A. 研究目的

神経変性疾患の治療を考えた場合、有用なアプローチの一つは、神経細胞の死のメカニズムを解明することである。本年度の研究の目的は、アポトーシスとネクローシスに関与することが示唆されている MPT の分子メカニズムの解明と疾患治療のための標的分子の同定のために Cyclophilin D に結合する因子を同定することと、MPT の分子機構解明と MPT が関与する疾患の治療薬探索に向け、Cyclophilin D の構造解析を行うことである。さらに、Cyclophilin D の生理機能解明のために Cyclophilin D 欠損マウスを詳細に解析することである。

B. 研究方法

（1）Cyclophilin D に結合する分子を同定するために、免疫沈降法を用いた。Cyclophilin D に対する良質な抗体が確立されていなかったため、Cyclophilin D にタグを付けた遺伝子を哺乳動物細胞に導入し、タグに対する抗体を利用し免疫沈降を行った。Cyclophilin D と免疫共沈するものを電気泳動により分離し、アミノ酸配列解析により結合因子を同定した。

（2）Cyclophilin D とその阻害剤の一つであるシクロスポリン A の複合体の結晶化を行い、構造解析を行った。大腸菌で作製したリコンビナントたんぱくを用いて結晶化を行った。

（3）種々のテスト法を駆使し、Cyclophilin D 欠損マウスの行動解析を行った。

動物を用いる実験は、全て大阪大学医学部の動物実験安全委員会の許可を得ている。動物愛護上の配慮からの審査基準は以下の通りである。（1）代替え手段がないこと（特定遺伝子 Cychlophilin D の生体内での機能解析のために利用できるのはマウスに限られている）、（2）苦痛を回避する手段を講じている。

C. 研究結果

（1）Cyclophlin D 結合因子の同定

Cyclophilin D は PPIase (Peptidyl prolyl cis-trans isomerase)の活性を有しているため、その機能発現に結合ターゲット分子が想定される。Cyclophlin D に結合し、MPT に関与する因子の候補を得るために、Cyclophilin D の C 末に FLAG タグを付加したコンストラクトを作製し、293T 細胞株に安定導入した。この細胞株に酸化ストレス（H2O2 処理）を加えて Cyclophlin D/MPT 依存的細胞死を誘

導し、そのライセートからFLAGタグに対する抗体を用いてCyclophlin Dを免疫沈降させた。その沈降物をSDS/PAGEで展開し、刺激特異的にCyclophlin Dに結合する分子を3種類（ゲル上のバンドとして）得た。これらの分子をMASS解析により同定した。これらの分子は全てミトコンドリアマトリックスのたんぱくであり、MPTの誘導（内膜の透過性亢進）という観点から予想されたようなミトコンドリア内膜のタンパクは検出されなかった。Cyclophlin D結合因子の探索を継続しつつ、得られた分子の解析を遂行中である。

（2）Cyclophlin D・シクロスポリンA複合体の構造解析

Cyclophilin DはPPIaseであるため、ターゲット分子の構造を変化させて機能することが予想されている。そのため、Cyclophilin Dの機能やMPTの分子機構を解くために、たんぱくの構造解析が必須である。また、Cyclophilin Dの構造解析はCyclophilin Dの特異的な阻害剤の探索にも有効であると考えられる。そこで、まず、大腸菌で作製したリコンビナントCyclophilin Dとその阻害剤シクロスポリンA複合体の結晶を作製し、それを用い構造解析を行った。その結果、０．９６オングストロームレベルの構造を解くことに成功した。Cyclophilin Dとシクロスポリン Aの結合様式も詳細に見る事が出来、Cyclophilin Dに特異的な阻害剤の探索に有用な情報となった。

（3）CyclophilinD欠損マウスの行動解析

記憶における役割を検討するために、モリス水迷路テストを行った。Cyclophilin D欠損マウスのプラットホームへのescape latencyは、野生型マウスに比べて有意に延長しており、陳述記憶障害が認められた。プローブ試験において野生型マウスは、プラットホームが設置してあった区間（trained quadrant）におけるpercent search timeは他のquadrantのそれに比べ有意に増加していたが、Cyclophilin D欠損マウスではそのような有意な増加が認められなかった。これらの結果は、Cyclophilin D欠損マウスは、海馬機能の異常に伴う陳述記憶が障害されたことによるものと考えられた。さらに、Cyclophilin Dの記憶における役割を検証するために「恐怖条件付け学習試験」を行った。マウスを訓練時に用いる測定ケージとは異なるケージ（ニュートラルケージ）に入れ、1分間無動時間を測定した。その後床がグリッドからなる装置（訓練ケージ）に入れ、2分間無動時間を測定した。その後、80 dBの音刺激を15秒間呈示し、その最後の5秒間に1.0 mAの電気刺激を与える作業を、連続して4回行った。24時間後、ニュートラルケージに入れ、1分間の音刺激（80 dB）を与え

ている間の無動時間を測定した。その後、訓練ケージに入れ、2分間の無動時間を測定した（電気および音刺激なし)。その結果Cyclophilin D欠損マウスの電気刺激に対する感受性は、野生型マウスと差はなかったが、テスト試行においてCyclophilin D欠損マウスと野生型マウスとも音刺激学習(cue)・文脈学習(contextual)を獲得した。しかしCyclophilin D欠損マウスのfreezing timeは、野生型マウスのそれに比べ有意に短縮されたので、Cyclophilin D欠損マウスの音刺激学習・文脈学習能は、野生型マウスより劣るものと示唆された。

