 2nd and 3rd hour after i.p. administrations. Control recordings were taken 2 days before (pre-drug control) and 2 days after (post-drug control) the drug trial. Data are shown as mean ± s.e.mean of eight dystonic hamsters.

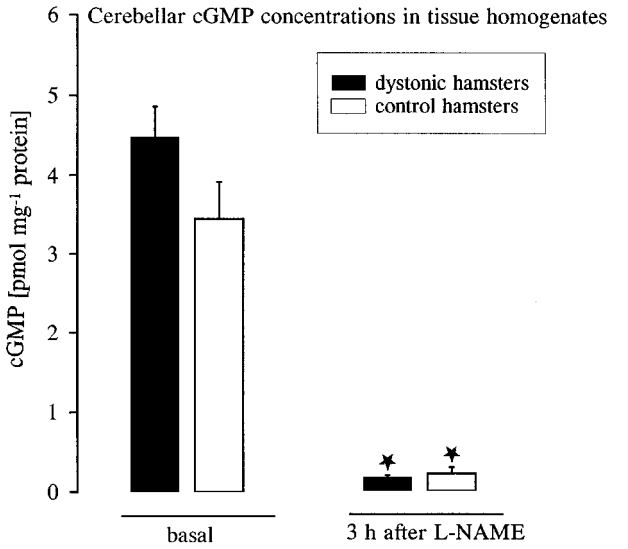

Figure 5 Levels of cyclic GMP in the cerebellum of mutant dt^{sz} hamster and control hamsters 3 h after administration of vehicle (basal) or after treatment with L-NAME (50 mg kg^{-1}). Asterisks indicate significant reduction of cyclic GMP after treatment with L-NAME in comparison to basal levels (*$P<0.001$). Data are shown as mean ± s.e.mean of 7–9 animals.

rodents, the duration of action of 7-nitroindazole, which shows a trend towards higher inhibition of neuronal NO synthase (Moore *et al*., 1991; Moore & Handy, 1997), is known to be shorter than that of L-NAME which inhibits endothelial and neuronal NO synthase (Iadecola *et al*., 1994; MacKenzie *et al*., 1994). Therefore, the more marked antidystonic effects of L-NAME in comparison with 7-nitroindazole is probably based on its longer action but not to the different affinity to the NO synthase isoforms, also indicated by the low antidystonic activity of L-NNA. The measurements of cerebellar cyclic GMP levels 3 h after administration of the antidystonic effective dose of L-NAME indicate that the beneficial effect was accompanied by a pronounced inhibition of neuronal NO synthase.

Since NO synthase inhibitors share the antidystonic action of NMDA receptor antagonists, previously described in mutant hamsters (Löscher & Richter, 1993; Richter & Löscher, 1997; Richter *et al*., 1991), NO could serve as a mediator of pathophysiologically increased glutamatergic transmission involved in the manifestation of a dystonic attack. Recent determinations of excitatory amino acid concentrations in discrete brain regions of dt^{sz} hamsters, examined in the absence of dystonia, failed to show significant changes (Löscher & Hörstermann, 1992). Furthermore, autoradiographic analysis of NMDA receptor density in 67 brain regions, using the ligand [^{3}H]*N*-(1-[2-thienyl]cyclohexyl)3,4-piperidine ([^{3}H]-TCP), which binds to the phencyclidine site in the ion channel of the NMDA receptor channel, demonstrated that NMDA receptor binding is not substantially altered in mutant hamsters in the absence of dystonic attacks compared to age-matched controls (Nobrega *et al*., 1997). However, during a dystonic episode there was a tendency towards enhanced binding in most regions, including a significant increase in the ventrolateral thalamic nucleus (Nobrega *et al*., 1997). The consequence of enhanced glutamatergic transmission during a dystonic attack, as suggested by the increased [^{3}H]-TCP binding in dystonic brains during manifestation of dystonia (Nobrega *et al*., 1997) and antidystonic effects of competitive and non-competitive NMDA receptor antagonists (Richter *et al*., 1991; Richter & Löscher, 1997), could be an enhanced NO synthesis, leading by increasing cyclic GMP levels to enhanced release of glutamate and dopamine, as shown by several *in vivo* and *in vitro* studies (e.g. Bowyer *et al*., 1995; Prast *et al*., 1994; Stewart *et al*., 1996). These considerations are not clearly supported by the measurements of cyclic GMP levels, but there was, at least, a tendency of enhanced cyclic GMP levels in brains of mutant hamsters which exhibited severe dystonia. In contrast, in the *dt* rat, a genetic animal model of permanent dystonia in which cerebellar dysfunctions play a critical role, decreased cyclic GMP levels were found in the cerebellum (Lorden *et al*., 1985). It should be noted that the cerebellum seems not to be the site of the primary defect in mutant hamsters and that cerebellar cyclic GMP levels are probably not important for dystonia in this animal model. The present study does not exclude significant increases of cyclic GMP levels in pathophysiologically more important brain regions, such as thalamic nuclei, or in subregions, such as the dorsal striatum (Richter & Löscher, 1998). Furthermore, it remains unclear whether the inhibition of endothelial NO synthase and effects on vascular tone are involved the antidystonic action of NO synthase inhibitors. Thus, the present pharmacological data give rise to further investigations of the role of NO in the pathophysiology of dystonia. Immunhistochemical examinations of endothelial and neuronal NO synthase in different brain regions of dt^{sz} hamsters are under way.

