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The neurochemical and behavioral effects of the novel cholinesterase–monoamine oxidase inhibitor, ladostigil, in response to L-dopa and L-tryptophan, in rats

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1 The novel drugs, ladostigil (TV3326) and TV3279, are R and S isomers, respectively, derived from a combination of the carbamate cholinesterase (ChE) inhibitor, rivastigmine, and the pharmacophore of the monoamine oxidase (MAO) B inhibitor, rasagiline. They were developed for the treatment of comorbidity of dementia with Parkinsonism. In the present study, we determined the effects of these drugs on both aminergic neurotransmitter levels and motor behavioral activity in naïve and in L-dopa- or L-tryptophan-induced rats.

2 Chronic treatment of rats with ladostigil (52 mg kg^{-1} for 21 days) inhibited hippocampal and striatal MAO A and B activities by >90%, increased striatal levels of dopamine and serotonin, and inhibited striatal ChE activity by ~50%.

3 Chronic TV3279 (26 mg kg^{-1} for 21 days) similarly inhibited ~50% of striatal ChE activity, but did not affect MAO activity or amine levels.

4 In sharp contrast to the inductive effect of the MAO A/B inhibitor, tranylcypromine (TCP), on stereotyped hyperactivity in response to L-dopa (50 mg kg^{-1}) or L-tryptophan (100 mg kg^{-1}), ladostigil completely inhibited these behavioral hyperactivity syndromes. Accordingly, acute rivastigmine (2 mg kg^{-1}) and chronic TV3279 abolished the ability of TCP to initiate L-dopa-induced hyperactivity, while scopolamine (0.5 mg kg^{-1}) reversed the inhibitory effect of chronic ladostigil on L-dopa-induced hyperactivity, suggesting that ladostigil may attenuate successive locomotion by activating central cholinergic muscarinic receptors.

5 Finally, while chronic ladostigil administration to naïve rats resulted in preserved spontaneous motor behavior, acute treatment with ladostigil decreased motor performance, compared to control animals. In contrast, chronic as well as acute treatments with TV3279 reduced spontaneous motor activity. Thus, the aminergic potentiation by ladostigil may counteract its cholinergic inhibitory effect on spontaneous motor behavior.

6 Our results suggest that potentiation of both aminergic and cholinergic transmission systems by ladostigil contributes equally to motor behavior performance, which is substantially impaired in comorbidity of dementia with Parkinsonism including dementia with Lewy bodies (DLB).
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Abbreviations: AD, Alzheimer's disease; ChE, cholinesterase; DA, dopamine; DHPG, 3,4 dihydroxyphenylglycol; DLB, dementia with Lewy bodies; DOPAC, dihydroxyphenylacetic acid; 5HIAA, 5-hydroxyindole-3 acetic acid; 5-HT, serotonin; HVA, homovanilic acid; MPTP, (*N*-methyl-4-phenyl-1,2,36-tetrahydropyridine); NA, noradrenaline; PD, Parkinson's disease; TCP, tranylcypromine

Introduction

The progressive neurodegenerative Alzheimer's disease (AD) and Parkinson's disease (PD) are characterized by a respective reduction in striatal acetylcholine (Ach) or dopamine (DA) levels, and correlative reduction in muscarinic or dopaminergic receptors. Indeed, several pathological diseases including dementia with Lewy bodies (DLB) involve dementia with an extrapyramidal disorder and are characterized by a reduction of striatal Ach and DA levels, as well as a parallel reduction in their striatal receptors (Piggott *et al*., 1999; 2003). Consequently, current pharmacological therapy for DLB includes L-3,4-dihydroxyphenylalanine (L-dopa) for the motor complications, as well as cholinesterase (ChE) inhibitors, which stabilize cognitive and psychotic symptoms (Mosimann & Mckeith, 2003; Kaufer, 2004). While treating each type of symptom with a different drug raises concern about possible drug interactions, the use of agents that address multiple core features of DLB would help reduce the risks of polypharmacy (Kaufer, 2004). Ladostigil (TV3326, (*N*-propargyl-(3*R*)aminoindan-5-yl) ethyl methyl carbamate)) and its S isomer, TV3279, are carbamate derivatives of rasagiline and its S isomer TVP1022 (Weinstock

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et al., 2000a). These novel drugs were developed recently in order to maintain the neuroprotective and monoamine oxidase (MAO) inhibitory properties of rasagiline with the ChE inhibitory activity of the pseudo-irreversible ChE inhibitor, rivastigmine, for the treatment of dementia comorbid with extrapyramidal disorder (Youdim *et al*., 2001; Sterling *et al*., 2002; Youdim & Weinstock, 2002; Group, 2004). However, while ladostigil is a ChE–brain selective MAO A and B inhibitor, TV3279 has only comparable ChE inhibitory activity. Both drugs have been shown to possess an anti-Alzheimer activity in preventing scopolamine-induced deficit in spatial learning (Weinstock *et al*., 2000b), while the brain selective MAO A and B inhibitory activity of ladostigil accounts for its anti-Parkinson activity in preventing *N*-methyl-4-phenyl-1,2,36-tetrahydropyridine (MPTP)-induced nigrostriatal dopaminergic neurodegeneration (Sagi *et al*., 2003).