また、情動反応への影響を検討した。まず、高架式十字迷路試験を行った。壁の高さが 20cm、長さ 25 cm、幅 8 cm のアーム（closed arm）と同様の長さと幅を持つ、壁のないアーム（open arm）を十字に組み合わせ、高さ 50 cm の位置に設置した装置を用いた。マウスをアームの交差する位置に置き、その後 5 分間隔で 10 分間にわたって装置内を自由に散策させ、open arm に入って留まっていた時間と回数を測定した。その結果、Cyclophilin D 欠損マウスは、野生型マウスと比較して、最初の 5 分間において open arm に入って留まっていた時間の有意な短縮と有意な回数の減少を示した。10 分間の測定においては、Cyclophilin D 欠損マウスでは、野生型マウスと比較して、open arm に入って留まっていた時間が有意に短縮した。また、驚愕反応テストを San Diego の Startle Response System を用いて行なった。すなわちマウスを驚愕反応測定ホルダーに入れて 10 分間装置に馴化させた後、驚愕反応試験を開始した。バックグランド（BG）は 60 dB、パルスは 80dB、90dB、100dB、110dB、120 dB の音圧とした。BG（バックグランドを聞かせる)、80dB、90dB、100dB、110dB、120 dB（パルスを聞かせる）の組み合わせを 1 試行として、試行内をランダムに 8 試行行い、試行間隔は 25 秒とした。その結果、Cyclophilin D 欠損マウスは、野生型マウスに比べて 110dB および 120dB の音圧に対して驚愕反応が有意に高く、音刺激に対する感受性が高まっているものと示唆される。これらのことから、Cyclophilin D 欠損マウスは、記憶障害と情動性に障害を呈することが分かり、Cyclophilin D の生理機能の一つが明らかになった。

D. 考察

今回、我々は、細胞死刺激導入時に特異的に Cyclophilin D と相互作用する分子を複数確認したが、これらの分子が Cyclophilin D の機能ターゲットである可能性があり、その詳細な解析は MPT のメカニズム解明に向け重要な情報を与

えるものと考えている。さらに、これらの分子は、MPTが関与する疾患の治療薬ターゲットになる可能性もあり、今後の解析が急務である。
Cyclophilin Dの特異的な阻害剤は、MPTが関与する疾患の治療薬候補と成り得るため、今回得られた詳細な構造に関する情報は、今後の薬剤探索に重要な情報を提供するものと考えられる。
MPTやCyclophilin Dの生理的な役割を明らかにするために、Cyclophilin D欠損マウスは有用な材料であるが、今回、このマウスの解析から、Cyclophilin D（あるいはCyclophilin D依存的MPT）が、記憶や情動性など幾つかの行動に関与することが明らかになった。その詳細なメカニズムは今後の課題であるが、Cyclophilin D（あるいはCyclophilin D依存的MPT）の生理的機能を考える上で重要な発見であると考えている。

E. 結論

MPTの分子メカニズムの解明と細胞死における役割を解明するために、MPTに必須分子である Cyclophilin D の結合分子の探索を免疫沈降法を用いて行い、Cyclophilin Dと相互作用する分子の存在を確認した。
MPT の分子機構解明と MPT が関与する疾患の治療薬探索を目的として、Cyclophilin D・シクロスポリンA複合体の詳細な高次構造の解明を行った。
Cyclophilin Dの生理的機能を明らかにする目的で、Cyclophilin D欠損マウスの解析を行い、Cyp D欠損マウスは、記憶障害や情動反応に異常を呈することが明らかになり、Cyclophilin Dの（恐らくはMPTの)生理機能の一つを明らかにすることが出来た。

F. 健康危険情報

特になし

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H. 知的財産権の出願・登録状況
特になし

研究成果の刊行に関する一覧表

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Neuroprotection by propargylamines in Parkinson's disease: intracellular mechanism underlying the anti-apoptotic function and search for clinical markers

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Summary In Parkinson's and other neurodegenerative diseases, a therapeutic strategy has been proposed to halt progressive cell death. Propargylamine derivatives, rasagiline and (−)deprenyl (selegiline), have been confirmed to protect neurons against cell death induced by various insults in cellular and animal models of neurodegenerative disorders. In this paper, the mechanism and the markers of the neuroprotection are reviewed. Propargylamines prevent the mitochondrial permeabilization, membrane potential decline, cytochrome c release, caspase activation and nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase. At the same time, rasagiline induces anti-apoptotic pro-survival proteins, Bcl-2 and glial cell-line derived neurotrophic factor, which is mediated by activated ERK-NF-κB signal pathway. DNA array studies indicate that rasagiline increases the expression of the genes coding mitochondrial energy synthesis, inhibitors of apoptosis, transcription factors, kinases and ubiquitin-proteasome system, sequentially in a time-dependent way. Products of cell survival-related gene induced by propargylamines may be applied as markers of neuroprotection in clinical samples.

Keywords: Apoptosis, propargylamine, rasagiline, mitochondria, permeability transition pore, GDNF, Bcl-2, nuclear transcription factor

Abbreviations

ANT	adenine nucleotide translocator
BDNF	brain-derived neurotrophic factor
BPAP	1-(benzofuran-2-yl)-2-propylaminopentane
CyP-D	cyclophilin-D
CsA	cyclosporin A
$\Delta\Psi m$	mitochondrial membrane potential
FACS	fluorescence-augmented flow cytometry
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GDNF	glial cell-line derived neurotrophic factor
R-2HMP	*N(R)*-(2-heptyl)-*N*-methyl-propargylamine
IL	interleukin
MAO-A and *MAO-B*	type A and B monoamine oxidase
MAP	mitogen-activated protein
MEM	Hanks' minimum essential medium
mPT	mitochondrial permeability transition
NM(R)Sal	*N*-methyl(*R*)salsolinol
PD	Parkinson's disease
PI	propidium iodide
TNF	tumor necrosis factor
VDAC	voltage-dependent anion channel

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disease and affects 1–2% of the aged population. PD is pathologically characterized by selective cell death of dopamine neurons in the substantia nigra pars compacta, and biochemically by depletion of dopamine neurotransmitter in the striatum. The etiology for the sporadic form of PD remains enigmatic, whereas a growing understanding of responsible genes for familiar forms of PD suggests that the processes leading to neuronal loss may be common with those in the sporadic form of PD (Eriksen et al., 2005; Vila and Przedborski, 2004). The loss of nigral dopamine neurons in PD is hypothesized as the mutations in genes detected in the familiar form sensitizes the neurons to intrinsic and extrinsic insults. Increased oxidative stress, mitochondrial dysfunction, impaired ubiquitine-proteasome system, abnormal inflammatory cytokines, and excitotoxicity are considered to cause cell death in dopaminergic neurons, in which dopamine itself should be involved by not fully clarified mechanisms. At present, available therapies for patients with PD are limited to ameliorate the symptoms. Dopamine replacement relieves the major symptoms at least for the beginning several years. However,

