Results

Inhibition of MAO-A by N-propargylamine

Inhibition of MAO-A activity in mitochondria by *N*-propargylamine is shown in Fig. 1. *N*-Propargylamine reduced MAO activity significantly until at 10 μM, whereas *N*-methylpropargylamine inhibited the activity only at 1 mM, and propiolaldehyde did not even at 1 mM. Kinetic analyses indicate that *N*-propargylamine inhibited MAO-A in competition to the substrate (Fig. 2). The apparent K_i value of *N*-propargylamine was estimated to be 28.0 μM, whereas the values of the apparent K_m and maximal velocity, V_{max}, were 45.5 μM and 2.87 nmol/min/mg protein, respectively. The dialysis experiments showed that the inhibition of MAO by *N*-propargylamine was irreversible (Fig. 3), which is similar to those reported often for (−)deprenyl, rasagiline and other propargylamine derivatives. The activity of MAO-A treated with or without *N*-propargylamine was reduced further by the dialysis procedure, which may be due to the marked un-stability of MAO-A activity.

Anti-apoptotic function of N-propargylamine

Treatment of SH-SY5Y cells with 250 μM *N*M(*R*)Sal resulted in apoptosis of 37.6 ± 3.9% of cells, whereas necrotic cells were virtually not detected. Apoptotic cells were almost negligible in the control or *N*-propargylamine alone-treated cells. Figure 4 shows typical FACS profiles of the cells treated with *N*M(*R*)Sal with or without 1 μM–1 nM *N*-propargylamine. *N*-Propargylamine reduced the number of apoptotic cells to 63–70% of that of *N*M(*R*)Sal-treated cells. The potency of anti-apoptotic function was compared with that of *N*-methylpropargylamine, propiolaldehyde, and rasagiline in a similar manner. Rasagiline was found to be most potent to prevent apoptosis followed by *N*-propargylamine (Fig. 5), but *N*-methylpropargylamine and propiolaldehyde were virtually not effective.

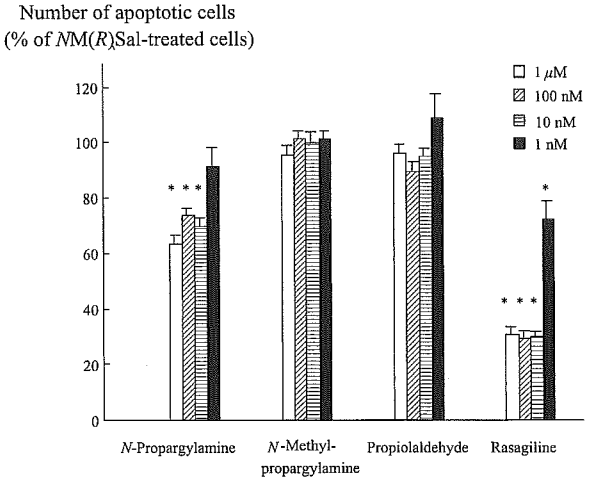

Fig. 5. The anti-apoptotic potency of *N*-propargylamine analogues and rasagiline. SH-SY5Y cells were treated with 1 μM, 100–1 nM propargylamine derivatives, and then with 250 μM *N*M(*R*)Sal overnight. The number of apoptotic cells was determined by FACS as sub G1 peak (Fig. 4) using PI as an indicator. The number of apoptotic cells was expressed as % of apoptotic cells after treated with 250 μM *N*M(*R*)Sal alone. The column and bar represent the mean and SD of three independent experiments measured in triplicate. *$p < 0.01$ from cells treated with *N*M(*R*)Sal alone

Fig. 4. The effects of *N*-propargylamine against apoptosis induced by *N*M(*R*)Sal. SH-SY5Y cells were treated with 1 μM–10 nM *N*-propargylamine at 37°C for 30 min, then with 250 μM *N*M(*R*)Sal for 24 h. Apoptotic cells were quantitatively determined by FACS, after staining the cells with PI. Three-color flow cytometry was used to analyze the frequency of PI positive cells. **A** The gate to differentiate singlet from doublet cells was determined by FL-2 (PI)-A (Area) and FL-2 (PI)-W (Width) characteristics of PI-stained cells. **B** The frequency of PI-positive cells. The cells in subG1 peak were assessed to be apoptotic, and the number of apoptotic cells was represented as percent of the total, as shown in **B**. #1; control SH-SY5Y cells. #2; cells treated with 250 μM *N*M(*R*)Sal. #3, 4, and 5; cells pretreated with 1 μM, 100 nM, 10 nM *N*-propargylamine, then 250 μM *N*M(*R*)Sal. #6; cells treated with 1 μM *N*-propargylamine

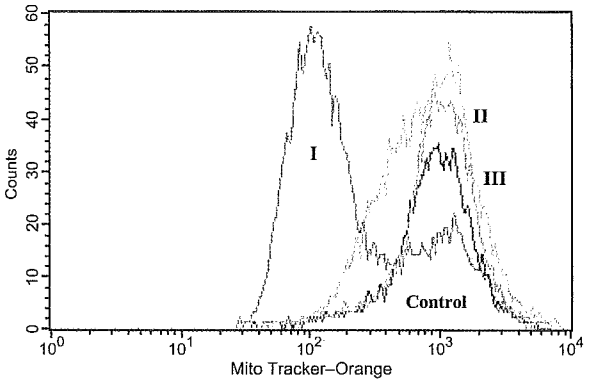

Fig. 6. The effects of *N*-propargylamine on ΔΨm decline induced by *N*M(*R*)Sal in mitochondria prepared from SH-SY5Y cells. Mitochondria were treated for 20 min without (I) or with 1 μM *N*-propargylamine (II), and then with 250 μM *N*M(*R*)Sal for 3 h at 37°C. III: Cells treated with 1 μM *N*-propargylamine alone. Mitochondria were gated by staining with MitoTracker Green, and the ΔΨm was quantitatively measured using MitoTracker Orange fluorescence

Stabilization of ΔΨm by N-propargylamine

*N*M(*R*)Sal at 250 μM induced ΔΨm decline in mitochondria isolated from SH-SY5Y cells, as shown by FACS MitoTracker Orange fluorescence representing ΔΨm (Fig. 6). Pre-

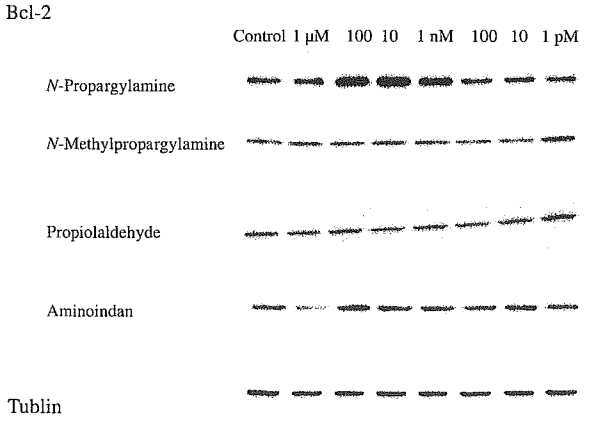

Fig. 7. The effect of *N*-propargylamine analogues and aminoindan on protein levels of Bcl-2 in SH-SY5Y cells. The cells were cultured in the presence of 1 μM-1 pM *N*-propargylamine analogues or aminoindan for 24 h, and Bcl-2 protein was quantified by Western blot analysis. Tublin in the cells was used as control

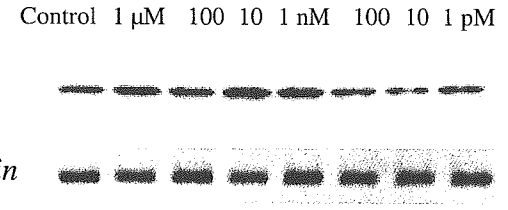

Fig. 8. The effect of *N*-propargylamine on mRNA levels of *bcl*-2 in SH-SY5Y cells. The cells were cultured in the presence of 1 μM-1 pM *N*-propargylamine for 24 h, and mRNA levels were measured by RT-PCR method, as written in Materials and methods. β-*Actin* mRNA was used as control

treatment of the cells with 1 μM *N*-propargylamine markedly prevented the decline in ΔΨm, and the protective effect was confirmed at until 10 nM of *N*-propargylamine. On the other hand, *N*-methylpropargylamine and propiolaldehyde did not prevent the decline in ΔΨm (data, not shown).

Induction of anti-apoptotic Bcl-2 by N-propargylamine

Bcl-2 is known to prevent apoptosis and promote survival, through regulating mitochondrial permeability transition. Bcl-2 protein levels in SH-SY5Y cells treated with *N*-propargylamine were estimated by Western blot analysis and increased levels of Bcl-2 protein were observed at the concentrations of *N*-propargylamine employed (Fig. 7). On the other hand, neither *N*-methylpropargylamine nor propiolaldehyde affected Bcl-2 protein levels. Aminoindan, a hydrophobic part of rasagiline, did not increase Bcl-2. The effects of *N*-propargylamine on the mRNA level of *bcl-2* were examined by RT-PCR method. As shown in Fig. 8, *N*-propargylamine significantly increased mRNA level of *bcl-2* at 1 μM–1 nM.