Within the three classes of striatal aspiny interneurons, the cholinergic neurotransmission system is of crucial importance in determining the final output from the striatal spiny neurons to other basal ganglia nuclei, as well as from the dopaminergic neurons to their effectors (Olianas *et al*., 1983; Izzo & Bolam, 1988; Kawaguchi, 1997; Calabresi *et al*., 2000). In particular, different subtypes of muscarinic receptors are implicated in Ach inhibitory control on the release of neurotransmitters from striatal spiny neurons (Sugita *et al*., 1991). Consequently, while treatment with central acting ChE inhibitors decreases motor behavior (Rastogi *et al*., 1982; Gonzalez & Ellinwood, 1984; Wolthuis & Vanwersch, 1984; Palumbo *et al*., 2001), antimuscarinic agents counteract this effect (Joyce & Koob, 1981; Shannon & Peters, 1990; Sipos *et al*., 1999). In contrast to spontaneous motor behavior, nonselective MAO inhibitors in rats and mice have been shown to induce stereotyped hyperactivity in response to L-dopa and L-tryptophan, resulting from increased synthesis and release of DA and serotonin, respectively (Grahame-Smith, 1971; Green & Youdim, 1975; Green *et al*., 1977). Cholinergic agents, in contrast, counteract the hyperactive motor behavior attributed to dopaminergic activation (Davis & Rosenberg, 1981; Gonzalez & Ellinwood, 1984; Gao *et al*., 1997). Nonetheless, the effect of a bifunctional dopaminergic-cholinergic-intensifying agent on motor behavior has not been studied yet.

The objective of the present work is therefore to study the effects of treatment with ladostigil and its optical isomer, TV3279, on neuronal aminergic neurotransmitter content as well as on spontaneous motor behavior. Additionally, we evaluated the effects of ladostigil on the neurochemical and behavioral responses to L-dopa and L-tryptophan, in order to determine the role of the ChE inhibitory activity of ladostigil on stereotyped motor behavior.

Methods

Animals

Animal care was in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and was approved by the Animal Ethics Committee of the Technion, Haifa, Israel. Male Sprague–Dawley rats (200–250 g) were purchased from Harlan (Rehovot, Israel) and housed under controlled temperature and lighting conditions. All experiments were performed between 0800 and 1400. The spontaneous motor behavior assay was performed in groups of 10 rats, while hyperactive activity assays were performed in groups of six.

Animal treatment

The doses of ladostigil, TV3279 and rivastigmine were chosen in order to reach a similar ChE inhibitory activity, according to previous work (Weinstock *et al*., 2000a). Thus, chronic ladostigil and TV3279 were administrated daily with oral doses of 52 and 26 mg kg^{-1}, respectively, for 21 days, while control rats received water. Additionally, 1 day acute treatment groups were added for the spontaneous motor and biochemical assays. In hyperactivity trials, L-dopa (50 mg kg^{-1} i.p.) or L-tryptophan (100 mg kg^{-1} i.p.) was administrated 2 h after the last dose according to the methods devised by Green & Youdim (1975) and Green *et al*. (1977), while TCP (10 mg kg^{-1} i.p.) was administered 30 min prior to L-dopa or L-tryptophan. In order to determine the effect of acute treatment with ChE inhibitors on motor hyperactivity, we used the carbamate cholinesterase inhibitor, rivastigmine (2 mg kg^{-1}), from which ladostigil and TV3279 were derived. Rivastigmine, which unlike its propargylamine-containing derivatives does not have active metabolites, was administered orally 30 min before L-dopa. In order to determine the involvement of the muscarinic receptors in mediating the effect of ladostigil on hyperactivity, scopolamine sulfate, a central acting muscarinic antagonist, was administered (0.5 mg kg^{-1} i.p.) 10 min prior to L-dopa.

Behavioral assays

All behavioral experiments were performed in an isolated, light-controlled room. A 5 min spontaneous motor activity was measured for each rat in a polypropylene cage, $58 \times 38 \times 18$ cm^3. Hyperactive motor activity studies were carried for paired rats in standard plastic cages, and activity was detected every 2 min, for 1 h. Motor activity was measured using a Columbus activity meter (OH, U.S.A., sensitivity 50 μA), and was normalized by the subtraction of the baseline motor activity.