progressive loss of dopamine neurons results in motor fluctuation and cognitive dysfunction, hallucinations, depression and dementia. A therapy intervening the disease progress itself is now seriously required, and "neuroprotective" therapy to rescue neurons from cell death and "neurorestrorative" therapy to restore deteriorated neurons to a normal state have been proposed (Dawson and Dawson, 2002). The therapy should target intracellular death cascade, which is activated rather slowly for decades to the end point showing the clinical signs and regulated by well-conserved and -regulated cell death system (Riederer, 2004). Using cellular and animal PD models, the molecular mechanisms behind neuronal loss have been intensively studied, and several agents have been confirmed to prevent the cell death processing. In order to ameliorate the pathogenic factors, neuroprotective agents have been proposed, including antioxidants, neurotrophic factors, anti-inflammatory drugs, mitochondria supplement, inhibitors of monoamine oxidase (MAO), and drugs interfering glutamate excitotoxicity. Since signal proteins for apoptosis increase in the nigral neurons of Parkinsonian brains, anti-apoptotic agents altering apoptotic signal pathway have been gathering attention (Maruyama et al., 2002a; Mandel et al., 2003; Simpkins and Jankovic, 2003; Youdim et al., 2006). The anti-apoptotic function is confirmed in inhibitors of type B MAO (MAO-B) and caspase inhibitors, immuno-modulators, Co-Q10, NMDA receptor antagonists and neurotrophic factors in cellular and animal model systems. Recently, several clinical trials were reported to examine effects of propargylamine MAO-B inhibitors, rasagiline [*N*-propargyl-1(*R*)-aminoindan] (Youdim et al., 2001) and (−)deprenyl [selegiline, *N*, α-dimethyl-*N*-2-propynylbenzene-ethanolamine], in Parkinsonian patients, and beneficial effects were confirmed to slow the progression of the symptoms (Parkinson Study Group, 2004, 2006; Pälhagen et al., 2006). However, the final conclusion about the neuroprotective efficiency remains to be clarified (Riederer et al., 2004; Schapira and Olanow, 2004; Suchowersky et al., 2006).

Rasagiline and (−)deprenyl were applied in PD to increase dopamine availability through inhibiting the oxidative deamination by MAO (Birkmayer et al., 1977). In addition, MAO-B inhibitors inhibit the oxidation of protoxicants to toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to 1-methyl-4-phemylpyridinium ion (MPP^+), scavenge reactive oxygen species, and prevent the lipid peroxidation and the formation of toxic dopamine quinone. Later clinical observations suggest that they may protect neurons against cell loss in PD, AD and other neurodegenerative disorders. We studied the mechanism behind protection of rasagiline against apoptotic or necrotic cell death induced in human neuroblastoma SH-SY5Y cells by oxidative stress (Maruyama et al., 2002c) and neurotoxins, such as *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal] (Naoi et al., 2002a) and 6-hydroxydopamine (6-OHDA) (Maruyama et al., 2001b, 2002b). *N*M(*R*)Sal binds to type A MAO (MAO-A) in mitochondrial outer membrane, opens a mega-channel called mitochondrial permeability transition (mPT) pore, initiates rapid reduction of mitochondrial membrane potential, $\Delta\Psi m$, and swelling of mitochondria (Akao et al., 2002a; Maruyama et al., 2002a; Naoi et al., 2006; Yi et al., 2006a). Induction of mPT results in the cytochrome c release signaling subsequent apoptosis, or the loss of ATP production leading to necrosis. Bcl-2 protein family in mitochondria directly regulates the apoptotic pathway, and intracellular signaling strictly regulates the synthesis and posttranslational modification. Neuroprotective agents intervene these apoptotic processes, either by suppressing apoptogenic factors or increasing pro-survival, anti-apoptotic factors in cells.

In this paper, our recent understanding on the mechanism underlying anti-apoptotic function of propargylamines is reviewed. The effects of propargylamine derivatives were examined in relation to the regulation of mPT and the induction of pro-survival proteins, Bcl-2 and neurotrophic factors. To confirm the involvement of cell signaling, gene expression by the propargylamines was studied by cDNA array analyses. Hitherto clinical studies indicate that the more quantitative, biochemical and molecular evaluation is required to confirm the neuroprotection in Parkinsonian patients. Our recent results by use of primate suggest that gene products increased by rasagiline in the CSF and serum may be used as clinical markers to quantify the potency of putative neuroprotective drugs in clinical samples. The expected future development of neuroprotective therapy is discussed.

Materials and methods

Materials

Rasagiline and related compounds were kindly donated by Teva Pharmaceutical (Netanya, Israel). *N*-Propargylamiine and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA); JC-1, Hoechst33342, MitoTracker Orange and Green, and Rhodamine 123 from Molecular Probes (Eugene, OR, USA). Anti-Bcl-2 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA); anti-β-actin antibody from Oncogene (Boston, MA, USA); mouse monoclonal anti-GAPDH antibody from Chemicon International (Temecyla, CA, USA). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in 95% air and 5% CO_2. Bcl-2 was overexpressed in SH-SY5Y cells as reported previously (Akao et al., 2002a). Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999).

Determination of apoptosis

Apoptotic and necrotic cell death were assessed quantitatively using fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Benton Dickinson, San Jose, CA, USA) (Yi et al., 2006a). To determine apoptotic cells, the cells were stained with PI solution in phosphate-buffered saline (PBS) containing 1% Triton X-100 and subjected to FACS analysis. Cells with a lower DNA content showing less PI staining than G1 were defined to be apoptotic (subG1 peak) according to Eckert et al. (2001).

Measurement of mitochondrial membrane potential, $\Delta\Psi m$

The $\Delta\Psi m$ in isolated mitochondria was quantified by FACS using MitoTracker Orange and Green. The mitochondria were treated with agents at 37°C for 3 h, and stained with 100 nM MitoTracker Orange and Green, then subjected to FACS. The laser emission at 560–640 nm (FL-2) and at shorter than 560 nm (FL-1) with excitation at 488 nm were used for the detection of MitoTracker Orange and Green fluorescence, respectively. In other experiments, mitochondria were prepared from male Donryu rat liver or transgenic mice expressing human Bcl-2 in the liver, as previously described (Shimizu et al., 1998). $\Delta\Psi m$ was assessed also by measurement of reduction in Rhodamine 123 fluorescence, which was ascribed to $\Delta\Psi m$-dependent uptake of Rhodamine 123 into the mitochondria (Narita et al., 1998).