With regard to antidystonic effects of the NO synthase inhibitors in mutant hamsters, aggravation of dystonia would have been expected to be caused by L-arginine, the biological precursor for NO synthesis (Moncada *et al*., 1991). By itself, L-arginine, however, failed to exert prodystonic effects even at a high dose of 600 mg kg^{-1} which reversed the beneficial effect of L-NAME. This result, which is difficult to explain, is consistent with observed effects on locomotor activity in normal mice (Starr & Starr, 1995). While L-arginine (500 mg kg^{-1}), administered alone, had no effect on locomotor activity in mice, the amino acid reversed the reduction of locomotor activity induced by a high dose (125 mg kg^{-1}) of L-NAME (Starr & Starr, 1995). Nevertheless, the lack of prodystonic effects of L-arginine in dt^{sz} hamsters argues against a primary role of enhanced glutamatergic activity and NO formation in the pathogenesis of primary dystonia, which is in line with the previous neuro-chemical findings (Löscher & Hörstermann, 1992; Nobrega *et al*., 1997), as described above. Actually, several lines of evidence suggest that dystonia in mutant hamsters is primarily caused by disturbed GABAergic inhibition (Richter & Löscher, 1998), leading to overactivity of the glutamatergic and dopaminergic system which both seem to contribute to the manifestation of a dystonic attack.

Since recent dopamine receptor autoradiographic studies and pharmacological examinations indicated that an enhanced striatal activity of dopamine is involved in the dystonic syndrome in mutant hamsters (Rehders *et al*., 2000; Richter & Löscher, 1998), the inhibitory effects of NO synthase inhibitors on striatal dopamine release (Bowyer *et al*., 1995) may be relevant for their antidystonic activity and for the reduction of locomotor activity, observed after administration of high doses of 7-nitroindazole in the present study and also described in mice (Starr & Starr, 1995). Furthermore, the anxiolytic effect of 7-nitroindazole is possibly related to antidopaminergic activity (Dunn *et al*., 1998). With regard to induction of dystonic attacks by stress and beneficial effects of benzodiazepines in dt^{sz} hamsters and in patients with paroxysmal non-kinesiogenic dystonic choreoathetosis, anxiolytic effects of NO synthase inhibitors may be an important component for the antidystonic efficacy.

In contrast to NMDA receptor antagonists (Richter *et al*., 1991; Richter & Löscher, 1997), NO synthase inhibitors exerted antidystonic effects at doses which did not cause marked side effects in mutant hamsters. Although L-NAME and L-NNA did not exert observable central adverse effects, these compounds are known to inhibit the activity of endothelial NO synthase and cause pronounced increases of arterial blood pressure at doses which exerted antidystonic effects in the hamster model (Dwyer *et al*., 1991) which limit the suitability for clinical treatment of movement disorders. However, more selective inhibitors of the neuronal NO synthase might be interesting candidates for treatment of dystonia.

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