Discussion

N-Propargylamine, *N*-methylpropargylamine and propiolaldehyde are metabolites of a relatively non-selective MAO inhibitor, pargyline [*N*-methyl-*N*-propargylbenzylamine]

(Shirota et al., 1979; DeMaster et al., 1981), even though such metabolites have never been reported for rasagiline at present. The results in this paper clearly demonstrate neuroprotective activity of *N*-propargylamine, whereas other two metabolites did not prevent apoptosis. The results may be relevant with the fact that the propargylamine moiety plays a key role in anti-apoptotic function of rasagiline and other propargylamine derivatives (Maruyama et al., 2003; Yogev-Falach et al., 2003). Rasagiline, the (*R*)-enantiomer of *N*-propargyl-1-aminoindan, has higher anti-apoptotic activity than its (*S*)-enantiomer, TV1022 (Maruyama et al., 2001c), and only the (*R*)-enantiomer of *N*-2HMP shows anti-apoptosis activity (Maruyama et al., 2001a). These results suggest that the stereo-chemical structure of the propargylamine moiety plays a decisive role in the neuroprotective function of complex propargylamines. The dependence of anti-apoptotic activity on the stereo-chemical configuration of propargylamine residue may explain the relative weak anti-apoptotic potency of free *N*-propargylamine as reported in this paper.

In addition, our previous results suggest that there may be a binding site in the outer membrane of mitochondria, which distinguishes the enantiomeric structure of propargylamines and activates the anti-apoptotic and pro-survival cascade (Maruyama et al., 2001b). The binding protein in mitochondria remains to be elucidated, but MAO may be one of the candidates, since rasagline (Youdim et al., 2001a) and (–)deprenyl (Magyar et al., 1998) are potent irreversible inhibitors of MAO-B, as a consequence of the formation of *N*(5)-flavocyanine adduct with the FAD moiety (Nagy and Salach, 1981). *N*-Propargylamine itself irreversibly inhibits MAO-A activity in a competitive way to substrate, as reported in this paper. However, it remains to clarify whether it binds with the FAD moiety covalently, as in the case with phenolic or indane propargylamine derivatives. The parallelism between the MAO-A inhibition and anti-apoptotic function was confirmed with *N*-propargylamine and related compounds, suggesting that MAO-A may be involved in the neuroprotective function of propargylamines. Indeed, our previous work has proved the anti-apoptotic function of rasagiline in SH-SY5Y cells, where only MAO-A is expressed. These results suggest that MAO-A may be involved in neuroprotection and MAO-B inhibition is not required for the anti-apoptotic function of propargylamine derivatives, as described in Introduction. However, the possibility that propargylamines bind MAO at the site other than the substrate-binding site cannot be excluded. In addition, it remains to be clarified how *N*-propargylamine and rasagiline interact PT pore components, such as voltage-dependent anion channel, adenine nucleotide translocator or peripheral benzodiazepine receptors, and stabilize $\Delta\Psi m$.

Rasagiline increases the expression of anti-apoptotic genes, including *bcl*-2 and *bcl-xL*, but not Bax and Bad (Akao et al., 2002a, b), and GDNF (Maruyama et al., 2004). The induction of pro-survival genes by rasagiline is mediated by nuclear NF-κB transcription factor and extracellular signal-regulated protein kinase (ERK) cascade (Maruyama et al., 2004). At the same time, the involvement of mitogen-activated protein kinase (MAPK)- and protein kinase C (PKC)-kinase (Yogev-Falach et al., 2002, 2003; Bar-Am et al., 2004; Weinreb et al., 2004) was also suggested by the observation that a PKC inhibitor, GF109203X and an ERK inhibitor, PD98059, prevent the neuroprotective activity of *N*-propargylamine and rasagiline (Bar Am et al., 2004; Weinreb et al., 2004). At present, the intracellular mechanisms how *N*-propargylamine and complex propargylamines activate the intracellular signaling and the transcription factors remains to be enigmatic.

These results in this paper point out that free *N*-propargylamine itself shows the anti-apoptotic activity, in a similar way as

rasagiline and other propargylamines (see Youdim 2003, for a review). If *N*-propargylamine is identified as a metabolite of rasagiline in humans, it may be involved, at least partially, in neuroprotective function of rasagiline, as shown by recent clinical trial (Parkinson Study Group, 2004). Further studies on the metabolites of rasagiline and other propargylamines in humans will clarify the pharmacodynamics of these neuroprotective and anti-apoptotic agents in PD and other neurodegenerative disorders.

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NEUROPHARMACOLOGICAL, NEUROPROTECTIVE AND AMYLOID PRECURSOR PROCESSING PROPERTIES OF SELECTIVE MAO-B INHIBITOR ANTIPARKINSONIAN DRUG, RASAGILINE

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CONTENTS

Summary

Rasagiline (*N*-propargyl-1*R*-aminoindan) is a novel, highly potent, irreversible monoamine oxidase (MAO)-B inhibitor designed for use as an antiparkinsonian drug. Unlike selegiline, rasagiline is not derived from amphetamine or metabolized to neurotoxic l-methamphetamine derivative, and it does not have sympathomimetic activity. Moreover, at selective MAO-B inhibitory dosage, it does not induce a "cheese reaction." Rasagiline is effective as monotherapy or as an adjunct to L-dopa for patients with early and late Parkinson's disease. Adverse events do not occur with greater frequency in subjects receiving rasagiline than in those on placebo. Its *S*-isomer, TVP1022, is more than a thousand times less potent as an MAO inhibitor. However, both drugs have neuroprotective activities in neuronal cell cultures in response to various neurotoxins, as well as *in vivo* (*e.g.*, in response to

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global ischemia, neurotrauma, head injury, anoxia, etc.), indicating that MAO inhibition is not a prerequisite for neuroprotection. The neuroprotective activity of these drugs has been demonstrated to be associated with the propargylamine moiety, which protects mitochondrial viability and mitochondrial permeability transition pore by activating Bcl-2 and downregulating the Bax family of proteins. Rasagiline processes amyloid precursor protein (APP) into the neuroprotective-neurotrophic soluble APPalpha (sAPPα) by protein kinase C- and mitogen-activated protein kinase-dependent activation of α-secretase, and increases nerve growth factor, glial cell–derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) expression and proteins. Thus, rasagiline may induce neuroprotection, neuroplasticity and long-term potentiation. Rasagiline has therefore been chosen by the National Institutes of Health (NIH) to study its neuroprotective effects in neurodegenerative diseases. Long-term studies are required to evaluate the drug's disease-modifying prospects in Parkinson's and Alzheimer's diseases.

Introduction

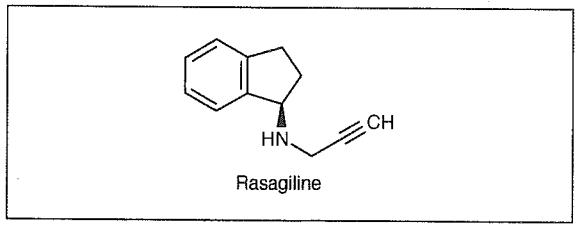
The knowledge that dopamine is oxidatively deaminated equally well by monoamine oxidase (MAO) types A and B (1), the dominance of MAO-B (80%) as compared to MAO-A in the extrapyramidal regions of human brain (1–3) and the absence of the "cheese reaction" in whole animal and isolated tissue preparations by the selective irreversible MAO-B inhibitor selegiline (deprenyl) (4) led us to introduction of this irreversible inhibitor as an adjunct to levodopa therapy for Parkinson's disease (5–7). Selegiline has been a useful antiparkinsonian drug, both in monotherapy (8) and as an adjunct to levodopa therapy. Additionally, it has levodopa-sparing action (6, 9, 10). Selegiline is a propargyl derivative of l-methamphetamine. It irreversibly inhibits MAO-B by binding mole per mole covalently to the N5 position of the isoalloxazine moiety of FAD, the cofactor of MAO-B (11, 12). *In vivo*, selegiline is metabolized to its major metabolites l-amphetamine and l-methamphetamine (13). In behavioral (14), pharmacological preparations and *in vivo*, it possesses amphetamine-like and sympathomimetic actions (15, 16), which result in its ability to increase blood pressure and heart rate. Its major metabolite, l-methamphetamine, has neurotoxic action. However, as a consequence of not having similar structural drugs, it has not been possible to evaluate selegiline's antiparkinsonian activity or its mechanism of action.

Rasagiline has been identified and developed as a highly potent irreversible selective inhibitor of MAO-B, and has the chemical formula *N*-propargyl-1*R*(+)-aminoindan (TVP-1012) (Fig. 1) (17–22). Rasagiline has been developed as an antiparkinsonian drug (17, 23–27). It is a propargylamine derivative of aminoindan, and unlike selegiline, it is not metabolized to amphetamine but to aminoindan, which has no amphetamine-like sympathomimetic properties (Fig. 1). Similarly to selegiline at its selective MAO-B inhibitory dosage, it does not produce the "cheese reaction" in isolated tissue preparations or *in vivo* in rats and cats (16, 28).

Three controlled phase III studies have demonstrated that rasagiline is effective as monotherapy in early Parkinson's disease patients and as an adjunct to L-dopa in moderate to advanced patients (26, 27, 29, 30, 31).

Monoamine oxidase inhibitory activity of rasagiline

Rasagiline's MAO-A and -B inhibitory activities have been compared to those of selegiline (l-deprenyl) *in vitro*, as well as acutely and chronically *in vivo* in various tissues of mice, rats, cats and monkeys (Fig. 2). The tissues that were studied for MAO inhibition included brain, liver and small intestine. Rasagiline is a highly potent, selective, irreversible inhibitor of MAO *in vitro*, with similar potency to that of selegiline (Fig. 2). The *in vitro* IC_{50} values for inhibition of rat brain MAO activity by rasagiline are 4.43 ± 0.92 nM (type B) and 412 ± 1.23 nM (type A). The ED_{50} values for *ex vivo* inhibition of MAO-B in the brain and liver by a single dose of rasagiline are 0.1 ± 0.01 and 0.042 ± 0.0045 mg/kg, respectively. These same values for MAO-A inhibition are 8.35 ± 2.2 and 2.42 ± 0.39 mg/kg, respectively. *In vivo* rasagiline is between 10–20 times more potent than selegiline, and it maintains its selective MAO-B inhibition in the liver and brain when administered as chronic (21-day)



Rasagiline

Fig. 1. The chemical structure of rasagiline.

