Table 3
Characteristics of enzymes related to the metabolism of *N*-methyl-(*R*)salsolinol

Enzymes	Characteristics	Substrate
(*R*)Salsolinol synthase	Molecular weight: 34.3 kDa Optimum pH: 7.4	Dopamine Acetaldehyde Pyruvic acid
Neutral (*R*)salsolinol *N*-methyltransferase	Methyl donor: SAM[a] Molecular weight: 35.6 kDa Optimum pH: 7.0	(*R*)Sal, norsalsolinol
N-Methyl(*R*)salsolinol oxidase	Sensitive to semicarbazide, not to clorgyline, (−)deprenyl	*N*M(*R*)Sal, *N*M(*S*)Sal, *N*-methylnorsalsolinol

These enzymes were isolated from the cytosol soluble fraction of human brain gray matter.
[a] *S*-Adenosyl-L-methionine.

weight) and (*R*)Sal (19–117 pmol/g wet weight). It is not yet clarified whether the synthesizing enzyme is identical with the enzyme catalyzing the synthesis of (*R*)Sal.

THE DISTRIBUTION OF SALSOLINOL DERIVATIVES IN BRAIN REGIONS AND FACTORS AFFECTING THE CONCENTRATIONS

The distribution of Sal, *N*MSal and $DMDHIQ^+$ was examined in four major regions of control brains, the frontal cortex, caudate, putamen and substantia nigra (Maruyama et al., 1997c). *N*M(*R*)Sal and its precursor, dopamine, were found to occur selectively in the nigro-striatum, whereas (*R*)Sal distributes uniformly among the brain regions, as shown in Table 2 and Fig. 3. On the other hand, $DMDHIQ^+$ with chemical structure similar to, a dopaminergic neurotoxin, 1-methyl-4-phenyl-pyridinium ion (MPP^+) was detected only in the substantia nigra (Table 1). These results suggest that their concentrations do not depend on those of dopamine, but on the activity of the synthesizing enzymes. The *N*-methylation of (*R*)Sal by an *N*-methyltransferase, whose activity is higher in the nigro-striatum than in other brain regions (Maruyama et al., 1992), brings about the selective distribution in dopamine neurons. Binding of $DMDHIQ^+$ to melanin may account for the selective accumulation of the isoquinolinium ion in the substantia nigra (Naoi et al., 1994a).

The factors determining the levels of Sal derivatives, the age, alcohol contents in blood, dopamine turnover, and the enzyme activities related to the metabolism, were examined in human brain samples. The levels of (*R*)Sal decreased in the caudate, putamen and substantia nigra according to the age (Maruyama et al., 1997c). A negative correlation was confirmed between the level of *N*M(*R*)Sal in the striatum and the age. The effects of alcohol level in blood on the concentrations of Sal derivatives were examined, and as shown in Fig. 3, the presence of alcohol did not affect the levels of (*R*)enantiomer of Sal and *N*MSal in any brain regions, whereas the (*S*)enantiomers were not detected even in alcoholic brains. These results suggest that the levels of acetaldehyde, a product of ethanol, do not determine those of Sal derivatives in the brain. The level of a dopamine product, homovanillic aid (HVA), or the ratio of HVA/dopamine, an indicator of dopamine turnover, did not correlate with the levels of catechol isoquinolines in the human brain.

We examined the effects of the activities of the enzymes related to the metabolism on the levels of Sal derivatives in brain regions, the frontal cortex, caudate, putamen, substantia nigra and thalamus (Naoi et al., 1997). The activities of (*R*)Sal synthase, (*R*)Sal *N*-methyltransferase with the optimal pH at 7.0 (neutral) and at 8.4 (alkaline), and of *N*M(*R*)Sal oxidase were compared with the levels of (*R*)Sal, *N*M(*R*)Sal and $DMDHIQ^+$. A good correlation was confirmed between the activity of neutral (*R*)Sal *N*-methyltransferase in the striatum (putamen and caudate) and the concentration of $DMDHIQ^+$ in the substantia nigra, as shown in Fig. 4. There was no significant correlation between the activities of other enzymes and the levels of isoquinolines in the these brain regions. These results clearly show that the enzymatic activity of *N*-methyltransferase regulates the level of *N*M(*R*)Sal, a neurotoxin, in the nigro-striatum of human brains. In addition, it is suggested that *N*M(*R*)Sal is synthesized in the striatum, transported to the substantia nigra and oxidized into $DMDHIQ^+$, which accumulates as conjugated with melanin. This is comparable with the results observed in a rat model of Parkinson's disease, where $DMDHIQ^+$ was detected in the substantia nigra