Catecholamine analysis

Rats were killed by decapitation and striata were quickly removed and frozen in liquid nitrogen, followed by homogenization in 600 μl of 0.1 M perchloric acid. Catecholamines and their metabolite levels were determined by electrochemical coupled-HPLC according to Keller *et al*. (1976), and as adopted by Ben-Shachar & Youdim (1990) consisted of an ESA Coulochem 5200A electrochemical detector, with a 5011 dual analytical cell and a 5021 conditioning cell and a hypersil column H30DS-125A, 12.5 cm $\times$ 4.6 mm (Hichrom, CA, U.S.A.). Standards of amines were carried out in order to evaluate recovery.

MAO activity measurement

MAO A and B activities were measured according to Tipton & Youdim (1976) with the following modifications: triplicates of striatal or hippocampal homogenate containing 70 μg protein in 0.32 M sucrose were incubated with ^{14}C-labeled serotonin (5-HT) for 30 min (final concentration 100 μM), as a substrate for MAO A, or ^{14}C-labeled phenylethylamine for 20 min (final concentration 10 μM), as a substrate for MAO B. For

determination of MAO A or B, homogenates were preincubated with 75 nM *l*-deprenyl or 75 nM clorgyline, respectively, for 1 h at 37°C prior to the addition of the substrates. The metabolites were extracted and determined by a liquid scintillation counter in c.p.m. units.

ChE activity measurement

ChE activity was measured in the striatum as previously shown (Weinstock *et al*., 2000b). The striata were rapidly weighed and 5.6 mg protein was homogenized in 1 ml of 0.1 M phosphate buffer, pH 8.0, containing 1% Triton. ChE activity was measured by the colorimetric method of Ellman *et al*. (1961) in 1 ml samples containing 25 μl of enzyme homogenate, following a 10 min preincubation with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB; 0.33 mM) at 37°C, using acetylthiocholine iodide (1.0 mM) as a substrate. Activity was detected by Ultraspec 2000 (Amersham-Biotec, Buckinghamshire, U.K.) at 412 nm.

Chemicals

Tranylcypromine (TCP), Bradford reagent, DA, homovanilic acid (HVA), 3,4 dihydroxyphenylacetic acid (DOPAC), noradrenaline (NA), 3,4 dihydroxyphenylglycol (DHPG), 5-HT, 5-hydroxyindole-3 acetic acid (5HIAA), scopolamine sulfate, L-dopa, L-tryptophan, acetylcholinesterase (EC 3.1.1.7), acetylthiocholine iodide and DTNB were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The protease inhibitor mixture (Complete™, EDTA free) was purchased from Boehringer-Mannheim (Mannheim, Germany). ^{14}C-labeled phenylethylamine·HCl (specific activity 44.13 mC mmol^{-1}) and ^{14}C-labeled 5HT binoxalate (specific activity 44.9 mC mmol^{-1}) were purchased from Perkin-Elmer (Boston, MA, U.S.A.).

Data analyses

All values are expressed as the mean ± s.e. mean (s.e.m.). All experiments were repeated twice and data analysis was performed on data from both experiments, using Analyse-it software for Windows Excel (Leeds, England). Differences in enzyme inhibition were evaluated with Student's *t*-test, while catecholamine level changes were analyzed using one-way ANOVA followed by Student's *t*-test. Spontaneous motor behavior values distributed normally, and differences between groups were analyzed using two-way ANOVA (drug × duration of treatment). Factors found to be significant were tested with the Tukey comparison. In the hyperactive assays, motor activity was normalized to the baseline activity level. Differences between various treatments before and after hyperactivity (10 and 46 min after L-dopa/L-tryptophan administration, respectively) were processed with the nonparametric Kruskal–Wallis ANOVA followed by the Mann–Whitney *U*-test. For all experiments, a *P*-value lower than 0.05 was considered significant.

Results

Effect of ladostigil and TV3279 on spontaneous motor activity

Rats were placed on activity meter 2 h after administration of ladostigil (52 mg kg^{-1}) or TV3279 (26 mg kg^{-1}), while control rats received water, and a 5 min spontaneous exploratory motor behavior in a novel environment was recorded. Spontaneous motor activity of control rats after acute and 21 days chronic water treatment was similar (Figure 1). Acute treatment with ladostigil or TV3279 significantly decreased spontaneous locomotion, compared to control. However, chronic ladostigil, but not TV3279, administration significantly increased spontaneous activity, resulting in activity similar to control. Indeed, a statistically significant interaction between drug treatments and duration of treatment was found ($P<0.02$), suggesting that the interaction is attributed to the distinctive effects of acute *versus* chronic treatment with ladostigil on spontaneous motor behavior (Figure 1).