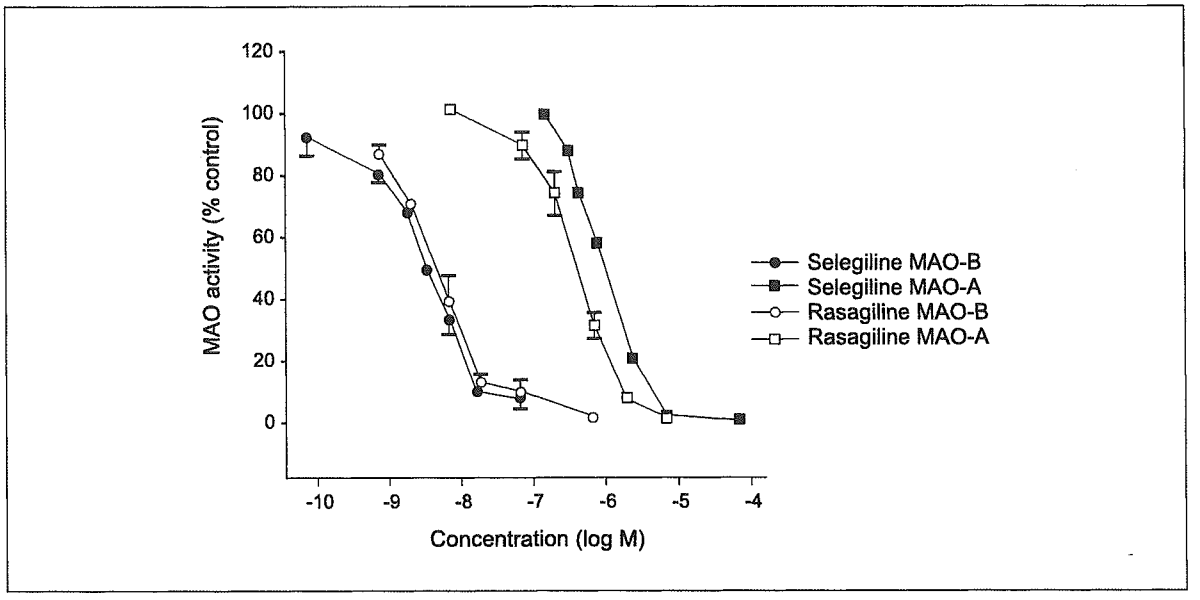


Fig. 2. Comparative *in vitro* MAO inhibitory activities of rasagiline and selegiline in rat brain mitochondrial preparations. Similar but lower effects were seen with liver MAO inhibition (ref. 22).

oral dosage, with ED_{50} values of 0.014 ± 0.002 and 0.013 ± 0.001 mg/kg, respectively, for these tissues. The degree of selectivity of rasagiline for inhibition of MAO-B as opposed to MAO-A *in vitro* is similar to that of selegiline, with rasagiline being significantly more potent *in vivo*. In fact, rasagiline produces near complete inhibition of MAO-A and -B at doses of 10 and 0.4 mg/kg, respectively (22). This may explain its greater potency for inhibition of human platelet MAO-B. In addition, a rasagiline dose as low as 0.5–2 mg/day has shown efficacy in parkinsonian subjects (27).

Mechanism of monoamine oxidase B inhibition by rasagiline

The inhibitory potency of rasagiline has been examined in rat brain, liver and intestine after acute and chronic oral treatment (22) and compared to that of selegiline (4). The results demonstrated a high degree of stereo selectivity for MAO inhibition by rasagiline. In contrast, selegiline shows only a small degree of stereo selectivity. The full explanation for this markedly different degree of stereo selectivity between closely allied chemical compounds has become apparent with three-dimensional modeling of the MAO active site.

Rasagiline, similar to selegiline, is a propargyl-containing drug. However, the propargyl component in rasagiline, unlike that of selegiline, is bound to an aminoindan moiety. The propargyl-containing MAO inhibitors such as clorgyline, pargyline and selegiline are known as suicide inhibitors since they are substrates of the enzyme (11, 32, 33). They inactivate MAO-A and -B selectively and irreversibly by time- and concentration-dependent mechanisms by interacting covalently with the cysteinyl-FAD cofactor of the enzymes at their active centers (34, 35). This interaction takes place between the propargyl group and the N5 of the FAD isoalloxazine component of liver (11) and brain MAO (36). It has been shown for these inhibitors that one mole of the inhibitor inactivates one mole of the enzyme (32), and since rasagiline has the same enzyme-inactivating moiety as those of the aforementioned propargylamines, its mechanism of MAO inactivation is considered to be identical to that of these inhibitors. The 1:1 interaction of propargylamine MAO inhibitors with MAO-A and -B has been used to determine the molecular turnover of these enzymes in the estrus cycle and their response to sex steroids (37), as well as their regulation with steroids (38, 39). The estrus cycle has a profound effect on MAO-A activity, with progesterone inducing and estradiol reducing MAO-A enzyme activity. Studies with endothelial and chromaffin cell cultures, which contain, respectively, MAO-A and -B only, have shown that these steroids affect only synthesis of active MAO-A and its turnover, and not those of

MAO-B (39). No adequate explanation has been put forward as to why steroids do not alter MAO-B expression, what regulates this enzyme, what its function is in the brain and whether this enzyme has other endogenous substrates, such as *N*-acetyl derivatives of diamine oxidase substrates (*e.g.*, spemine spemidine, puetrecine) (40), besides dopamine and phenylethylamine.

A comparison of turnover of rat striatal MAO-B inhibition by rasagiline and selegiline shows remarkable similarities between the two compounds (41). Striatal MAO-B has a significantly slower turnover as compared to MAO-A. It is apparent that rasagiline is a very potent selective MAO-B inhibitor and has a good uptake across the blood-brain barrier, as shown by the similarity of inhibition curves between liver and brain (42). Although when compared *in vitro*, rasagiline had similar potency to selegiline for inhibition of MAO-B, the *in vivo* study showed a greater potency for rasagiline. This greater potency of rasagiline is even more marked if, instead of 50% enzyme inhibition, the dose required for 80% inhibition is measured. The reason for this is not currently known, but it may be due to different rates of metabolism of the parent compounds *in vivo* or to improved tissue penetration of rasagiline. Studies in humans show an approximately 5- to 10-fold greater potency for rasagiline over selegiline for inhibition of platelet MAO-B (unpublished data). Although rasagiline has a greater potency than selegiline, its selectivity for MAO-A and -B inhibition is very similar to what has been reported for selegiline.

The results from studies on rat tissues complement findings in nonhuman primate (monkey) brains (43), where rasagiline was given chronically for 7 days at various doses and MAO-A and -B activities were measured in several brain regions, including caudate nucleus, globus pallidus, cerebral cortex and hippocampus. Rasagiline was shown to be a potent selective inhibitor of MAO-B in the caudate nucleus and globus pallidus, where the activity of MAO-B, similarly to in the human brain, is 4-fold higher than that of MAO-A (43). In human volunteers and parkinsonian subjects, a 0.5, 1.0 or 2.0 mg daily dosage given chronically is sufficient to completely inhibit platelet MAO, which is solely type B. This potency will most likely be reflected in the human brain, since Riederer and Youdim (44) showed that a 10 mg daily dose of selegiline given to parkinsonian subjects fully inhibited platelet MAO-B with a similar inhibitory potency in caudate nucleus, substantia nigra, globus palllidus and thalamus, in contrast to a limited MAO-A inhibition in these brain regions, as determined at autopsy from subjects on chronic selegiline. Unlike the studies in rat brain, where MAO-A or -B inhibitors do not affect dopamine levels (45), in human brain there was a significant, but moderate, increase (45%) in dopamine levels (enriched by MAO-B), as well as a highly significant increase (3,000%) in the MAO-B substrate phenylethylamine in these regions (46). In contrast, the limited MAO-A inhibition was observed in these regions, and there were no appreciable effects on serotonin or noradrenaline and their metabolites in these regions. Thus, selective inhibition of brain MAO-B can be maintained with the right dosages of selegiline, and it would not be expected to be different for rasagiline, as discussed above regarding studies on rat brain *in vivo* (22).

MAO-A and -B activity recovery after *in vivo* inhibition by rasagiline, which is related to the synthesis of enzyme apoprotein, differs significantly between tissues such as liver, small intestine and brain. The small intestine MAO-B activity has the fastest recovery, while the brain MAO-B activity shows the slowest recovery. In comparison, MAO-A activity recovery in rat brain is significantly faster and the half-life is 3–5 days. These differences in rat brain enzyme activity recovery after rasagiline treatment are not unusual: similar findings have been reported for enzyme recovery after inhibition by selegiline and clorgyline (47, 48). Indeed, a recent comparative study of rasagiline and selegiline showed almost identical results, with a half-life in the striatum of 7–9 days. However, the half-life for recovery of MAO-B after selegiline treatment has been reported to be well over 30 days (38) in primate (monkey and human) brains and 13 days in the rat brain (47, 48).