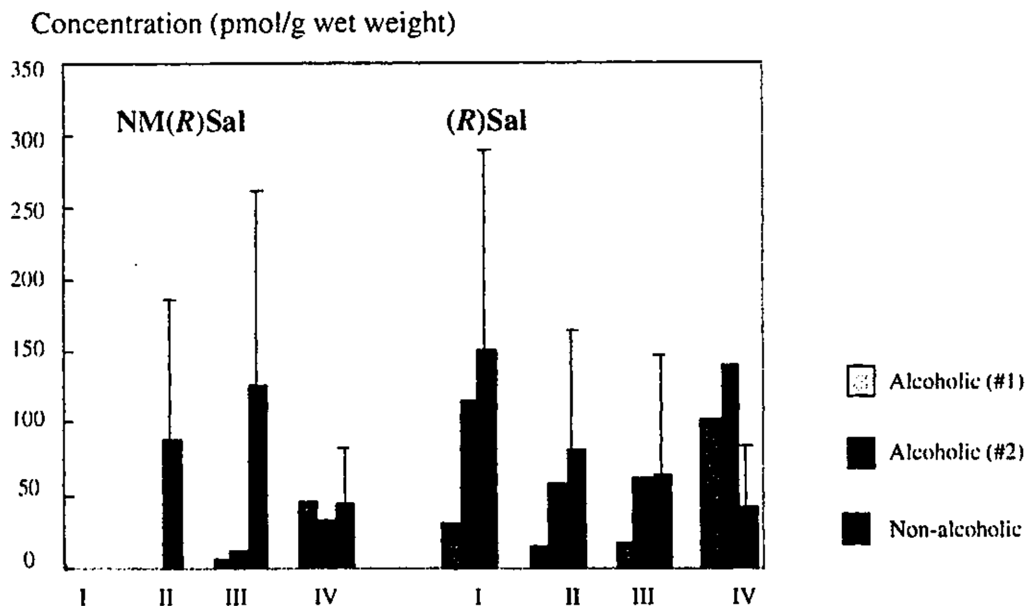

Fig. 3. Concentration of (*R*)salsolinol ((*R*)Sal) and *N*-methyl(*R*)salsolinol (*N*M(*R*)Sal) in the four regions of control human brains, and the effects of ethanol in blood. I: frontal cortex; II: caudate; III: putamen; IV: substantia nigra. In peripheral blood of alcoholic case 1 and 2, 3.3 and 0.1 mg/ml of ethanol were detected. The results of non-alcoholic were the mean ± S.D. of seven cases without ethanol in blood. In frontal cortex, *N*M(*R*)Sal was not detected in any sample.

