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# Possible anti-Parkinson properties of *N*-(α-linolenoyl) tyrosine A new molecule

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**Abstract**

Tyrosine is unable to cross the blood–brain barrier and is therefore unable to improve the status of brain dopamine (DA) and to provide relief for patients with Parkinson's disease (PD) or other DA-insufficient disorders. We report the creation of an amide bond molecule [*N*-(α-linolenoyl)tyrosine (NLT)] that combines tyrosine with a fatty acid mixture. NLT significantly improves the rotational behavior of rats [following unilateral striatal lesions (as a model for Parkinson's)] and overcomes the exaggerated eye-blinking induced by a potent DA-depleting agent (as a model for essential blepharospasm). These results are supported by the finding that NLT's mode of action, in striatum, is the same as the mode of action of D-amphetamine. They both induce an increase in the DA level, DA turnover and release. 

*Keywords:* Parkinson's disease; Animal model; Tyrosine; α-Linolenic acid; Amide bond; Dopamine

## 1. Introduction

The role of essential fatty acids (EFA) in the brain of patients with Parkinson's disorder (PD) is not known. Das (2000) claimed that $n-3$ fatty acids are potent neuroprotectors. In addition, dietary $n-3$ fatty acids can prevent PD. Data from experimental models and from human postmortem studies indicate that a significant decrease in polyunsaturated fatty acids (PUFA) in the neural membrane was found in the brains of PD, as well as an increased level of lipid peroxidation in the substantia nigra (Youdim et al., 2000). Those findings support the hypothesis that oxidative stress is implicated in PD. On the other hand, recent studies showed that the incorporation of arachidonic acid from blood to selected brain regions is increased in a 6-hydroxydopamine-HCl (6-OH-DA) animal model of PD (Hayakawa et al., 1998).

We have already shown that a mixture of EFA can restore learning deficits following AF64A (cholinergic toxin), 6,7-DHT (serotonergic toxin) and Ro-1284 [a potent dopamine (DA) depletion agent]. This mixture also restores the decrease in PUFA in the neuronal membrane and improves neuronal membrane and receptor functions (Yehuda et al., 1999a,b).

One of the major behavioral methods to measure the increase in DA activity in the rat brain is rotational behavior. Unilateral ablation of the striatum will result in walking in circles, as the intact striatum is still functioning. Ungerstadt and Arbuthott (1970) were the first to lesion one striatum and to measure the increase in rotational behavior. Since then, this technique has been used to screen molecules that induce increase dopaminergic activity and to screen potential anti-Parkinson drugs (Mele et al., 1998).

The compound Ro4-1284 (Hoffman-LaRoche, Basel) has been established as a potent DA-depleting agent (Fuller and Hemrick-Luecke, 1985) and provides an animal model of benign essential blepharospasm. Burkard et al. (1989) demonstrated that challenging rats with a dose of 20-mg/kg Ro4-1284 was sufficient to induce blepharospasms.

This study investigated the possibility that an amide bond molecule, comprised of L-tyrosine (which is unable to cross the blood–brain barrier by itself) and α-linolenic acid [i.e., *N*-(α-linolenoyl)tyrosine (NLT)], will cross the blood–brain barrier and be an effective DA agonist. We examined the dopaminergic activity of NTL in an animal model of PD (via rotational behavior, following unilateral

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Table 1
Effects of the various treatments on DA and DA metabolite levels in the unlesioned striatum

| | DA | HVA | DOPAC | HVA/DA | DOPAC/DA |
|---|---|---|---|---|---|
| Control ($n=11$) | 10.840 ± 0.12 | 0.657 ± 0.018 | 1.456 ± 0.034 | 0.06 | 0.13 |
| Sham ($n=12$) | 11.244 ± 0.13 | 0.717 ± 0.019 | 1.278 ± 0.028 | 0.06 | 0.11 |
| 6-OH-DA + L-tyrosine ($n=11$) | 11.290 ± 0.14 | 0.629 ± 0.015 | 1.555 ± 0.031 | 0.05 | 0.13 |
| 6-OH-DA + α-linolenic acid ($n=12$) | 11.832 ± 0.12 | 0.609 ± 0.022 | 1.374 ± 0.038 | 0.05 | 0.11 |
| 6-OH-DA + NTL ($n=12$) | 13.220 ± 0.18 | 0.508 ± 0.013 | 1.114 ± 0.025 | 0.03 | 0.08 |
| 6-OH-DA + D-amphetamine ($n=11$) | 14.943 ± 0.16 | 0.435 ± 0.025 | 1.031 ± 0.021 | 0.03 | 0.07 |

Cell entries represent the number of rats for the separate experimental groups. The level of DA, HVA and DOPAC expressed as ng/mg wet weight of tissue ($M$ ± S.E.M.).