Neuropharmacology of rasagiline

The effects of rasagiline on CNS monoamine levels, behavioral response to fluoxetine, reserpine reversal and cardiovascular responses to tyramine have been studied in a range of doses in rats (49). Rasagiline reverses reserpine-induced ptosis at doses above 2 mg/kg, which are nonselective for MAO-B, but not at MAO-B-selective doses. Combination of rasagiline, at doses up to 20 mg/kg, which is well above its selectivity for MAO-B, with fluoxetine (10 mg/kg) does not induce the behavioral serotonergic syndrome seen following nonselec-

tive MAO-A + -B inhibition by traylcypromine, and unlike the latter inhibitor, it does not induce behavioral serotonergic nor dopaminergic syndrome in response to l-tryptophan or L-dopa, respectively (49). Similarly to selegiline (14, 45), when administered orally it does not alter hippocampal or striatal levels of noradrenaline, 5-hydroxytryptamine (5-HT) or dopamine after single or chronic doses of rasagiline up to 2 mg/kg. At doses higher than this, it causes an increase in hippocampal 5-HT and a reduction in 5-hydroxyindole acetic acid (5-HIAA). Striatal levels of dihydroxyphenylacetic acid (DOPAC) are reduced at doses above 1 mg/kg, suggesting inhibition of both MAO-A and -B. However, with chronic oral administration of doses up to 1 mg/kg daily for 21 days, levels of noradrenaline, 5-HT and dopamine in hippocampus and striatum, respectively, are unaffected, but 5-HT increases at 2 mg/kg and striatal dopamine content increases at 2 and 5 mg/kg. It is most likely that at these doses MAO-A is also inhibited, leading to the increase in these neurotransmitters, which are metabolized by MAO-A (14, 45). Rasagiline, similarly to selegiline and reversible MAO-B inhibitors (*e.g.*, lazabemide and milacemide), does not potentiate pressor responses to oral tyramine when administered as single oral doses of up to 5 mg/kg. However, doses of 10 mg/kg do significantly potentiate this response. At this dose, both MAO-A and -B are inhibited, and it is the irreversible inhibition of MAO-A that induces the cheese reaction (16, 50). Similarly, pressor responses to tyramine (5, 10 and 20 mg/kg) are not significantly potentiated following 21-day chronic treatment with rasagiline up to 2 mg/kg daily. Thus, rasagiline does not modify CNS monoamine tissue levels or pressor responsiveness to tyramine at doses selective for inhibition of MAO-B. The greater potency of rasagiline in comparison to selegiline is also reflected in humans, since a 1–2 mg daily dose of rasagiline inhibits platelet MAO-B. Subjects with early Parkinson's disease respond to rasagiline monotherapy at doses as low as 0.5–2 mg daily, and those with moderate to advanced disease respond to rasagiline as an adjunct to L-dopa therapy (26, 27, 51). This value is significantly lower than the 10 mg daily dosage employed with selegiline. These results, together with the lack of tyramine sympathomimetic potentiation by rasagiline at selective MAO-B inhibitory dosage, indicate that this inhibitor may be a more efficient drug than selegiline in the treatment of Parkinson's disease as either symptomatic or levodopa adjunct therapy. Furthermore, its lack of amphetamine-like metabolites could confer a therapeutic advantage to rasagiline.

***In vivo* dopamine release and turnover**

Acute inhibition of MAO-B with selegiline or rasagiline in rats does not alter striatal dopamine metabolism (45), but both drugs chronically increase *in vitro* and *in vivo* release of dopamine (52). The incremental release of striatal dopamine induced by depolarizing concentrations of K^+ is significantly greater with chronic clorgyline (a MAO-A inhibitor), selegiline and rasagiline treatments, where MAO-A and -B are selectivity inhibited (53). Single doses of selegiline and rasagiline have no significant effects on striatal dopamine efflux (54). In these studies, only clorgyline reduced dopamine metabolism, whereas dopamine release was enhanced by both chronic MAO-A and -B inhibitor administration. The possibility that an inhibition of high-affinity dopamine transporter by selegiline and rasagiline may be the resultant effect has also been investigated (55). Striatal levels of dopamine transporter are elevated with acute and chronic selegiline administration, but not with rasagiline, clorgyline, nomifensine or amphetamine. Thus, an increase rather than a decrease in dopamine transporter expression appears to be a special property of selegiline and not of other inhibitors. Selegiline is a derivative of l-amphetamine and its major metabolite is l-methamphetamine. It has been suggested that dopamine release may result from this metabolite. However, since rasagiline is not metabolized to amphetamine and has a similar action on striatal dopamine release, this effect is not dependent on the production of amphetamine. The most likely explanation for this effect is the metabolic inhibition of phenylethylamine, a selective substrate of MAO-B, which is highly elevated in response to selegiline in rat brain (56) and parkinsonian brain obtained at autopsy (46). In contrast, neither dopamine, serotonin or noradrenaline metabolism are altered by rasagiline or selegiline in rat brains (14, 45, 57, 58). Although selegiline has been shown to cause a modest but significant increase in parkinsonian striatal dopamine (44), it also profoundly affects phenylethylamine metabolism, with a more than 3,000% increase in parkinsonian striatum levels obtained at autopsy in patients on l-deprenyl therapy for between 4 weeks and 4 years (46). As a specific substrate of MAO-B (56, 59), phenylethy-

lamine is increased in rat brains treated with selegiline. Phenylethylamine is a highly potent releaser of dopamine, as well as an inhibitor of neuronal uptake and a competitive inhibitor of MAO-B. Indeed, at their selective *in vivo* MAO-B inhibitory doses, both selegiline and rasagiline potentiate the behavioral response to exogenous treatment with phenylethylamine (41). This response is only observed when MAO-B is inhibited by more than 80% (60, 61). These results may have significant implications for understanding the therapeutic action of rasagiline when administered as monotherapy in early and late Parkinson's disease, or when used as an adjuvant to L-dopa. The continuous release of dopamine after chronic rasagiline and selegiline in rat brain (52), together with the long-term selective inhibition of MAO-B in extrapyramidal regions (*i.e.*, caudate nucleus, substantia nigra, globus pallidus and thalamus) (44), may explain why the initial clinical response to rasagiline monotherapy in parkinsonian subjects continues even 8 weeks after the termination of the drug (29), whose half-life is considered to be greater than 30 days (38). There could also be another explanation, such as adaptive postsynaptic dopamine receptor sensitization, but this has not so far been shown for either selegiline or rasagiline.

Neuroprotective activity of rasagiline

In cell culture and in vivo

Rasagiline possess neuroprotective activity against cell death, as demonstrated *in vitro* (in neuronal cell cultures) and in several *in vivo* preparations in response to neurotoxins and neurotoxic events and serum and nerve growth factor withdrawal in neuronal cell culture (Tables I and II). Rasagiline is among a handful of agents chosen for further study as potential neuroprotective compounds in clinical neuroprotection trials in Parkinson's disease (68). Clinically as an antiparkinsonian drug (26, 27) and experimentally as a MAO-B inhibitor (22, 49), rasagiline is 10–20 times more potent than other agents studied. Similarly, selegiline and akyl-propargylamines exhibit neuroprotective activities in several neuronal cell culture models, as well as *in vivo* in response to a number of neurotoxins (69–75). The property is not related to the MAO-B–inhibiting activity of either rasagiline or selegiline (42, 76–79), since the *S*-optical isomer of rasagiline, TVP1022, which has poor MAO-B inhibitory activity, exhibits similar neuroprotective activity (70, 71, 74, 75, 80). Clinical trials with selegiline (81) failed to establish its neuroprotective activity because its symptomatic effect could not be distinguished from a possible neuroprotective action. One reason why neuroprotection was not observed with selegiline in clinical trials (81) may be a consequence of l-methamphetamine generation, which could interfere with selegiline's neuroprotective activity (77, 79, 82–84). Although rasagiline

Table I: In vivo *neuroprotective activity of rasagiline in models of neurodegenerative diseases.*

- MPTP model of Parkinson's disease (62)
- Globa ischemia (63, 64)
- Neurotruma model of head injury (65)
- Amyotrophic lateral sclerosis (66)
- Eae model of multiple sclerosis?
- Heart ischemia?
- 6-Hydroxydopamine? (67)

Table II. In vitro *neuroprotective activity of rasagiline in neuronal cell cultures.*

	Cell type	
Neurotoxic agents	PC-12	SHSY-5Y neuroblastoma
Glutamate	+	+
SIN-1	ND	+
N-methyl-(*R*)-salsolinol	ND	+
6-Hydroxydopamine	ND	+
Serum and nerve growth factor depravation	+	ND
Glucose and oxygen depravation	+	ND
Okdanoic acid+	+	
Aβ-amyloid aggregate	+	+
α-Synuclein-Fe aggregate	+	+
TNF-α		

(70) and selegiline have similar structures, selegiline's major metabolites l-amphetamine and l-methamphetamine are thought to be neurotoxic (82–84). However, rasagiline's metabolite is aminoindan, which has been reported to have neuroprotective activity in its own right (85, 86). Indeed, Abu-Raya *et al.* (83) and Bar Am *et al.* (84) have demonstrated that l-methamphetamine interferes with the neuroprotective action of selegiline and rasagiline in oxygen-glucose deprivation-induced cell death in nerve growth factor-differentiated PC-12 cells. These findings were confirmed with PC-12 cell cultures in the absence of serum and nerve growth factor. Furthermore, in these studies aminoindan, the major metabolite of rasagiline, prevented the neurotoxicity of l-methamphetamine, the major metabolite of selegiline (84), supporting the results from *in vivo* neuroprotective studies with aminoindan (85, 86) (Fig. 3).