Effect of ladostigil, TV3279 and tranylcypromine on striatal MAO and ChE activities

Chronic oral administration of ladostigil and TV3279 resulted in a significant striatal ChE inhibition (Table 1). Ladostigil inhibited ChE activity by ~50% of control activity, similar to TV3279. Chronic but not acute treatment with ladostigil almost completely inhibited (>90%) both striatal and hippocampal MAO A and B activities. Similarly, acute treatment with the nonselective irreversible MAO inhibitor, tranylcypromine (TCP, 10 mg kg^{-1} i.p.), resulted in nearly total blockade of both MAO A and B activities (Table 1).

Effect of ladostigil, TV3279 and TCP on striatal amines

Ladostigil treatment significantly increased striatal levels of DA, 5-HT and NA by 36, 46 and 220% of control values, respectively, and significantly reduced transmitter metabolites HVA, DOPAC and 5HIAA (Table 2a). TV3279, which did not inhibit MAO, had no effect on striatal levels of the three neurotransmitters or their metabolites, while TCP increased DA, 5-HT and NA levels in the striatum by 45, 150 and 75% of control, respectively. Consequently, TCP significantly

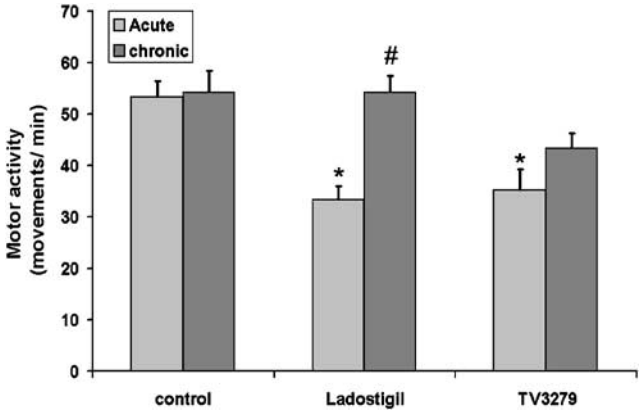

Figure 1 Effect of ladostigil on spontaneous motor activity. Rats were treated orally with either ladostigil (52 mg kg^{-1}), TV3279 (26 mg kg^{-1}) or water (control), for 21 days. At 2 h after treatment, a 5 min spontaneous motor activity was assessed for each rat randomly. Data at day 1 (acute) or day 21 (chronic) present the mean movements per minute ± s.e.m. ($n=10$ rats). Two-way ANOVA (drug × duration), for both levels $P<0.001$ and for the interaction $P<0.02$. For both factors, Tukey test was applied, $^{*}P<0.05$ *versus* respective control, $^{\#}P<0.05$ *versus* respective acute treatment.

Table 1 Effect of chronic ladostigil and TV3279 on striatal ChE and MAO activities in rats

	Enzyme inhibition (% of control activity)		
Enzyme	*Ladostigil*	*TV3279*	*TCP*
MAO A	92±0.8** (91±1.7)	ND	95±1.4**
MAO B	91±1.1** (92±2.4)	ND	98±0.3**
ChE	41±7.2**	52±5.3**	ND

Rats were injected chronically with either ladostigil (52 mg kg^{-1}, orally), TV3279 (26 mg kg^{-1}, orally) or water for 21 days, while TCP (10 mg kg^{-1} i.p.) was given acutely. At 2 h after the last dose, the rats were killed. Results represent percentage inhibition ±s.e.m. ($n=7$). Results in parentheses represent hippocampal values. Student's *t*-test: **$P<0.01$ *versus* control. Control MAO A and B activities were 6888±249 and 55,389±4297 c.p.m. mg protein^{-1}, respectively. Control ChE activity was 4.6±0.6 U ml^{-1} mg protein^{-1}. ND: not determined.

Table 2 Effect of chronic ladostigil - TV3279 and L-dopa induction on striatal transmitter levels in rats

(a) Effect of chronic ladostigil and TV3279 on striatal catecholamines in rats[a]

	Neurotransmitter content (pmol mg tissue^{-1})			
Transmitter	*Control*	*Ladostigil*	*TV3279*	*TCP*
Dopamine	79±8.6	100±14.1*	80±2.3	100±5.8*
HVA	5±1	1±0.3**	6±0.2	0.2±0.1**
DOPAC	8±1.6	1±0.4**	8±0.2	0.1±0.0**
5-HT	4±0.7	6±0.3*	4±0.2	10±1.1**
5HIAA	4±0.5	2±0.3**	4±0.2	1±0.1**
NA	0.3±0.1	1±0.2*	0.6±0.05	0.7±0.01*

(b) Effect of L-dopa induction on striatal transmitter levels[b]