6-OH-DA lesion) and in a brain DA depletion model (via administration of Ro4-1284). The levels of DA and its metabolites were measured in the intake striatum to evaluate the effects of NLT on the DA level, DA turnover and release (Table 1).

## 2. Methods

### 2.1. Test material

NLT (mass: 456.310, $C_{28}H_{42}N_1O_4$) was prepared as follows: 1-g L-tyrosine (Sigma T 8909), 1-ml α-linolenic acid (Sigma L 2376), 0.25-ml acetonitrile and 1-ml methanol dissolved in ether (5 ml). The solution was transferred to a test tube by vacuum distillation (liquid nitrogen). The test tube had inlet/outlet caps. There was anhydrous calcium sulfate in the tube. The solution was saturated with anhydrous, gaseous HCl at 0 °C. The solution was stored for 2 h at 0 °C, and 50 ml of dry ether was added with shaking. The product crystalled out and, after standing for 1 h at 0 °C, was collected by decantation and washed with 210-ml cold dry ether. The crystals were dried under vacuum and stored for 24 h in a tightly stoppered bottle under anhydrous condition at − 20 °C.

High-resolution mass spectrum analysis of NLT yield the following results: CI by $CH_4$: 456.310112 (M+, 100%), $C_{28}H_{42}NO_4$ calculated mass: 456.311384. $^1$H-NMR ($CDCl_3$): 0.97 (t, $J=7.5$ Hz; 3 H), 1.28 (broadened; 10 H), 1.58 (m; 2 H), 2.07 (m; 4 H), 2.19 (t, $J=5.6$ Hz; 2 H), 2.80 (m; 2 H), 3.03 (m; 2 H), 3.37 (s; 3 H), 4.87 (m; 1 H), 5.37 (m; 6 H), 6.04 (d, $J=8.0$ Hz; 1 H), 6.10 (broadened; 1 H), 6.83 (m, 4 H) ppm. $^{13}$C-NMR ($CDCl_3$): 14.24; 20.51; 25.49; 25.52; 25.57; 27.16; 29.08; 29.15; 29.54; 36.48; 37.18; 52.40; 53.17; 115.53; 126.94; 127.07; 127.70; 128.21; 130.21; 131.93; 155.45; 172.30; 173.53 ppm.

### 2.2. Experimental animal

For the rotational (PD) study, seven groups ($n=16$), male Sprague–Dawley rats, weighing 150–200 g, were housed six per cage in a well-ventilated and air-conditioned room, with an ambient temperature of 22 ± 2 °C and a relative humidity of 45%. Food and water were supplied continuously. The seven experimental groups were Control (not operated) + saline, Sham operated + saline, 6-OH-DA groups treated with either saline, L-tyrosine, α-linoleic acid, NLT or D-amphetamine. Two other groups ($n=12$) of rats were treated daily for 14 days with 25-mg/kg NLT (intraperitoneal) followed by a challenge of 40-mg/kg Ro4-1284 (intraperitoneal) or saline, 30 min after the last injection.

### 2.3. Lesions

One group served as a no treatment control group, and a second group was a sham-operated group. The other four groups were surgically operated and served as experimental groups. All surgery was performed under sodium pentobarbital anesthesia (pentobarbital sodium, 50 mg/ml as required). The rats were pretreated with 10 mg/kg ip of desimipramine (in order to prevent damage to the noradrenergic neuron) and fixed in a Kopf stereotaxic instrument (Model 900). Left unilateral 6-OH-DA (8 μg in 4 μl of saline containing 0.05% ascorbic acid) lesions were made. In order to verify the location of the injection, the site was marked by electrical current, which was delivered by the needle of the injection. The electrical current was constant (0.5 mV for 1 s) and was passed through a stainless-steel insulated needle, bared only in the tip. The coordinates (modified from Konig and Klippel, 1963) for the caudate nucleus lesion were A 8.5, L 2.2, and B + 1.8 from the interaural line. The chemical lesion was made in the left side of the brain. Sham-operated animals were treated as lesioned animals, i.e., they were placed in a stereotaxic instrument with a needle placed in the brain but without injection or electric current passing through the electrode. After surgery, each rat was placed in an individual cage. Ten days after surgery, the experimental groups were given (intraperitoneally) 1-ml saline (0.9% NaCl), 100-mg/kg NLT, 100-mg/kg L-tyrosine, 100-mg/kg α-linolenic acid or 5-mg/kg D-amphetamine sulfate (Sigma A 5880), respectively.

At the end of the experiment, the rats were sacrificed, and the brains were removed as quickly as possible (less than 30 s) and divided into the two hemispheres. The left side was used for the histological examination and the unlesioned side for biochemical studies.