Similarly to selegiline, rasagiline protects against serum and nerve-growth-factor withdrawal-induced apoptotic death in partially differentiated PC-12 cells (and SHSY-5Y neuroblastoma cells, data not shown). Moreover, their activity appears to be superior to that of selegiline (Fig. 3). The results of these studies are very similar to the neuroprotective actions observed for rasagiline and selegiline in the prevention of PC-12 cell death during oxygen-glucose deprivation. Serum and nerve growth factor withdrawal in partially neuronally differentiated PC-12 cells results in decreased mRNA and protein of Bcl-2 and SOD, while those of Bax increase (22, 87). Rasagiline can substitute for serum and nerve growth factor to restore these proteins, an action that can be prevented with transcriptional and translational inhibitors cycloheximide and actinomysin D (Fig. 3). These findings suggest that rasagiline induces the gene-dependent synthesis and degradation of mitochondrial cell survival proteins. Furthermore, the neuroprotective activity of rasagiline, both *in vitro* in PC-12 and SHSY-5Y cells and *in vivo*, is associated with its induction of antiapoptotic SOD and catalase (88). The *in vivo* induction of Mn-zinc SOD and catalase by rasagiline was mainly associated with substantia nigra and kidney, two dopamine-rich tissues, without a substantial effect in the hippocampus (88). The induction of these enzymes was concentration and time dependent. The potent MAO-B inhibitory activity of rasagiline, which resides in the interaction of its propargylamine moiety with FAD co-factor of the enzyme (22), is unrelated to its neuroprotective activity (42, 70, 71, 74, 75). Furthermore, PC-12 cells and human SH-SY5Y neuroblastoma cells contain only MAO-A (89, 90), and the concentration of rasagiline employed in neuroprotective studies would not be sufficient to inhibit this enzyme. It is apparent that the neuroprotective ac-

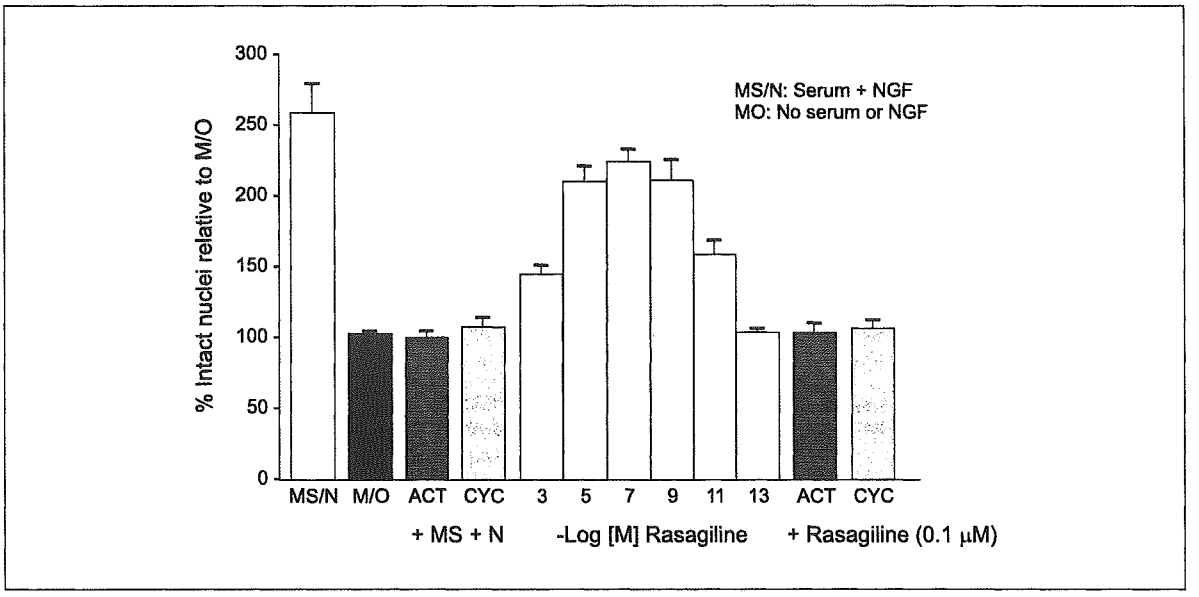


Fig. 3. The prevention of apoptotic death in serum- and nerve growth factor (NGF)-deprived PC-12 cells by rasagiline. The effects of transitional and transcriptional blocked by cycloheximide and actinomycin D (ref. 42).

tivity of rasagiline may be superior to that of selegiline, as reported by Abu-Raya *et al.* (83), Maruyama *et al.* (74, 75) and Bar Am *et al.* (84) (Fig. 4). This superior neuroprotective activity may result from the differences between the structure of selegiline and that of rasagiline. Selegiline is a derivative of amphetamine and is metabolized to methamphetamine *in vivo*, and similarly to amphetamine, it is a sympathomimetic compound (15, 25). Methamphetamine is known to be neurotoxic to dopaminergic neurons *in vivo* and to PC-12 and neuroblastoma cells *in vitro* (84, 91). Not only do the studies with l-methamphetamine confirm its neurotoxicity in cell culture, but we have also shown that unlike the aminoindan metabolite of rasagiline, l-methamphetamine prevents the neuroprotective activity of selegiline as well as that of rasagiline (Fig. 4). However, rasagiline is an aminoindan derivative, and neither it nor aminoindan is vasoactive (49). Unlike methamphetamine, aminoindan has little effect on sympathetic nervous function. Furthermore, both compounds demonstrate neuroprotective activity in serum- and nerve growth factor-free PC-12 cell cultures. Against l-methamphetamine, the major metabolite of selegiline (79, 84) (Fig. 4), it has been established that the neuroprotective activity of propargylamines such as rasagiline and selegiline resides in the propargylamine moiety. Our recent study on the neuroprotective activity of rasagiline in PC-12 cells (84), together with previous observations (74, 75, 79, 83), indicate that rasagiline may have a greater neuroprotective potency. This may be attributed to a combination of the propargylamine moiety and the aminoindan backbone of the drug. Indeed 4-hydroxyaminoindan was reported to be protective in global ischemia in gerbils. Rasagiline prevents the neurotoxicity of glutamate in PC-12 cells (unpublished data) and primary cultures of rat hippocampal neurons (25), as well as in global ischemia in gerbils (85). The metabotropic glutamate 1 (mGlu1) receptor antagonist, 1-aminoindan-1,5-dicarboxylic acid (AIDA), which reduces neuronal death following oxygen-glucose deprivation in murine cortical cells and rat organotypic hippocampal cultures and after *in vivo* global ischemia in gerbils, possesses an aminoindan to which the dicarboxylic moiety is attached at its 1 position (92). The

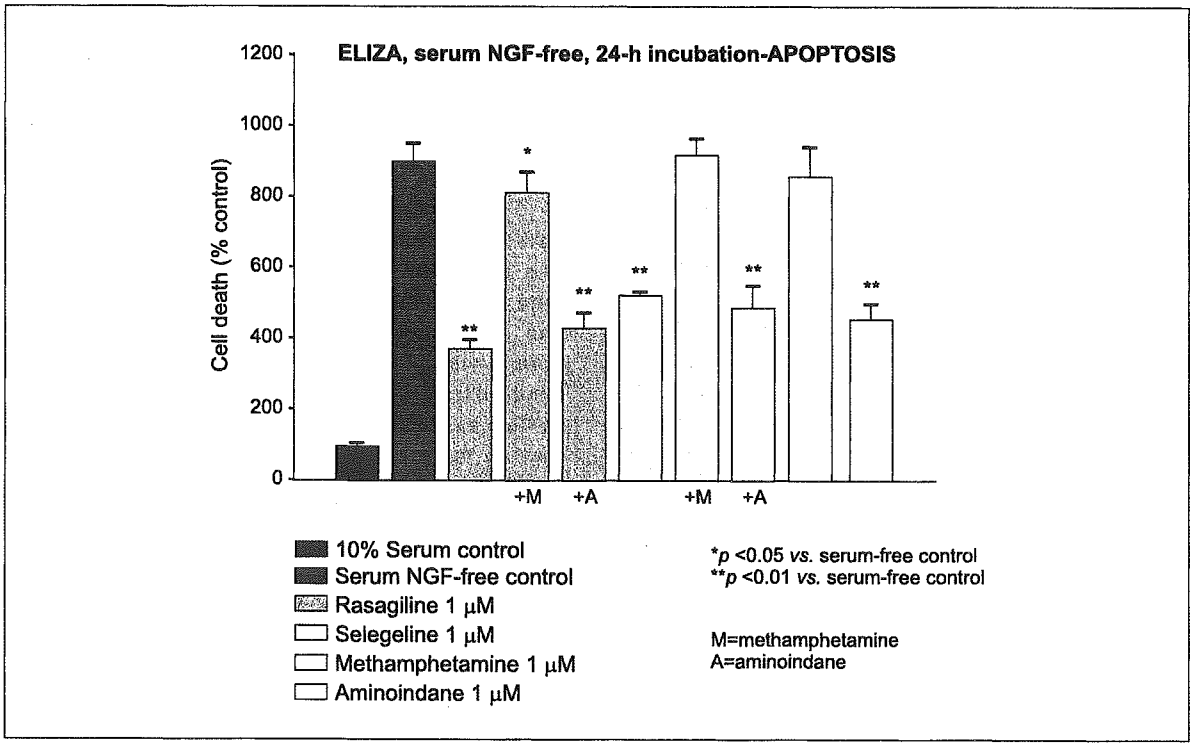


Fig. 4. The neuroprotective effect of rasagiline's metabolite aminoindan and the neurotoxicity of selegiline's metabolite methamphetamine in PC-12 cells (ref. 84).

structural similarity in propargylamine attachment to position 1 of aminoindan of rasagiline and its pharmacological neuroprotective activity to AIDA cannot go unnoticed. These results may point to a possible role for rasagiline as an mGlu1 antagonist. Support for this has come from cDNA microarray gene expression and proteomics with chronic rasagiline treatment in mice. Rasagiline significantly decreases the expression as well as the proteins of the glutamate and AMPA receptor in the mid brain of mice (93). Furthermore, rasagiline's metabolite aminoindan, unlike selegiline's metabolite methamphetamine, not only has no toxic effects, but also has neuroprotective activity under the conditions studied. Its generation *in vivo* may have an additive neuroprotective effect to that of rasagiline. As one of a few compounds identified for neuroprotective studies in Parkinson's disease (68), rasagiline may have the added advantage of neuroprotective properties arising from both its nontoxic metabolite and the parent compound. The involvement of the protein kinase C (PKC)-dependent mitogen-activated protein (MAP) kinase pathway in the neuroprotective activity of rasagiline in the absence of serum and nerve growth factor has recently been demonstrated. The ERK inhibitor PD-98096 and the PKC inhibitor GF109203X prevent the neuroprotective activity of rasagiline normally observed in PC-12 cells in the absence of serum and nerve growth factor (84) and will be discussed later.