Measurement of mRNA and protein of Bcl-2 family proteins

SH-SY5Y cells were cultured in the presence of various concentrations (10 μM–1 pM) of rasagiline for 24 h or for a various incubation time with 100 nM rasagiline. The whole cells were gathered and the total RNA was extracted by the phenol/guanidinium thiocyanate method. The cDNA was generated by reverse transcription of the total RNA, and the cDNA fragments were amplified using the PCR primers (Akao et al., 2002b). PCR products were analyzed by electrophoresis on 3% agarose gels, and β-actin cDNA was used as an internal standard.

Quantitative measurement of mRNA and protein of GDNF

SH-SY5Y cells were treated with rasagiline in 96 well plates with Hanks' minimum essential medium (MEM). The effect of sulfasalazine (100 μM), an inhibitor of IκB, was examined by adding the inhibitor 30 min before the treatment with rasagiline. The protein amount of GDNF was quantified as reported previously using the enzyme immunoassay (EIA) (Nitta et al., 2002). Samples or standard were added to GDNF antibody-coated wells, and incubated for 12–18 h at 4°C. The biotinylated secondary antibody was reacted in avidin-conjugated β-galactoside (Boehringer Mannheim) for 1 h. The enzyme activity in each well was measured by incubation with a fluorescent substrate, 4-methylumbelliferyl-β-D-galactoside. The fluorescence intensity of produced 4-methylumbelliferone was measured at 360 nm with excitation at 448 nm. The mRNA of GDNF was measured by reverse transcription-polymerase chain reaction (RT-PCR), as reported (Maruyama et al., 2004a).

Quantitation of activated NF-κB

Activation of NF-κB was determined by NF-κB binding to κB sites using NF-κB p65 transcription assay kit (Active Motif, Carlsbad, CA, USA) (Maruyama et al., 2004a). Five μg of the extract of Hela cells stimulated with TNF-α for 30 min was used as a positive control. The activation of NF-κB was expressed as % of the positive control.

cDNA array for gene expression in apoptosis

The cells were incubated with 100 nM rasagiline for 6, 12, and 24 h, and the total RNA was extracted. Using AMV reverse transcriptase, total RNA isolated from the sample and control was labeled with Cy3- or Cy5-dUTP. The levels of gene expression were quantitatively analyzed by cDNA expression array using TaKaRa IntelliGene Human Apoptosis CHIP (Takara Biomedicals, Ohtsu, Japan).

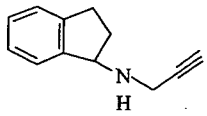
Statistics

Experiments were repeated at least 4 times and the results were expressed as mean and SD. Difference was statistically evaluated by analysis of variance (ANOVA) followed by Sheffe's F-test. A p-value less than 0.05 was considered to be statistically significant.

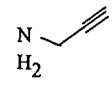
Results

Stabilization of mitochondrial contact sites by propargylamines

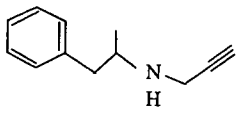
A series of propargylamines, rasagiline, (−)deprenyl, aliphatic (*R*)*N*-(2-heptyl)-*N*-methylpropargylamine (*R*-2HMP) and free *N*-propargylamine, prevent the activation of apoptotic cascade and protect SH-SY5Y cells against apoptosis induced by neurotoxins, *N*M(*R*)Sal and 6-OHDA, and oxidative stress caused by dopamine oxidation and a peroxynitrite-generating agent, SIN-1 (Akao et al., 2002a; Maruyama et al., 2002a, b, c; Yi et al., 2006b). Figure 1 shows the chemical structure of examined propargylamines. An endogenous neurotoxin *N*M(*R*)Sal induces the mPT and apoptosis (Naoi et al., 2002b, 2006). As summarized in Fig. 2, these propargylamines completely suppress opening of mPT pore caused by neurotoxins and oxidative stress. Rasagiline inhibits mitochondrial swelling and $\Delta\Psi m$ reduction (Akao et al., 2002a), and prevents release of cytochrome c, caspase 3 processing and nuclear translocation of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Maruyama et al., 2002a). Rasagiline protected MAO-A-expressing SH-SY5Y cells from apoptosis and transfection-enforced expression of MAO-B did not increase the sensitivity to rasagiline, indicating that neuroprotective function does not depend on the MAO-B inhibition (Yi et al., 2006a). On the other hand, clorgyline [*N*-methyl-*N*-propargyl-3(2,4-diclorophenpxy)-propylamine] did not prevent, but induced mPT. Table 1 shows the results on the structure-activity relationship for direct stabilization of mPT among propargylamine derivatives with different hydrophobic structure, indanyl (rasagiline), phenyl (deprenyl), aliphatic (2-HMP) and benzofuranyl groups [1-(benzofuran-2-yl)-2-propylaminopentane, BPAP]. The aminoindan derivatives are the most active followed by the phenyl derivatives, and the derivatives with aliphatic and benzofuranyl structures require rather high concentrations for preventing mPT. The modification of aminoindan ring does not affect the potency to stabilize mPT pore, as shown



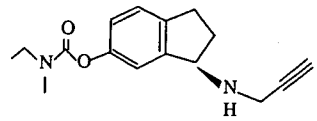
N-Propargl-1(*R*)-aminoindan (Rasagiline)
(*S*)- (TV1022)



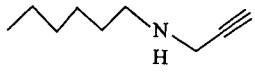
N-Propargylamine



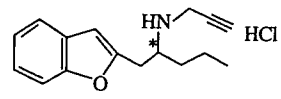
(-)Deprenyl (selegiline)



TV 3326 (*R*-), TV 3279 (*S*-)



N-(2-Heptyl)-*N*-methylpropagylamine
(2-HMP)



FPFS-1180 (*R*-), FPFS-1182 (*S*)