### 2.4. Neuroanatomy

Serial coronal slices were made at 40 μm using a freezing microtome. Representative slides were stained with cresyl violet for lesion verification. The results presented here are from rats with the histology confirmed that the site of the injection was in the striatum.

### 2.5. Neurochemical measurements

The dissection was performed free hand on an ice-cold table. The striatum was very quickly separated, and the tissue was frozen on dry ice and stored at −80 °C. The levels of DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined in the right striatum using HPLC methods. Tissue preparations were according to Berridge et al. (1999).

### 2.6. Rotational behavior

Rotational behavior was tested in a rotometer 30 min after injection for 30 min (i.e., between 30 and 60 min after injection), with each rat mounted in a special harness and placed in an acrylic, transparent dome of 41-cm radius. Rotational movements were transuded from the harness via a stainless-steel tube (1/8 in.) and a precision universal joint (Pic BC 12) to a 5-K linear potentiometer (Spectrol), which received an excitation current from Sanborn polygraph. The potentiometer (preamplifier 8805A) measured and recorded the changes in the amount of current resulting from the rotational movements. A turn to the right deflected the recording pen upward, while a leftward turn deflected the pen downward. Continuous recordings were made at a speed of 1 mm/s.

### 2.7. Blepharospasm

Blepharospasm (involuntary spasm of the orbicular muscles of the eye, causing forceful closure of the eyes, with significant increases in eyelid closures per minute) is considered a type of dystonia, resulting from a decrease in brain DA level. In saline-treated animals, Ro4-1284 (a powerful DA-depleting agent) is able to induce an animal model of benign essential blepharospasm. The rats were monitored by a video camera, for 10 min, 30 min after challenged with Ro4-1284. Two independent observers rated the number of eye-blinking, without knowledge about the treatment the rat received.

## 3. Results

### 3.1. Rotational behavior

Sham-operated rats behaved as control rats. Unilateral treatment with 6-OH-DA (+saline) modified the rotational behavior of the rats. The treated rats turned away from the lesion side and increase the number of turns compared with the "control + saline" group. ($\chi^2 = 12$, $P \leq .05$).

Table 2
Rotational behavior

| | L | R | L&R | %L | %R |
|---|---|---|---|---|---|
| Control + vehicle ($n = 11$) | 12 ± 0.9 | 8 ± 0.4 | 20 | 60 | 40 |
| Sham operated + saline ($n = 12$) | 12 ± 0.9 | 16 ± 1.0 | 28 | 44 | 56 |
| 6-OH-DA + saline ($n = 11$) | 4 ± 0 | 26 ± 2.0 | 30 | 13 | 87 |
| 6-OH-DA + L-tyrosine ($n = 11$) | 3 ± 0 | 27 ± 1.8 | 30 | 10 | 90 |
| 6-OH-DA + α-linolenic ($n = 12$) | 8 ± 0.9 | 32 ± 4.1 | 45 | 20 | 80 |
| 6-OH-DH + NTL ($n = 12$) | 98 ± 10 | 2 ± 0 | 100 | 98 | 2 |
| 6-OH-DA + D-amphetamine ($n = 10$) | 78 ± 7 | 4 ± 0 | 82 | 95 | 5 |

Cell entries represent the number and direction of rats' turns, during the measurement period (see text), for the separate experimental groups. ($M \pm$ S.E.M.) See text for significant statistical differences between the experimental groups.

Replacement of saline treatment with L-tyrosine or α-linoleic acid to the 6-OH-DA-treated groups did not modify their rotational behavior, e.g., an increased number of turning to the right — the contralateral side of the lesion [ANOVA, $F(3,44) = 9.41$, $P < .001$]. In contrast, NLT and D-amphetamine treatment to 6-OH-DA-treated rats had induced an increase in motor activity and reversed the rotational direction (ipsilateral turns to the side of the lesion). These treatments were different from all other treatments [ MANOVA, $F(6,72) = 13.4$, $P < .0001$; Table 2]. There are no statistical differences between the results of the NLT and D-amphetamine groups.

### 3.2. Blephrospasm

The rate of eye-blinking of a normal rat is 5.16 ± 1.58 ($M \pm$ S.D.). Acute administration of Ro4-1284 induced an increase in the rate of eye-blinking 30 min after treatment (19.08 ± 3.4). Pretreatment with NLT reduced the rate to 5.00 ± 1.95 [$t$ test, $df(11,11)$, $F = 4.876$, $P < .001$].