Mitochondrial permeability transition pore and neuroprotective activity of rasagiline

Mitochondria are potent integrators and coordinators of cell death and survival. Apoptosis and necrosis are modes of cell death that play an integral part in a variety of biological processes, and their demise has been implicated in variety of neurodegenerative diseases, including Parkinson's, Alzheimer's and Huntington's diseases, as well as in non-neurodegenerative diseases such as cardiovascular disease and diabetes (for reviews, see refs. 94–98). The participation of mitochondrial-induced apoptosis in, for example, neuronal and cardiovascular disease-related cell death has not been fully established and therefore remains a controversial subject. Nevertheless, mitochondrial-induced apoptosis has been a target for study of toxin-induced cell death and neuroprotection in progressive loss of neurons and cytoprotection in cardiovascular diseases with variety of pharmacological agents. The initial phase of apoptosis is triggered in response to an induction phase resulting from a variety of initial insults, including xenobiotics, parkinsonism endogenous (*N*-methyl-(*R*)-salsolinol) and exogenous (6-hydroxydopamine and *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]) neurotoxin radiation, oxidative stress, etc. This results in an accompanying change in mitochondrial membrane permeability and a decline in mitochondrial membrane potential ($\Delta\psi m$), swelling of the mitochondria and opening of the mitochondrial permeability transition pore (MPTp) complex, inhibition of the ubiquitin-proteasome complex, release of mitochondrial cytochrome *c* and activation of caspases, especially caspase 3, resulting in cell death by apoptosis (Fig. 5). Mitochondrial permeability transition pores play a central role in the induction and prevention of apoptosis-induced cell death and consists of mitochondrial multiprotein complex, the exact nature of which is not yet fully known. However, it is known to include porin, hexokinase, peripheral benzodiazepine receptor, creatine kinase and cyclophilin D (Fig. 6).

The direct involvement of mitochondrial permeability transition pores in apoptotic-induced death of mammalian cells, including neurons, in cell culture and *in vivo* has been well documented for a variety of agents. The important role of mitochondrial permeability transition pores is also supported by the findings that the mitochondrial permeability transition pore complexes, particularly VDAC (voltage-dependent anion channel) and ANT (adenosine nucleotide translocase), are direct functional targets for the Bcl-2 family of proteins (Fig. 6). Mitochondrial phase function is controlled by oncogenes and antioncogens of the Bcl-2-Bax family. Antiapoptotic members (*e.g.*, Bcl-2, Bcl-XI, etc.) stabilize mitochondrial permeability transition pores, while pro-apoptotic members (*e.g.*, Bax, Bak, Bad and Bid) promote and increase mitochondrial membrane permeability (for reviews, see refs. 94–98). For example, we have shown that in response to the endogenous dopaminergic neurotoxin, *N*-methyl-(*R*)-salsolinol (SIN-1 peroxynitrite donor and 6-hydroxydopamine not shown), in the integration phase, the following responses occur in SHSY-5Y neuroblastoma cell mitochondria: a loss of $\Delta\psi m$, swelling of the mitochondria matrix (Fig. 7) and oxidative stress, and opening of mitochondrial membrane permeability (Fig. 8). Pretreatment of these cells with rasagiline prevents these effects (Fig. 6–8). In addition, there is inhibition of the ubiquitin–proteasome system by the neurotoxin, resulting in release

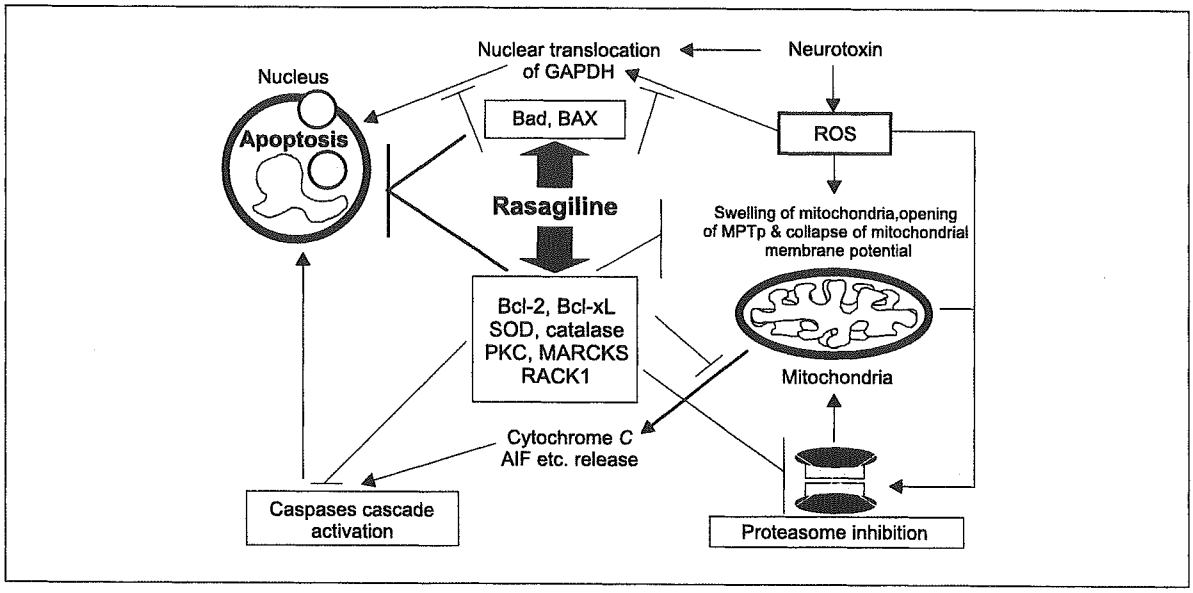


Fig. 5. The mechanism by which various neurotoxins induce neurotoxicity- and apoptosis-induced neuronal death.

of toxic intermembrane proteins such as cytochrome *c*, which rasagiline inhibits (72, 73). These responses are followed by a complex set of events which include activation of pro-caspases and caspase 3 activation (which rasagiline prevents [70, 71]), resulting in nuclear chromatin condensation, DNA fragmentation and changes in the plasma membrane that culminate in the death of SHSY-5Y cells (Fig. 5). Rasagiline-induced neuroprotection exhibited in response to *N*-methyl-(*R*)-salsolinol is also seen with a number of other neurotoxic events and compounds that induce neuronal death (Table I, Fig. 5). This has provided the first evidence that propargylamines and rasagiline antiapoptotic drugs regulate the anti- and pro-apoptotic signaling pathway in mitochondria. The ability of rasagiline to inhibit *N*-methyl-(*R*)- salsolinol-induced collapse of mitchondrial membrane potential ($\Delta\psi m$), the swel-

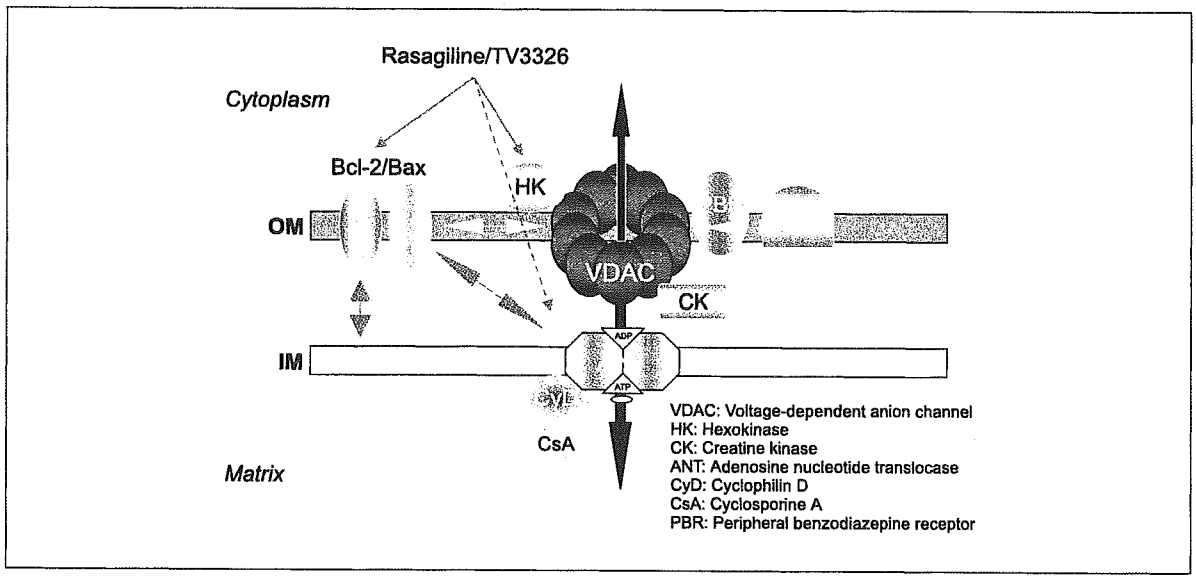


Fig. 6. Sites of rasagiline and TV3326 neuroprotective actions in preventing neurotoxin (sin-1, 6-hydroxydopamine and *N*-methyl-(*R*)-salsolinol) induced opening of mitochondrial permeability transition pore (MPTp) (ref. 51).