Fig. 1. Chemical structures of propargylamines with neuroprotective potency

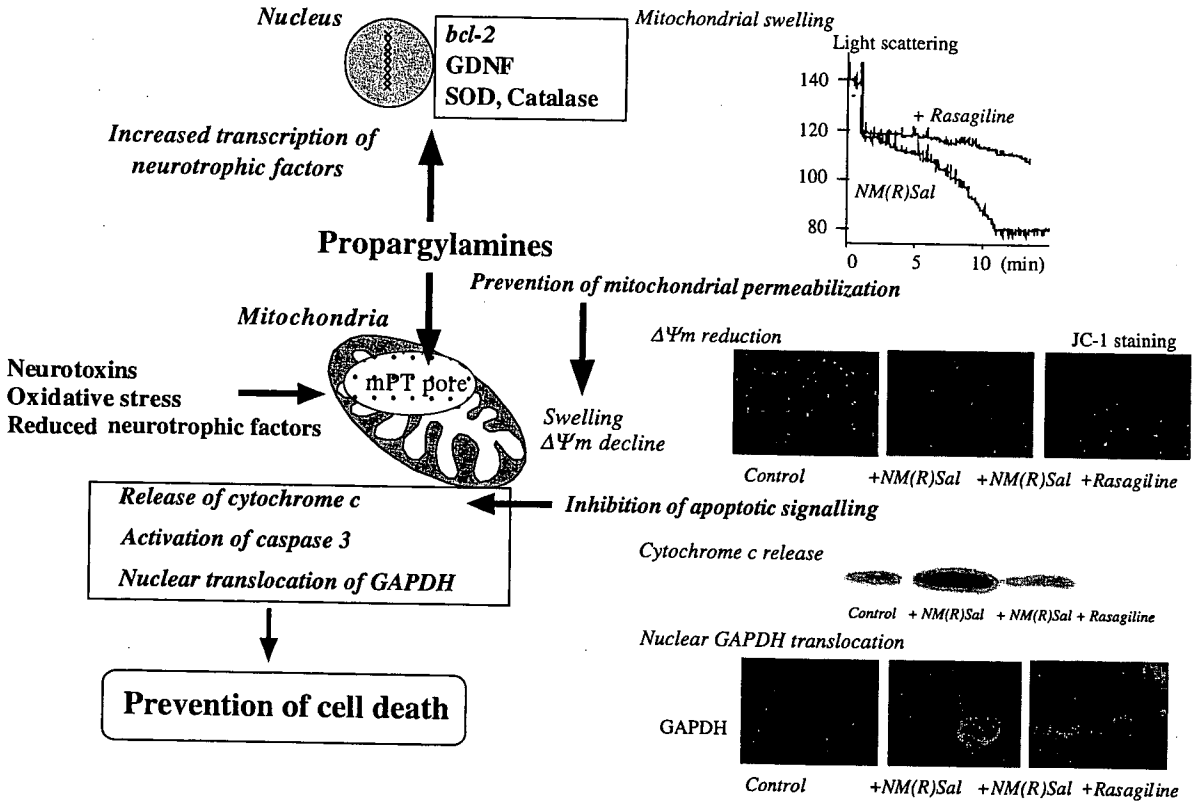


Fig. 2. Target sites of neuroprotective propargylamines in apoptosis cascade. Rasagiline and related compounds suppress mPT, as shown by prevention of mitochondrial swelling and ΔΨm reduction. They inhibit also cytochrome c release, caspase 3 activation and nuclear GAPDH translocation. In addition, the propargylamines increase the expression of anti-apoptotic Bcl-2 family protein, neurotrophic factors (GDNF, BDNF), and antioxidant enzymes (SOD, catalase)

with TV 3326 [(*N*-propargyl)-(3*R*)-aminoindan-5-yl]-ethymethyl carbamate and its hydroxyl metabolite, TV 3294 (Maruyama et al., 2003). In general, the *R*-enantiomers are more potent to prevent the mPT than the *S*-enantiomers (Maruyama et al., 2001a, b). The *S*-enantiomer of rasagiline, TV1022, lacks the MAO inhibiting function, but it still

Table 1. *Structure and neuroprotective characteristics of propargylamines*

Name [Structure]	Prevention of mPT	Induction of Bcl-2	Induction of GDNF
Rasagiline [*R*(+)-*N*-propyl-1aminoindan]	10 μM–1 nM	10 μM–1 nM, 10–1 pM	1 μM–100 pM
TV1022 [*S*(−)-*N*-propyl-1-aminoindan]	1 μM–100 nM	–*	–
Aminoindan	–	–	–
N-Propargylamine	1 μM–10 nM	100–1 nM	N.D.**
N-Methylpropargylamine	–	–	N.D.
Propiolaldehyde	–	–	N.D.
(−)Deprenyl	1 μM–100 nM		1 μM–10 nM
(+)Deprenyl	10 μM	–	–
Desmethyldeprenyl	10–1 nM		1 μM–10 nM
TV3326 [5-ethyl ethyl carbamate-rasagiline]	100–10 nM	–	–
TV3294 [5-hydroxyl-rasagiline]	100–10 nM	–	–
R-*N*-(2-Heptyl)-*N*-methylproparylamine	1 μM–100 nM	N.D.	N.D.
S-*N*-(2-Heptyl)-*N*-methylproparylamine	10 μM	N.D.	N.D.
R-*N*-(2-Heptyl)-propargylamine	1 μM–100 nM	N.D.	N.D.
R-3-(2-Heptylamine)-*N*-methylpropionic acid	–	N.D.	N.D.
R-(−)-BPAP	–	100–1 nM	1 nM***
S-(+)-BPAP	1 μM–10 nM	–	N.D.
R-(+)-*N*-(2-propynyl)-BPAP	1 μM–10 nM	100–1 nM	N.D.
S-(−)-*N*-(2-propynl)-BPAP	–	–	N.D.

* Not affective, ** not determined, *** Hirai et al. (2005).

prevents mPT, suggesting again that the anti-apoptotic function is not related to the MAO inhibition. In the case of the benzylfuranyl derivatives, the stabilization of mPT pore depends on the absolute structure of propargylamines. The compounds with dextro-rotation prevented $\Delta\Psi m$ decline by neurotoxins, whereas the corresponding enantiomer with levo-rotation did not (Maruyama et al., 2004b). The propargylamine group is essentially required for the activity as in the case with free *N*-propargylamine itself, whereas the analogues without a propargyl residue, aminoindan and *R*-3-(2-heptylamino)-propionic acid, did not prevent mPT. The methylation of the amino residue in *N*-propargylamine abolished the activity to prevent $\Delta\Psi m$ reduction (Yi et al., 2006b).