### 3.3. Neurochemical studies

NLT induced an increase of 32% ($P < .05$) in the DA level, a decrease of 21% in the HVA level ($P < .05$) and of 24% in the DOPAC level ($P < .05$), compared with the DA, HVA and DOPAC levels in intact rats. D-Amphetamine induced similar effects. There were no significant statistical differences between the NLT- and D-amphetamine-treated rats. The three other experimental groups did not differ from the control group (Duncan's $t$ post hoc test). The ratio of HVA/DA ($P < .05$) and DOPAC/DA ($P < .01$) were significantly reduced [MANOVA, $F(5,72) = 14.4$, $P < .0001$ and Duncan's $t$ post hoc test].

## 4. Discussion

The rotational behavior test is one of the most powerful tools to screen potential anti-Parkinsonian agents. The logic

of this screening test is the finding that the level of the dopaminergic activity in the striatum of Parkinson's patients is very low. Molecules, which can stimulate the activity of this dopaminergic system, are candidates to be anti-Parkinsonian agents.

This study indicates that NLT behaves as a potent dopaminergic agonist. Administration of NLT (a) increases the level of motor activity, (b) reverses the turning direction of unilateral striatal lesioned rats, (c) overcomes DA-deleting symptoms induced by Ro4-1484 and (d) increases the DA and decreases the DOPAC and HVA levels.

In the analysis of the biochemistry results, it should be noted that DOPAC primarily reflects intraneural metabolism, while HVA is a product of intra- and extraneuronal metabolism of the released DA. The decreased level of DOPAC/DA represents greater DA turnover, and HVA/DA represents greater release. The results indicated that NLT and D-amphetamine potentate the rate of DA turnover in greater extent than the DA release. However, the net result is an increase in the DA level. The correlation between the increased DA level and numbers of rotations is +.79.

It is interesting to note that while NLT and D-amphetamine share some dopaminergic activity, NLT does not modify the body temperature and the basic motor activity level among intact NLT (100 mg/kg)-treated rats. D-Amphetamine (5 mg/kg) induced hyperthermia and hyperactivity (unpublished results). Therefore, NLT should be devoid of the side effects of D-amphetamine.

Supportive evidence to the ability of NLT to cross the blood–brain barrier can be found in some preliminary studies. One such study shows that $^{14}$C-labeled NLT (148 Ci/mmol) can be found in the brain after an intraperitoneal injection. Another preliminary study showed that NLT treatment induced an increase in fatty acid amide hydrolysis (FAAH) activity in the striatum of NLT-treated rats. FAAH was increased from $1.5 \pm 0.3$ to $1.9 \pm 0.3$ nmol/(min mg) ($M \pm$ S.E.M.; Egertova et al., 1998).

In addition, several relationships between DA and α-linolenic acid have been reported. α-Linolenic dietary deficiency induces a significant decrease in DA level and in the density of dopaminergic D2 receptors in rat brains (Delion et al., 1996). The same enzyme (cytochrome *P*450) is involved in the metabolism of DA and EFA such as α-linolenic acid and arachidonic acid [cf. Yehuda et al., 1998 and Thompson et al., 2000 for additional speculations]. It is of interest to note that one of the hypothesis on the etiology of PD is that either genetic or environmental factor(s) induced changes in the cytochrome *P*450 (Riedl et al., 1998).

On the other hand, synthetic amides of PUFA affect the dopaminergic system (Bisogno et al., 2000; Baldessarini et al., 2001). One of the possible modes of action for the PUFA is by the action of the FAAH enzyme. This enzyme is unevenly distributed in the brain and catalyzes the hydrolysis of ananamide and oleamide. High levels of FAAH were found in the pyramidal and extrapyramidal systems, including the basal ganglia. In addition, cannabinoid receptors are highly concentrated in the basal ganglia (Tsou et al., 1998) and are involved in PD (Sanudo-Pena et al., 1998), and FAAH has an effect on cannabinoid and on cannabinoid receptors (Ueda et al., 2000). One can speculate that NLT effects are mediated via FAAH activity.

Given the high level of melanin in the substantia nigra, it is most interesting that α-linolenic acid can modify the peripheral melanin concentration (Ando et al., 1998). However, no interaction between fatty acids and melanin in the brain was found. The results of this study indicate that combining an essential amino acid with an essential fatty acid, via a special bond, produces an active biological molecule. More specifically, the newly combined α-linolenic acid with L-tyrosine reveals potent dopaminergic activity in a rat model of PD and in blepharospasm. The results of this study showed that NLT is as potent as D-amphetamine, and both dopaminergic drugs increase the level, the turnover and the release of DA. The working hypothesis, to explain the dopaminergic mode of action of NLT, is that (a) the α-linolenic acid plays a dual role, viz. a carrier for tyrosine and a membrane- and receptor-improving agent, with (b) the amide bond directing the molecule to FAAH-rich areas and with (c) tyrosine, correspondingly increasing the DA level, turnover and release.

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