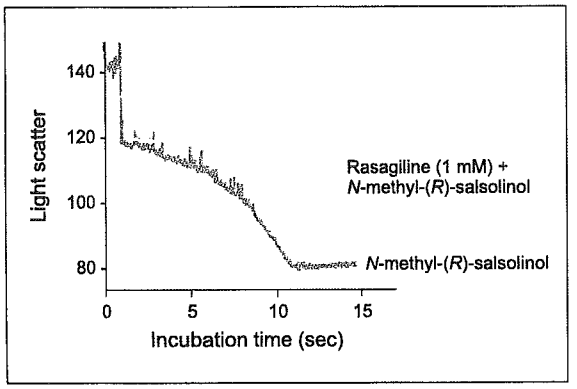


Fig. 7. Prevention of rat liver or brain mitochondrial swelling by rasagiline as induced by neurotoxin *N*-methyl-(*R*)-salsolinol (ref. 99).

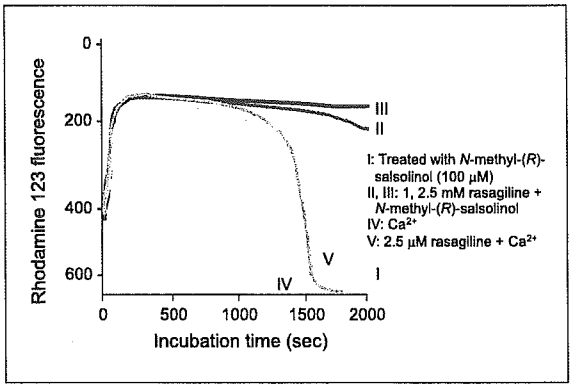


Fig. 8. Prevention of the permeability transition by rasagiline in isolated mitochondria as induced by *N*-methyl-(*R*)-salsolinol, but not of that induced by Ca^{2+} (ref. 99).

ling of mitochondria (Fig. 7) and mitochondrial membrane permeability opening (Fig. 8) in SHSY-5Y cells is associated with activation of antiapoptotic Bcl-2 family proteins. Evidence for this has come from the studies demonstrating that rasagiline increases mRNAs and proteins of Bcl-2 and Bcl-XI, while decreasing those of Bax and Bad in SHSY-5Y and PC-12 cells (submitted for publication). Furthermore, Bcl-2-overexpressed SHSY-5Y cells behave identically to rasagiline-treated cells by being resistant to neurotoxicity with *N*-methyl-(*R*)-salsolinol (SIN-1 and 6-hydroxydopamine not shown) (Figs. 6, 7) (51, 99). The neurotoxicity induced by *N*-methyl-(*R*)-salsolinol in SHSY-5Y cells, resulting in the inhibition of the ubiquitin-proteasome system, is associated with cytosolic translocation of pro-apoptotic GAPDH. However, it is not known whether the two are linked or independent pathways leading to apoptosis and cell death. Both rasagiline- and Bcl-2-overexpressed cells prevent these processes (72, 73). Further support for the participation of Bcl-2 family proteins in the neuroprotective activity of rasagiline has come from cDNA microarray gene expression in rat striatum with chronic rasagiline treatment. In these studies, cell survival (Bcl-2, Bcl-xl, AKT, PKC) and death (FAS, Bax and Bad, JNK) are respectively up- and downregulated (93, 100).

Structure-activity relationship studies with propargylamines have indicated that the propargyl moiety is crucial for the neuroprotective activity (63, 101–103), since although the aminoindan metabolite of rasagiline is not antiapoptotic, in certain other circumstances it has neuroprotective activity, as in the case of serum withdrawal in partially neuronally differentiated PC-12 cells (79, 84). The mechanism responsible for the neuroprotective action of rasagiline in some respects resembles that of cyclosporine A and immunophilins, which have been shown to have neuroprotective activity against a variety of neurotoxins, including *N*-methyl-(*R*)-salsolinol and MPTP (104). However, rasagiline is unable to fully suppress the Ca^{+2}-induced mitochondrial membrane permeability opening, as seen with BCL-2 overexpression (Fig. 7) and with the action of bonkrekic A and propargylamine, all of which target respective components of the mitochondrial permeability transition pore complex. Nevertheless, in many respects the neuroprotective response of SHSY-5Y cells to rasagiline is very similar to that of cells with overexpressed Bcl-2 (72, 99, 105). Such cells are also resistant to apoptosis by neurotoxins such as *N*-methyl-(*R*)-salsolinol, and there is no collapse of $\Delta\psi m$, no inhibition of the ubiquitin-proteasome system, no release of cytochrome *c* and no GAPDH translocation from the cytoplasm to the nucleus in response to the neurotoxin (Fig. 5) (72, 99, 105). Our current studies are directed at identifying the target protein(s) upstream and determining at which VDAC protein site rasagiline acts upon the mitochondria (72, 73). Preliminary proteomic studies suggest that upstream rasagiline acts to downregulate FAS and FAS receptor while upregulating PKC (106).

Amyloid precursor protein processing activity of rasagiline

Activation of PKC-dependent MAP kinase pathway

PKCs, which are abundant in neuronal tissues (107), are involved in neuronal survival and func-

tions of neuronal trophic factors (108, 109). They are critical in the formation and consolidation of different types of memory (110), suggesting a crucial role for PKC in the aberrant signal transduction occurring in Parkinson's and Alzheimer's disease brain (111). Indeed, several reports document a deficit in PKC isoform levels in Alzheimer's disease (112–114), which might lead to a reduced responsiveness of brain tissues to growth factors and neurotransmitters, including acetylcholine (111, 115). Furthermore, a defect in PKC activation in Alzheimer's disease has been documented as a marked loss of redistribution of cytosolic PKC to the particulate fraction in response to phorbol esters and K^+ depolarization in tissue slices from hippocampus, temporal and frontal cortex (116). *In vitro* studies have established the involvement of PKC and PKC-coupled receptors in the nonamyloidogenic -secretase pathway of the amyloid precursor protein (APP) cleavage (117–120). There is also evidence for the regulation of secretory APP processing by PKC-dependent mechanisms under *in vivo* conditions (121–123).

Some evidence suggests that rasagiline induces the release of the nonamyloidogenic α-secretase form of soluble APP (sAPP) from SH-SY5Y neuroblastoma and PC-12 cells by mitogen-activated protein kinase (MAPK)- and PKC-dependent mechanisms (101, 124). These processes have been shown to be directly dependent on the propargylamine moiety of rasagiline since propargylamine itself is as effective in these mechanisms (99). Indeed, the neuroprotective activity is also dependent on the propargylamine moiety (79, 101–103). Considering these findings, the effect of rasagiline on the regulation of PKC-dependent mechanisms and APP processing under *in vivo* conditions was investigated.

Evidence has been provided that rasagiline induces significant increases in p-PKC levels and in the expression of α and ε PKC isoforms in the mouse and rat hippocampus (84). Additionally, rasagiline treatment significantly elevated the levels of p-MARCKS, a major substrate for PKC, as well as levels of RACK1 (Fig. 9). Similar results have been obtained with propargylamine alone, indicating that the PKC activation by rasagiline is the pharmacological property of the propargylamine moiety (101, 125).

Studies investigating the role of the PKC family in the regulation of cell death have suggested that activation of PKC can prevent apoptosis via two

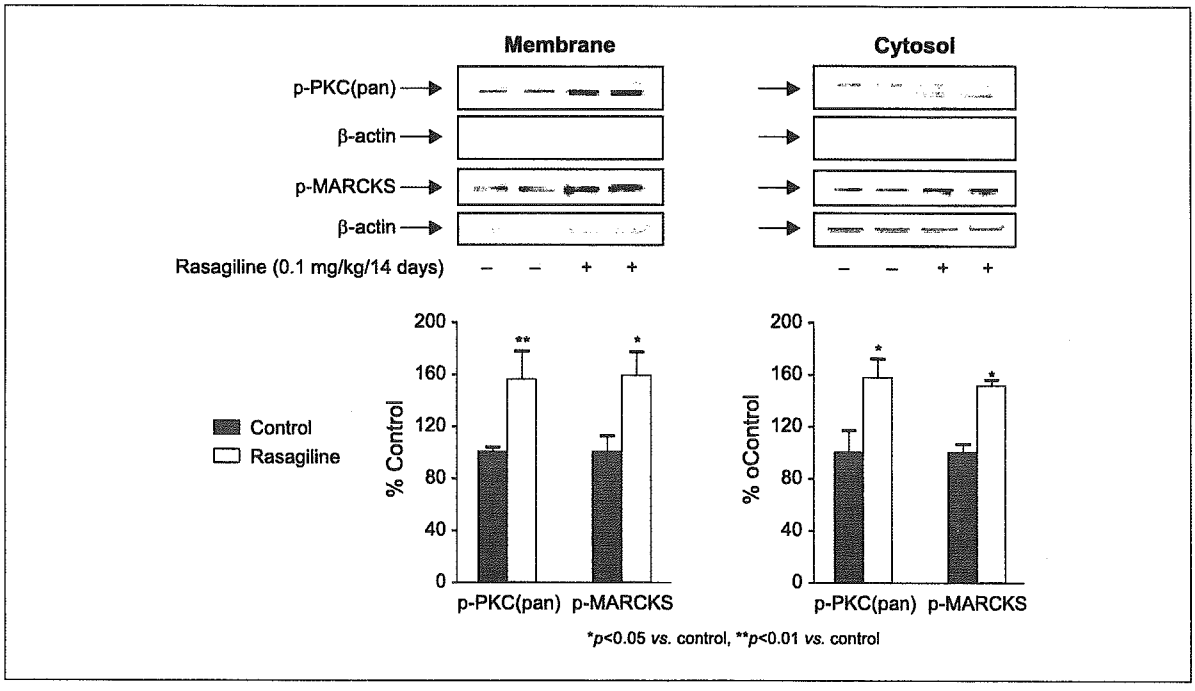


Fig. 9. Effects of rasagiline on p-PKC(pan) and p-MARCKS levels in mouse hippocampus after chronic treatment with rasagiline (ref. 125).