	Neurotransmitter content (pmol mg tissue^{-1})			
Transmitter	*Control*	*L-dopa*	*Ladostigil/L-dopa*	*TCP/L-dopa*
Dopamine	79±8.6	83±2.5	104±9.6$^{\#}$*	138±21.8$^{\#}$*
HVA	5±1	12±2.7**	6±2.4$^{\#}$	1±0.1$^{\#\#}$**
DOPAC	8±1.6	11±2.3	4±0.6$^{\#\#}$**	1±0.0$^{\#\#}$**
5-HT	4±0.7	5±0.3	7±1.0$^{\#}$*	7±1.2$^{\#}$*
5HIAA	4±0.5	4±0.3	3±0.4	2±0.1$^{\#}$*
NA	0.3±0.1	0.4±0.2	1±0.3*	1±0.2*

[a]Rats were injected chronically with either ladostigil (52 mg kg^{-1}, orally), TV3279 (26 mg kg^{-1}, orally) or water (control) for 21 days, while TCP (10 mg kg^{-1} i.p.) was given acutely. At 2 h after the last dose, the rats were killed. Results represent striatal catecholamine content ±s.e.m. ($n=7$). One-way ANOVA followed by Student's *t*-test: *$P<0.05$ *versus* control, **$P<0.01$ *versus* control.
[b]Rats were orally given ladostigil (52 mg kg^{-1}) or water (control) for 21 days, while TCP (10 mg kg^{-1} i.p.) was given acutely. This was followed by administration of L-dopa (50 mg kg^{-1} i.p.) as described in the text. At 1 h after L-dopa administration, the rats were killed. Results represent striatal mean transmitter content ±s.e.m. ($n=6$). One-way ANOVA followed by Student's *t*-test: *$P<0.05$ *versus* control, **$P<0.01$ *versus* control, $^{\#}P<0.05$ *versus* L-dopa, $^{\#\#}P<0.01$ *versus* L-dopa.

reduced the levels of the monoamine transmitter metabolites (Table 2a).

Effects of ChE inhibitors on L-dopa- and L-tryptophan-induced hyperactive motor behaviors

TCP induced a substantial stereotyped hyperactivity in response to L-dopa, expressed by a significant increment in locomotion, compared with the control. On the contrary, ladostigil did not provoke hyperactivity behavior as reflected by motor activity similar to that of L-dopa alone (Figure 2). Furthermore, chronic or acute pretreatment of rats with the ChE inhibitors TV3279 or rivastigmine, respectively, attenuated the TCP- plus L-dopa-induced hyperactivity motor behavior, with activity similar to that of L-dopa alone. Ladostigil also attenuated L-tryptophan-induced hyperactivity syndrome, in sharp contrast to TCP, which increased motor activity significantly in response to L-tryptophan. (Figure 3). Similar to ladostigil, chronic TV3279 prior to TCP and L-tryptophan attenuated hyperactivity motor behavior, resulting in activity similar to that of L-tryptophan-treated rats.

Striatal catecholamine in response to ladostigil and L-dopa

TCP (10 mg kg^{-1}) pretreatment followed by L-dopa (50 mg kg^{-1}) resulted in 66% increase in striatal DA, relative to L-dopa alone, along with a significant decrease in DA metabolites, HVA and DOPAC (Table 2b). Similarly, chronic treatment with ladostigil followed by L-dopa induction increased DA levels in the striatum by 31%, with a significant decrease in striatal DOPAC and HVA levels. L-dopa alone significantly increased striatal HVA by 140%, as well as insignificantly increasing DOPAC levels by 38% of control. Since neither MAO isoenzymes were inhibited, DA, NA, 5-HT and 5HIAA levels were not altered by L-dopa (Table 2b).

Effect of ladostigil and L-tryptophan on striatal amine levels

Ladostigil (52 mg kg^{-1}, 21 days) given with L-tryptophan (100 mg kg^{-1}) resulted in elevation of 5-HT levels in the striatum by 65% as compared to L-tryptophan treatments

(Table 3). Similarly, TCP (10 mg kg^{-1} i.p.) elevated striatal 5-HT levels. Additionally, both drugs reduced 5HIAA levels following L-tryptophan. Ladostigil reduced 5HIAA levels by 66% of L-tryptophan treatment, whereas TCP reduced 5HIAA levels by 83% of L-tryptophan treatment.

Effect of scopolamine on L-dopa-induced hyperactivity by ladostigil

Figure 4 shows that a low dose of scopolamine (0.5 mg kg^{-1} i.p.) did not affect baseline motor activity when given prior to L-dopa. On the other hand, the same dosage of scopolamine given to rats chronically treated with ladostigil (or TV3279 plus TCP although not shown) and L-dopa (50 mg kg^{-1}) reversed the cholinergic inhibition of stereotyped motor activity (46 min after L-dopa administration). Also, scopolamine significantly increased motor activity even before hyperactivity was initiated (10 min after L-dopa administration), suggesting that basal activity increment is also involved in the increase in locomotion induced by scopolamine.