The precise mechanism leading to the permeabilization of mitochondria is still unclear, even though several models have been proposed. The mPT pore is primarily composed of adenine nucleotide translocator (ANT) in the inner membrane and voltage-dependent anion channel (VDAC) in the outer membrane, which binds to ANT at the contact sites between the inner and outer membrane. In addition, peripheral benzodiazepine receptor (PBR) and MAO in outer membrane and hexokinase at the contact site are associated with the mPT pore. Cyclophilin-D (CyP-D) binds to the matrix site of ANT and induces conformation change to form a non-specific pore leading to release of any molecules of less than 1.5 kDa, and metabolic gradients across the inner membrane are dissipated, with accumulation of Ca^{2+}. Opening of the mPT pores results in swelling of the matrix and rupture of the outer membrane, which leads to the release of apoptogenic factors (cytochrome c, apoptosis-inducing factor, Smac/DIABLO, Omi/HtrA2) resulting in activation of caspase system. Oxidative stress and other insults facilitate the mPT pore opening though cross-linking of thiol groups of cysteine residues in ANT and increases the binding of CyP-D to the ADP binding site (McStay et al., 2002). Neurotoxins, PBR ligands (PK11195, protophorphirin IX), bax and other pro-apoptotic Bcl-2 protein family, heavy metals, inorganic phosphate, fatty aids, quinones and uncouplers of mitochondrial oxidative phosphorylation system induce mPT. On the other hand, viral proteins, such as HIV viral protein R (Jacotot et al., 2001) and myxoma poxvirus protein, M11L (Everett et al., 2002), bind to the CyP-D binding site and prevent the pore formation. Another model of mPT is that Bcl-2 interacts directly with VDAC and regulates ANT activity, which was proved in a model system composed of VDAC in liposomes (Shimizu et al., 1999; Tsujimoto and Shimizu, 2000). According to this model, VDAC interacts with apoptogenic Bax and Bak, functions as "VDAC modulators", changes its conformation leading to formation of a megachannel to allow cytochrome c to pass through, whereas anti-apoptotic Bcl-xL closes the channel. In this case, the outer membrane might be intact without rupture. More recently, lipid bilayer was proposed to play an important role in mPT by interacting with ANT or other mitochondrial components (Lucken-Ardjomande and Martinou, 2005).

*N*M(*R*)Sal binds to MAO-A in the outer membrane and opens mPT pore, which CsA and bongkrekic acid antagonize through binding to CyP-D and ANT. *N*M(*R*)Sal, dopamine and its oxidation product quinone, neuromelanin, and peroxynitrite modify sulfhydryl (SH) groups in mitochondria

and induce mPT (Yi et al., in preparation). Rasagiline prevents the reduction of free SH residues in mitochondria and the mPT, regardless of the types of insults leading to mPT (toxins, PBR ligands and oxidative stress). Rasagiline is bound to MAO-B, MAO-A, or other components in mPT pore, stabilizes the contact site and prevents the conversion of ANT into a pro-apoptotic pore. The study is under way whether rasagiline can bind directly to ANT or CyP-D. In addition, propargylamines bind to several other proteins in cells. (−)Deprenyl and its analogue TCH346 [CGP3466, dibenzo(*b,f*)oxepin-10-yl-methyl-methyl-prop-2-ynyl-amine], bind to GAPDH, and prevent the *S*-nitrosylation of GAPDH, the binding to Siah and its nuclear translocation (Hara et al., 2006). Another candidate binding site is poly(ADP-ribose)-polymerase-1 (Brabeck et al., 2003). However, in apoptotic processes these putative binding sites are downstream of mPT and our results demonstrate that the binding of rasagiline to mitochondrial protein and the regulation of mPT are the primary events in preventing apoptosis.

Induction of neuroprotective Bcl-2 family proteins

It is well known that some kinds of protein, Bcl-2 family protein, anti-oxidants and neurotrophic factors, alleviate neuronal loss through suppression of oxidative stress, prevention of apoptotic signal transduction and promotion of cell survival. Rasagiline, and (−)deprenyl increase the activity of anti-oxidative enzymes, superoxide dismutase (SOD) and catalase, in the rat brain after the systemic administration (Carrillo et al., 2000, Kitani et al., 2000). (−)Deprenyl and desmethyldeprenyl increase mRNA level of SOD 1 and 2, Bcl-2 and Bcl-xL, nitric oxide synthase, c-JUN, and NAD dehydrogenase in PC12 cells (Tatton et al., 2002). Our and Youdim's group have clarified the detailed mechanism underlying the induction of anti-apoptotic proteins by rasagiline analogues.

The family of Bcl-2-related proteins constitutes one of biologically most relevant regulatory gene products against apoptosis through controlling mitochondrial permeabilization (Kroemer, 1997). Bcl-2 family proteins are subdivided into three groups on the basis of the pro- and anti-apoptotic function and the Bcl-2-homology (BH) domains (BH1 to BH4). Anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) have 4 BH domains, whereas pro-apoptotic multidomain protein (Bax, Bak, Bok/mtd) lacks BH4. BH3 only proteins (Bid, Bim/Bod, Bad, Bmf) are also pro-apoptotic and link specific apoptotic stimuli to mPT. Bcl-2 is mainly localized in the mitochondrial inner membrane, and the family proteins form homo- or hetero-dimers between anti- and pro-apoptotic members and determine cellular sensitivity to apoptotic stimuli by titrating one another's function. Anti-apoptotic Bcl-2 family proteins prevent apoptosis either by inhibiting pro-apoptotic Bcl-2 members directly, controlling endoplasmic reticulum and mitochondrial homeostasis, or defending against oxidative stress. On the other hand, pro-apoptotic Bcl-2 family proteins induce mPT and trigger the release of mitochondrial apoptogenic factors into the cytosol, as discussed above.