main survival pathways: 1) the antiapoptotic protein BCL-2; and 2) the MAPK/extracellular regulated kinase (ERK) cascades. PKC can phosphorylate Bcl-2 at a site that increases its antiapoptotic function (126), and overexpression of PKC results in increased expression of Bcl-2 (127, 128). Furthermore, MAPK/ERK cascades, which have been shown to inhibit apoptosis in a number of systems, can be activated by PKC. For example, PKC phosphorylates and activates raf-1, an upstream kinase in the MAPK/ERK pathway (129), and pharmacological inhibition of MAPK/ERK signaling blocks phorbol ester-induced protection of neuronal cells against glutamate toxicity (130). Indeed, both of these pathways have been identified in the neuroprotective mechanism of action of rasagiline *in vitro*, where treatment in PC-12 cells with the ERK inhibitor PD98059 and the PKC inhibitors GF109203X and calphestin c (submitted for publication) prevented the neuroprotective activity of rasagiline in serum- and nerve-growth-factor-free system-induced apoptosis (125). Rasagiline induced cytoprotective gene expression (*e.g.*, Bcl-2 and Bcl-xl), which may rescue neurons from apoptosis, as mediated by the mitochondrial death cascade, and therefore protect declining neurons in neurodegenerative disorders and aging (131). Additionally, rasagiline dose-dependently (0.1–10 μM) increases the immunoreactivity of the phosphorylated MAPK in PC12 cells. The MEK inhibitor, PD98059, antagonized rasagiline-induced MAPK activation, indicating that MEK phosphorylated MAPK in the presence of rasagiline (101, 124). Thus, the activation of PKC by rasagiline may play a crucial role in its neuroprotective activity, which also involves the regulation of Bcl-2/Bcl-Xl/Bad and Bax.

One of the major downstream substrates of PKC is the MARCKS, which has been implicated in cell motility, cell adhesion, membrane traffic and mitogenesis. PKC phosphorylates serines 152, 156 and 163 of MARCKS (132, 133), which regulates MARCKS's calcium/calmodulin binding activity and filamentous actin cross-linking activity (132, 134). MARCKS is developmentally regulated, being highly expressed in select regions of the developing and adult rat brain (135), and it plays a significant role in spatial learning processes (136). The location of MARCKS in senile plaques along with PKC and Aβ fibrils points to a possible involvement in Aβ-induced neuronal dysfunction (137). Since the phosphorylation of MARCKS has been used as a marker for activation of PKC, the effect of rasagiline on p-MARCKS levels was investigated. The results of these studies showed that p-MARCKS levels were elevated in the hippocampus of rasagiline-treated mice as compared to controls, further supporting the induction of PKC activation by rasagiline.

Owing to the reputed role of RACK1 (receptor for activated C kinase 1) in PKC-mediated events in different cellular systems, the effect of rasagiline treatment on RACK1 levels was also determined. Rasagiline treatment increased the levels of RACK1 in the hippocampus, indicating that this compound may also affect other factors controlling PKC activation. RACK1 is emerging as important in targeting activated PKC to different intracellular sites, where substrates can be phosphorylated and thus modulate PKC-dependent functions (138). In brain, RACK1 mRNA and protein show developmental changes; a parallel in RACK1 and PKC protein ontogenesis suggests that they are interdependent and involved in synaptogenesis and myelination (139). In addition, RACK1 immunoreactivity is reduced in brain cortex of the aged rat, concomitantly with an impaired translocation in PKC activity and immunoreactivity for βII, indicating that RACK1 deficit contributes to age-dependent impairment in PKC activation/compartmentalization (139). Interestingly, it has been shown that in Alzheimer's disease, a reduced level of RACK1 protein can be observed in the brain of affected patients (140), a finding that links to previous observations concerning defective PKC machinery in Alzheimer's brain and peripheral tissues (139, 141). Thus, it can be suggested that upregulation of PKC by rasagiline may maintain appropriate PKC activity, an important task in the management of Alzheimer's disease and in parkinsonian subjects with dementia pathophysiology.

In Alzheimer's disease, a growing body of evidence suggests that increased expression and/or altered processing of APP and the ensuing increase in generation of β-amyloid peptides play a central role in amyloidogensis processes (142). Thus, the observation that rasagiline markedly decreased the levels of cell-associated, full-length APP in the mouse hippocampus could be of value towards accelerating nonamyloidogenic APP processing, thereby reducing β-amyloid levels. Since we have recently demonstrated the involvement of PKC in the release of sAPPα by rasagiline in PC-12 cells and SH-SY5Y neuroblastoma cells (101, 124), it is possible that the mechanism by which these drugs affect APP processing *in vivo* may be related to PKC-associated signaling. Indeed, among the various sig-

naling cascades known to participate in Alzheimer's disease pathophysiology and APP processing, PKC has received much attention. *In vivo*, it was shown that specific cholinergic lesions within the basal forebrain of rats lead to reduced secretory APP processing in the cholinergically deafferented neocortex, which strongly correlates with reduced activation of PKC-coupled M1 mAChR (122). Also, Lin *et al.* (123) observed reduced secretory APP processing in neocortex after specific cholinergic lesions of the basal forebrain and increased APP secretion (*i.e.*, less cell-associated APP but higher levels of cerebrospinal fluid secretory APP) after muscarinic agonist treatment. In an animal model of permanent hyperactivation of PKC in neocortex and hippocampus (*i.e.*, in the offspring of rats receiving *in utero* methylazoxymethanol acetate treatment), secretory APP processing was increased (121).

It is currently unknown which isoenzyme of PKC plays a major role in modulating APP processing. Nevertheless, several lines of evidence suggest the involvement of PKCε and PKC in APP processing. Thus, it was demonstrated that the EC_{50} for PMA regulation of sAPP release was lower in Swiss 3T3 fibroblast cells overexpressing PKCα (119), and a specific inhibitor of PKCα, GO-6976, reduced constitutive and phorbol ester regulation of sAPP in human fibroblasts (143). Furthermore, in a rat fibroblast cell line, sAPP was increased after stable overexpression of PKCα and PKCε isoenzymes (144). It was also shown that blockade of PKCε activation attenuated phorbol ester-induced increase of α-secretase-derived sAPP (145). This finding further supports previous studies on brains of patients with Alzheimer's disease, where PKCε activity in the membrane fraction was reduced (114). In accordance with these findings, we have shown that chronic administration of rasagiline for 14 days causes significant increases in the protein expression of PKC isoenzymes α and ε in the mouse and rat hippocampus. These results are consistent with our recent finding demonstrating induction of PKCα and PKCε mRNAs in PC-12 cells by rasagiline (manuscript in preparation).

In summary, rasagiline reduced the levels of APP and upregulated the levels of p-PKC, PKCα and PKCε in the mouse hippocampu and in PC-12 cells (125). Furthermore, the presence of a propargylamine group in this compound is essential for its APP processing to neuroprotective-neurotrophic sAPPα through α-secretase regulation and PKC activation-dependent neuroprotective activities (51). Moreover, studies on structure-activity relationships among rasagiline-related compounds have shown the crucial role of the propargyl moiety in these molecules with respect to these processes. This is supported by the recent observation that propargylamine itself was neuroprotective and able to regulate APP processing and MAPK phosphorylation with similar potency to that of rasagiline (101). Rasagiline is now being developed as an antidementia drug in Alzheimer's disease, Lewy body disease and parkinsonian dementia.

Neurotrophic activity of rasagiline: The involvement of GDNF, nerve growth factor and NF-κB

Rasagiline increases the mRNA and protein levels of GDNF (Fig. 10) (146), in addition to those of antiapoptotic Bcl-2 and Bcl-xL, as previously reported (99) in SH-SY5Y cells in culture. Selegiline and desmethylselegiline, propargylamines structurally related to rasagiline, were previously shown to increase mRNA levels of neuroprotective proteins, bcl-2, SOD, glutathione peroxides and GDNF in PC-12 cells (80). It is apparent that the activation of nuclear factor (NF)-κB by rasagiline may mediate the increase in the transcription of pro-survival genes.

In Parkinson's and Alzheimer's diseases, particular neurons deteriorate in a slow and continuous process, in which not only apoptotic, but also pro-survival factors may be activated. NF-κB may be a common regulator collecting information regarding upstream signal transduction events to determine the survival or death of the cells (147). Increased levels of NF-κB were detected in the brains of patients with Alzheimer's disease (148) and Parkinson's disease (149). Apoptosis, as induced by excitotoxicity (glutamate or NMDA) and oxidative stress, activates NF-κB (150), whereas cytotoxicity can be mediated by the activation of pro-apoptotic members of the Bcl-2 family (151). On the contrary, NF-κB has been reported to be cytoprotective against apoptosis as induced by oxidative stress and excitotoxic insults (152). These opposing results suggest that the cell types, the conditions of cytotoxic stimuli and the concomitant activation of other transcription factors may determine whether activated NF-κB promotes cell survival or cell death. The molecular mechanisms of NF-κB activation have been elucidated by Karin and Ben-Neriah (153). NF-κB is activated by IκB phosphorylation by kinase complex (I-B kinase,