Discussion

The nonselective irreversible MAO inhibitor, TCP, or a combination of irreversible MAO A (clorgyline) plus MAO B (*l*-deprenyl) inhibitors induces hyperactivity behavioral syndromes in response to L-dopa or L-tryptophan (Green & Youdim, 1975; Maitre *et al*., 1976; Green *et al*., 1977; Olds *et al*., 1981). This occurs as a result of increased basal levels of functional DA and 5-HT, respectively. However, the novel bifunctional ChE-MAO inhibitor, ladostigil, failed to induce DA- or 5-HT-dependent hyperactivity syndrome in response to these amino acids, even though it inhibited both MAO A and B activities by >90% and subsequently increased striatal catecholamine and 5-HT levels. The fact that ~50% inhibition of striatal ChE activity inhibited the hyperactivity syndrome

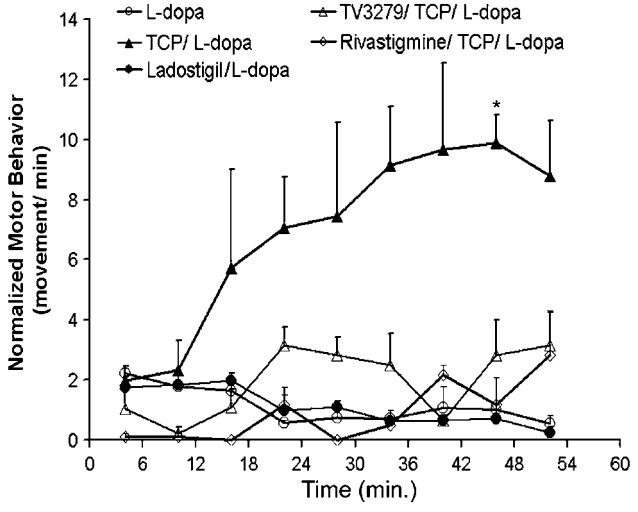

Figure 2 Effect of ChE inhibitors on L-dopa-induced stereotyped hyperactivity. Rats were treated chronically with ladostigil, TV3279 (52 and 26 mg kg^{-1} day^{-1}, orally, respectively) or water for 21 days, while rivastigmine (2 mg kg^{-1}, orally) was administered acutely. L-dopa (50 mg kg^{-1} i.p.) was administrated 2 h after the last dose (time 0). TCP (10 mg kg^{-1} i.p.) was given 10 min before L-dopa. Total activity was measured every 2 min. Data represent mean normalized movements ±s.e.m. ($n=6$ rats). Kruskal–Wallis analysis of variance followed by the Mann–Whitney *U*-test, $^{*}P<0.001$ *versus* L-dopa-treated rats at 46 min.

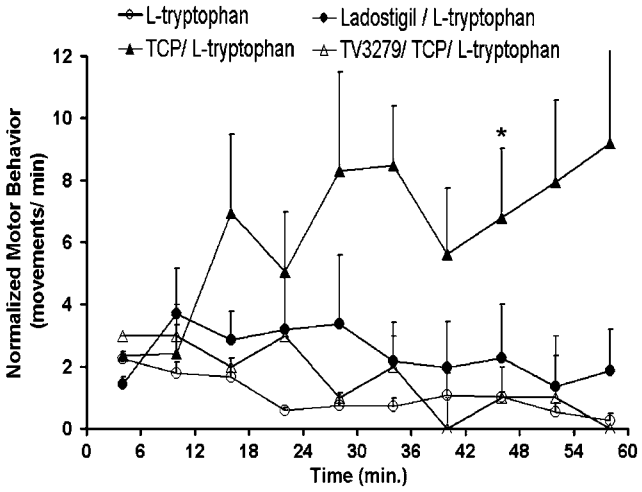

Figure 3 Effect of ladostigil on L-tryptophan-induced stereotypy. Rats were treated chronically for 21 days with either ladostigil, TV3279 (52 and 26 mg kg^{-1} day^{-1}, orally, respectively) or water, while TCP (10 mg kg^{-1} i.p.) was given acutely. At 2 h after the last dose of ladostigil, L- tryptophan (100 mg kg^{-1}) was administrated (time 0). Total motor activity was measured every 2 min. Data represent mean normalized movements ±s.e.m. ($n=6$). Kruskal–Wallis analysis of variance followed by the Mann–Whitney *U*-test, $^{*}P<0.002$ *versus* L-dopa-treated rats at 46 min.