Overexpression of Bcl-2 protects various neuron paradigms *in vivo* and *in vitro* from death induced by neurotoxins and other insults. Bcl-2-overexpression in SH-SY5Y cells prevented apoptosis induced by *N*M(*R*)Sal, which is relevant with the results that $\Delta\Psi$m decline induced by *N*M(*R*)Sal was suppressed in mitochondria prepared from Bcl-2 overexpressed mouse liver (Akao et al., 2002a; Maruyama et al., 2002a). These results suggest that rasagiline may induce Bcl-2 protein, in addition to the direct stabilization of the mPT pore. We found that rasagiline increases the mRNA and protein levels of *bcl-2* and *bcl-xL* in SH-SY5Y cells, as shown in Fig. 3 (Akao et al., 2002b). Rasagiline showed a reverse-bell shape curve of the concentration-activity relationship and the increase of Bcl-2 was detected at 10 μM–10 nM, and also at 10–1 pM. Bcl-2 protein level increased from 6 to 24 h of the treatment. Rasagiline induced mRNA levels of anti-apoptotic *bcl-2* and *bcl-xL*, but not those of pro-apoptotic *bax* and *mcl-l*. Other MAO-A and -B inhibitors, clorgyline and pargyline, did not affect the mRNA level at the concentrations examined.

The results of structure-activity relationship of propargylamine derivatives to Bcl-2 induction are summarized in Table 1. Rasagiline and *N*-propargylamine increased Bcl-2 mRNA and protein, whereas aminoindan and *N*-methylpropargylamine did not (Maruyama et al., 2002b; Yi et al., 2006b). The structure required for Bcl-2 induction is the propragylamine group, as in the case for preventing mPT. Also among BPAP derivatives, *R*(−)-*N*-propynyl compound, FDFS-1180, induced Bcl-2, more than FDFS-11169 without proynyl group (Maruyama et al., 2004b). For Bcl-2 induction, *R*-propargylamines are more potent than the *S*-enantiomers.

Induction of neurotrophic factors by propargylamines

Neurotrophic factors, including nerve growth factor, glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor, prevent cell death in specified type neurons. GDNF is a member of the transforming growth factor-β superfamily and effectively protects dopaminergic neurons against

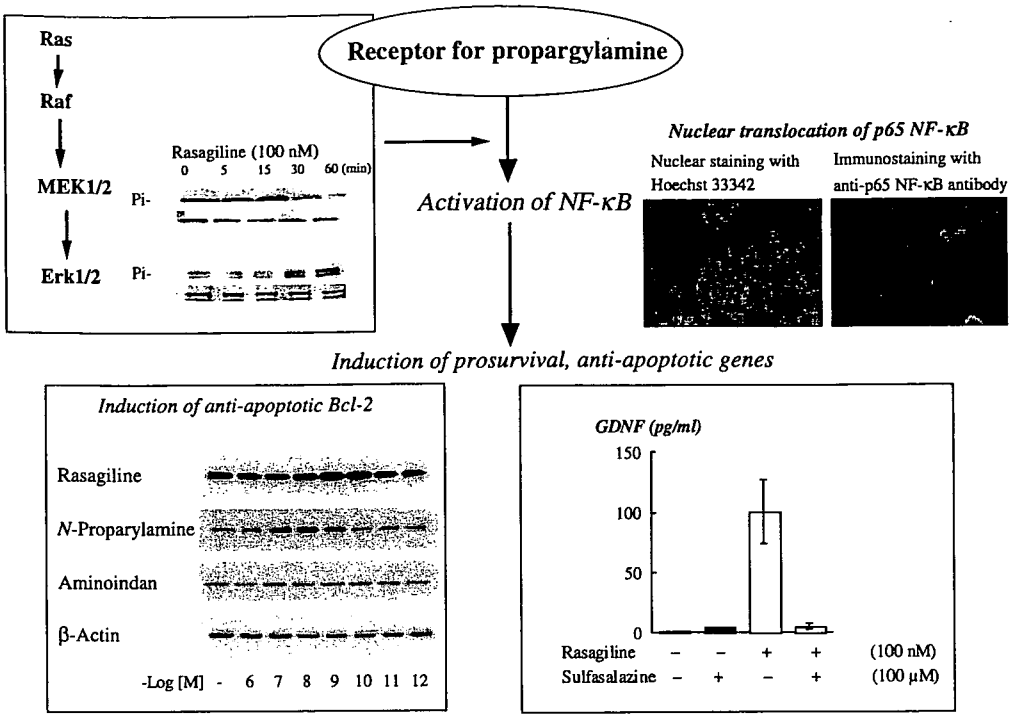


Fig. 3. Rasagiline increases anti-apoptotic Bcl-2 family and GDNF, a dopamine neuron-specific neurotrophic factor, through activation of ERK-NF-κB pathway. Anti-apoptotic propargylamines bind to the putative receptor on the membrane and activate the MEK1/2-ERK1/ERK2 pathway. The activated phosphorylated forms of ERK1/2 were detected after 30 min incubation with 100 μM rasagiline. After 3 h treatment with rasagiline, NF-κB was activated and p65 subunit was translocated into nuclei, as shown by staining using anti-p65 antibody for GAPDH and Hoechst 33342 for nuclei. The involvement of NF-κB in the induction of GDNF and Bcl-2 was also confirmed by use of an inhibitor of IκB kinase, sulfasalazine, which inhibited the increase of GDNF protein in SH-SY5Y cells treated with 100 nM rasagiline. The structure required for the Bcl-2 induction is a propargylamine structure, since aminoindan without a propargyl residue did not increase Bcl-2 levels

cell death in various animal PD models prepared with 6-hydroxydopamine and MPTP. Since GDNF and other neurotrophic factors cannot penetrate into the brain though the blood-brain barrier, several trials have been reported, delivering GDNF in the substantia nigra by direct administration, gene therapy, and cell implant (Bauer et al., 2000; Gill et al., 2003).

As shown in Fig. 3, rasagiline increases GDNF in SH-SY5Y cells. GDNF mRNA was virtually not detectable in SH-SY5Y cells, but after the treatment with 100 nM rasagiline for 3 h considerable amount of GDNF mRNA was detected. GDNF protein level in the control cells was less than 1 pg/ml and increased to be more than 100 pg/ml after rasagiline treatment. Induction of neurotrophic factors, GDNF, BDNF, NGF and neurotrophin-3 (NT-3), by propargylamines was examined in SH-SY5Y cells. Depending on the type of propargylamines, different neurotrophic factors were induced; rasagiline induced GDNF, and (−)deprenyl BDNF (Maruyama et al., in preparation). This result suggests that a specified propargylamine compound can induce a definite neurotrophic factor beneficial for selective type of neurons.