Table 3 Effect of L-tryptophan induction on striatal transmitter levels

		Neurotransmitter content (pmol mg tissue^{-1})		
Transmitter	*Control*	*L-Tryptophan*	*Ladostigil/L-tryptophan*	*TCP/L-tryptophan*
Dopamine	78±6.4	78±6.4	99±6.6$^{\#}$*	102±4.8$^{\#}$*
HVA	5±0.5	4±0.4	2±0.3$^{\#}$*	0.1±0.1$^{\#\#}$**
DOPAC	7±1.0	7±1.0	1±0.2$^{\#\#}$**	0.4±0.0$^{\#\#}$**
5-HT	5±0.5	5±0.5	8±0.6$^{\#}$*	8±0.6$^{\#}$*
5HIAA	5±0.8	6±0.4*	2±0.4$^{\#\#}$*	1±0.6$^{\#\#}$*
NA	0.1±0.05	0.3±0.1	1±0.3$^{\#}$*	1±0.3$^{\#}$*

Rats were injected chronically with ladostigil (52 mg kg^{-1}, orally for 21 days), water or TCP (10 mg kg^{-1}, acute i.p.) , followed by L-tryptophan (100 mg kg^{-1}) as described in the text. At 1 h after L-tryptophan administration, the rats were killed. Results represent striatal mean transmitter level ±s.e.m. ($n=6$). One-way ANOVA followed by Student's *t*-test: $^{*}P<0.05$ *versus* control, $^{**}P<0.01$ *versus* control, $^{\#}P<0.05$ *versus* L-tryptophan, $^{\#\#}P<0.01$ *versus* L-tryptophan.

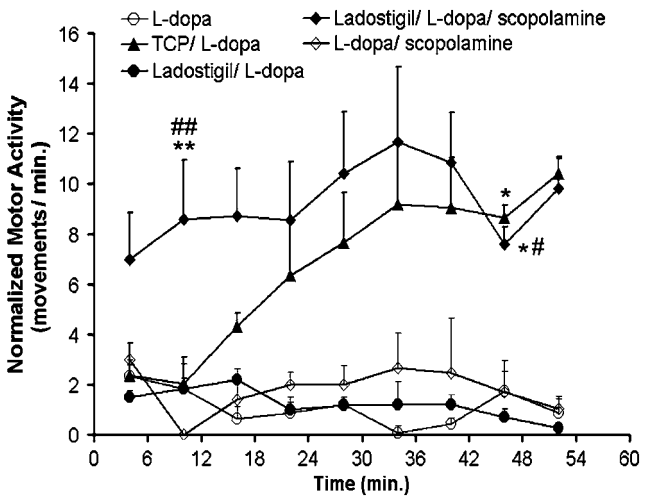

Figure 4 Effect of ladostigil on L-dopa-induced stereotyped hyperactivity. Rats were treated chronically with ladostigil ($52\,mg\,kg^{-1}\,day^{-1}$) or water for 21 days. L-dopa ($50\,mg\,kg^{-1}$ i.p.) was administrated 2 h after the last dose (time 0). TCP ($10\,mg\,kg^{-1}$ i.p.) was given 10 min before L-dopa. Scopolamine ($0.5\,mg\,kg^{-1}$ i.p.) was administrated 1 h prior to L-dopa ($50\,mg\,kg^{-1}$ i.p.). Total activity was measured every 2 min. Data represent mean normalized movements $\pm$s.e.m. ($n=6$ rats). Kruskal–Wallis analysis of variance followed by the Mann–Whitney *U*-test,$^{*}P<0.01$, $^{**}P<0.001$ *versus* L-dopa-treated rats, $^{\#}P<0.01$, $^{\#\#}P<0.001$ *versus* scopolamine plus L-dopa-treated rats at 10 or 46 min.

response supports the well-established inhibitory influence of Ach on DAergic-mediated stereotypy, and may be related to cholinergic activation postsynaptic to dopaminergic neurons (Davis & Rosenberg, 1981; De Souza & Palermo-Neto, 1982; Rastogi *et al*., 1982). This is in line with Gonzalez & Ellinwood (1984), who showed that physostigmine inhibits stereotypies generated by either apomorphine or amphetamine. Moreover, the inhibitory effects of ladostigil, its optical isomer TV3279, and rivastigmine on these hyperactivity syndromes are most likely mediated by the enhanced functional Ach levels. Indeed, Scali *et al*. (2002) have shown that 50% inhibition of ChE by the ChE inhibitors, rivastigmine, metrifonate and donepezil, is accompanied by a subsequent increase in central Ach release, with significantly greater effect by rivastigmine. Accordingly, brain Ach levels were induced following ladostigil treatment in rats (G. Pepeu, 2004, personal communication to Teva Pharmaceutical Co., Israel).