Signal transduction and gene expression by rasagiline for neuroprotection

These results on Bcl-2 and GDNF induction suggest that rasagiline may activate intracellular signals for induction of genes coding these anti-apoptotic proteins. NF-κB is the common transcription factor to induce anti-apoptotic *bcl-2*, neurotrophic GDNF and anti-oxidative SOD, all of which were increased by rasagiline (Carrillo et al., 2000; Akao et al., 2002b; Maruyama et al., 2004a). NF-κB consists of 2 subunits of 65 kDa (p65: RelA) and 50 kDa (p50) or 52 kDa (p52), and is sequestered in the cytoplasm as an inactive complex with NF-κB inhibitory subunit (IκB). Upon stimulation, IκB is phosphorylated, dissociated from the complex and degraded by the ubiquitin-proteasome system. This reaction allows translocation of free, active NF-κB complex into nuclei, where it binds to specific DNA motifs in the promoter/enhancer regions of target genes and activates transcription, as shown by the p65 binding assay. The translocation of activated p65 subunit into nuclei by rasagiline was confirmed by Western blot analysis of the subcelluar fractions and also by immunohistochemical

observation using the p65 antibody and Hoechst 33342 for nuclear staining (Fig. 3) (Maruyama et al., 2004a). The involvement of phosphorylation of inhibitory IκB subunit on the activation of NF-κB, was demonstrated by use of sulfasalazine, an inhibitor of by IκB kinase (Fig. 3). Sulfasalazine inhibited also the increase of mRNA of *bcl-2* and *bcl-xL* as in the case with GDNF, suggesting the involvement of NF-κB transcription factor in the induction of neuroprotective proteins in common.

Rasagiline and related propargylamines protect cellular and animal models of neurodegenerative disorders, including PD, AD and ischemia (Mandel et al., 2003, 2005). By screening the signal factors activated rasagiline, we found that extracellular-regulated kinase-1/2 (ERK1/ERK2) was activated as an upper signal of NF-*k*B activation (Maruyama et al., 2004a) (Fig. 3). After treatment with 100 nM rasagiline, phosphorylated ERK1/ERK2 was increased in a time-dependent way, which PD98059, an inhibitor of mitogen-activated protein (MAP) kinase/ERK kinase-1 (MEK 1/2), inhibited. CF10923x and Calphosin, inhibitors for protein kinase C (PKC), suppressed the increase of Bcl-2 and activated NF-κB by rasagiline, suggesting the involvement of the pathway through activation of PKC, Ras/Raf and MEK 1/2 in the induction of these proteins. Youdim and his group reported detailed data concerning the activation PKC system by rasagiline, which up-regulates MAP kinase/ERK cascades (Youdim et al., 2003a; Mandel et al., 2005; Weinreb et al., 2004). Recently, in mice treated with MPTP rasagiline was reported to activate signal pathway from neurotrophic factor responsive-tyrosine kinase receptor to phosphatidylinositol 3 kinase protein (Sagi et al., 2007). However, as shown later in DNA array studies, kinases may be activated not only primarily by rasagiline itself, but also secondarily by the following death-regulating processes. At present, it requires further studies to identify the initial signal to induce anti-apoptotic genes.

To screen the gene induction by rasagiline, we examine the time-dependent expression of genes by rasagiline. SH-SY5Y cells were treated with 100 nM rasagiline for 6, 12 and 24 h and mRNA was extracted and reverse-transcribed with biotylated dUTP (Roche Diagnostics) and gene-specific primer mixture reported as the manufacture's instruction (Takara Bio Co., Otsu, Japan). The relative expression level of a given mRNA was assessed by normalizing to a housekeeping gene, β-actin, and comparing to the control values obtained by the cells without treatment of rasagiline (Table 2). Rasagiline increased 108, 57 and 82 genes (>1.5 compared to control) and reduces 37, 54 and 104 genes (<0.5) after 6, 12 and 24 h treatment, respectively. Rasagiline affected genes with different cellular function in a time-dependent way. After 6 h treatment, mRNA of *bcl-2*, and genes related to NF-κB related transcription factors, cytokines and the receptors [interleukin (IL) receptors], mitochondrial ATP synthesis (cytochrome c oxidase, NADH-coenzyme Q reductase, ATP synthase, aconitase) and the ubiquitin-proteasome system were increased. In addition, genes of mPT pore components (ANT, VDAC and MAO-A) were also increased. On the other hand, genes coding growth factor (BDNF, transforming growth factor), cytokines and receptors [tumor necrosis factors (TNF), IL, fibroblast growth factor] were reduced. At 12 h of the treatment, most marked increase was observed in MAP-KK and cytokine receptors. In addition, rasagiline increased mRNA for ANT, VDAC and mitochondrial proteins (complex I–IV, mitochondrial transcription factor A). On the other hand, kinases associated with death signal (MAP kinase activating death domain, MAPKKK 4, TNF receptor associated factor 5, death-associated protein kinase-1), growth factors (NGF), and cytokines decreased. It is interesting that mRNA of *bcl-2*, MAO-B and also transcription factors were reduced significantly at this point. Rasagiline treatment for 24 h enhanced significantly the genes for *bcl-2*, apoptosis inhibitors (apoptosis inhibitors 1, 2 and 4, neuronal apoptosis inhibitory protein) and cell signals, including kinases (MAPK, MAPKK, cyclin-dependent kinase), cytokines and the receptors, and the transcription factors. It may be hypothesized that rasagiline sequentially increases ATP-dependent activation of kinases and transcription factors, the ubiquitine-proteasome system, which degrades the cleaved phosphorylated inhibitors of kinases and transcription factor, increases cytokines and the receptors, and finally induces pro-survival genes.

Table 2. *Gene induction in SH-SY5Y cells by rasagiline*

Rasagiline (100 nM) treatment for		
6 h	12 h	24 h
Increased genes	Increased genes	Increased genes
ATP-synthesis-related mitochondrial mPT pore related	Kinases	Bcl-2
Cytokine receptors	Cytokine and IL receptors	Apoptosis inhibitors
NF-κB related transcription factors	Mitochondrial complex I–IV	TNF and receptors
Ubiquitin-proteasome system	mPT pore related	Growth factors
Reduced genes	Reduced genes	
IL and TNF	Bcl-2	
Cytokin-related transcription factors	Kinases	
Growth factors	IL and TNF	
	Transcription factors	
	Growth factors	