Although the role of Ach in the activity of striatal projection neurons is still largely unknown, recent findings suggest that cholinergic transmission, *via* muscarinic receptors, ensures the correct processing mechanisms of cortical inputs forward from the cortex to projection cells (Izzo & Bolam, 1988; Calabresi *et al*., 2000). Thus, the ability of scopolamine to reverse the inhibitory effects of ladostigil and TV3279 plus TCP on L-dopa-induced hyperactivity is in line with the hierarchical structure of the motor loops in the basal ganglia. Indeed, muscarinic receptors are located on the perikarya, dendrites and spines of striatal spiny neurons (Calabresi *et al*., 2000), and inactivation of these receptors by scopolamine may induce hyperkinetic motor behavior, like the behavior generated by selective M1 antagonists or receptor deletion (Calabresi *et al*., 1999; Sipos *et al*., 1999; Gerber *et al*., 2001).

Similar to its inhibitory effect on stereotyped locomotion in the hyperactivity model, TV3279 reduced basal motor activity in the spontaneous motor paradigm. This is in line with Carriero *et al*. (1997) and Palumbo *et al*. (2001), who found that the carbamate ChE inhibitors aldicarb and tacrine reduce basal motor and open field activities, respectively. In sharp contrast to TV3279, chronic treatment with ladostigil was able to maintain spontaneous motor behavior to control levels. These results are consistent with those of Weinstock *et al*. (2000a), who previously found that chronic ladostigil, but not TV3279, preserved open field mobility. The partial hyperkinetic effect of chronic treatment with ladostigil on spontaneous locomotion is attributed to its MAO inhibitory activity, which results in a substantial rise in striatal DA levels. The fact that spontaneous motor behavior was significantly higher following chronic treatment as compared to acute treatment with ladostigil further supports the correlation between the biochemical and behavioral effects of this drug. We previously found that brain MAO A and B are inhibited only after chronic but not acute treatment with ladostigil (Sagi *et al*., 2003), whereas ChE is inhibited after an acute treatment (Weinstock *et al*., 2000a). This is attributed to the formation of active MAO inhibitory metabolites as a consequence of hydrolysis of the carbamate moiety in ladostigil by ChE, which yields the 6-OH derivative (Sterling *et al*., 2002). Indeed, the physiological effect of ChE or MAO inhibitors on Ach and DA release from their respective neurons is a complex aspect of their action, especially when one wishes to relate this with behavioral effects. Although electrophysiological experiments are yet to be performed, we speculate that the chronic treatment with ladostigil, which increases functional DA levels, may also increase striatal DA release. This is in line with Lamensdorf *et al*. (1996), who showed that on chronic treatment, MAO A and B inhibitors, including rasagiline, increased the tonic release of DA in rat striatum. Consequently, Ach release may be reduced and a normal level of motor activity will be achieved (Lehmann & Langer, 1983).

Finally, ladostigil was developed in order to induce monoaminergic and cholinergic neurotransmission in DLB patients. Although the present *in vivo* study with models of DA- and 5-HT-dependent motor behavior suggests that the cholinergic neurotransmission overrides the dopaminergic transmission system, it may not accurately reflect the overall effects of the drug in the clinic. Indeed, recent clinical evidence has shown that rivastigmine treatment in DLB patients improves cognitive and motor signs, and does not reduce motor response to L-dopa (Grace *et al*., 2001; Kaufer, 2004). This safer profile of rivastigmine may be related to its pharmacological properties. Although within all brain areas, the highest level of ChE activity is found in the striatum, this activity is mainly attributed to acetylcholinesterase, with 15-fold higher activity than that of butyrylcholinesterase (Meneguz *et al*., 1992). Moreover, the relative activity between the detergent-soluble fraction of acetylcholinesterase (G4) and the salt-soluble fraction (G1) was found to be the highest in the striatum, with a ratio higher than 7 (Meneguz *et al*., 1992; Das *et al*., 2001). The fact that rivastigmine preferentially inhibits the G1, but not the G4, Ach fraction and has a higher selectivity toward the butyryl form suggests that its main target is outside the striatum (Inglis, 2002; Rakonczay, 2003). Ladostigil inhibits both forms of the ChE, but similar to rivastigmine, it is about 100 times more potent against the butyryl isoform (Weinstock *et al*., 2003). Thus, it would be

expected to possess both cognitive and motor enhancing properties with the advantage of increasing dopaminergic neurotransmission.

In conclusion, both cholinergic and dopaminergic neurotransmission systems are activated following oral treatment to rats with the novel multifunctional drug ladostigil. The inhibitory effects of the drug on both striatal ChE and MAO activities may equally account for the ability of the drug to both attenuate stereotyped motor behavior and also maintain normal spontaneous motor performance. Taken together with the selectivity of the drug to cholinergic sites outside the striatum, these results suggest that ladostigil may be safe and effective for the treatment of impaired cognitive and motor performance in DLB patients.

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