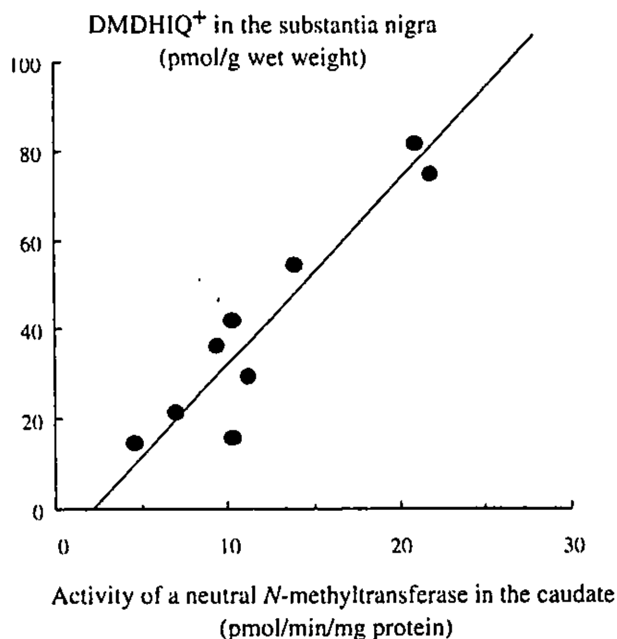

Fig. 4. The effect of the activity of a neutral (*R*)salsolinol *N*-methyltransferase in the caudate on the levels of 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion ($DMDHIQ^+$) in the substantia nigra. The frontal cortex, caudate, putamen and substantia nigra of nine control human brains were analyzed for the levels of (*R*)Sal, *N*M(*R*)Sal and $DMDHIQ^+$, and the activities of (*R*)Sal synthase, neutral and alkaline (*R*)Sal *N*-methyltransferases. Significant correlation was confirmed between the $DMDHIQ^+$ level in the substantia nigra and the activities of neutral *N*-methyltransferase in the caudate ($r = 0.905$, $P < 0.001$) and putamen ($r = 0.616$, $P < 0.05$).

after injection of *N*M(*R*)Sal in the striatum (Naoi et al., 1996a).

SALSOLINOL DERIVATIVES AS INHIBITORS OF TYPE A MONOAMINE OXIDASE

The inhibition of MAO by Sal was first reported by Yamada (1971). Sal inhibited MAO activity in rat brain stem and liver, and the inhibition was competitive to serotonin, a substrate specific for type A. Sal and THP inhibited rat brain MAO in vitro and the inhibition was competitive to serotonin and non-competitive to benzylamine, a substrate of type B MAO (Collins et al., 1973; Meyerson et al., 1976). A series of (*R*)- and (*S*)Sal were examined for the effects on the activities of type A and B MAO, as summarized in Table 4. The (*R*)enantiomers of Sal and *N*MSal inhibited MAO-A more potent than the (*S*)enantiomers, and the inhibition to type A MAO was competitive to the substrate, and to type B was noncompetitive (Dostert et al., 1989; Minami et al., 1993). The oxidized $DMDHIQ^+$ is the most potent inhibitor of MAO-A, followed by *N*M(*R*)Sal, (*R*)Sal and *N*-methylnorsalsolinol (Naoi et al., 1994b). The presence of hydroxyl groups at sixth and seventh position and substitution of a hydrogen group at first position with a methyl or dihydroxybenzyl group are required for the inhibition, whereas

Table 4
The K_i value of salsolinol derivatives to type A and B monoamine oxidase in human brain synaptosomes

Salsolinol derivatives	K_i values (μM) for monoamine oxidase	
	Type A	Type B
(*R*)Salsolinol	37.9	68.3
(*S*)Salsolinol	149.5	149.5
2(*N*)-Methyl(*R*)salsolinol	36.1	433.3
2(*N*)-Methyl(*S*)salsolinol	81.3	No inhibition
Norsalsolinol	No inhibition	No inhibition
2(*N*)-Methynorsalsolinol	61.4	289
1,2(*N*)-Dehydrosalsolinol	322.3	No inhibition
1-Carboxyl(*R*)salsolinol	421.3	No inhibition
1,2(*N*)-DM-6,7-DHIQ^{+a}	9.21	No inhibition
2(*N*)-M-6,7-DHIQ^{+a}	44.4	No inhibition
2(*N*)-M-1-carboxyl(*R*)salsolinol[b]	No inhibition	No inhibition

[a] 1,2(*N*)-Dimethyl- and 2(*N*)-methyl-6,7-dihydroxyisoquinolinium ion.
[b] 2(*N*)-Methyl-1-carboxyl(*R*)salsolinol.

the absence of a methyl group or a presence of a carboxy group at first position in addition to a methyl group depletes the inhibitory activity. The structure–activity relationship of isoquinoline derivatives was reported in details by Bembenek et al. (1990) and Thull et al. (1995).

To examine the effects of Sal derivatives on the neurotransmitter levels in the brains, in vivo microdialysis studies were carried out. The analyses of monoamine metabolites in the microdialysate in the rat striatum indicated that Sal derivatives inhibited MAO and COMT activities in situ and the levels of 3,4-diphenylacetic acid (DOPAC) and homovanillic acid (HVA) reduced markedly, and 5-hydroxyindolacetic acid (5-HIAA) most significantly, as shown in Fig. 5 (Maruyama et al., 1993). The (*R*)enantiomers were more potent than the (*S*)enantiomers, and the presence of a catechol structure and a methyl group at 1 or 2(*N*) position increased the effects, as in the case of the in vitro experiments. These results demonstrate

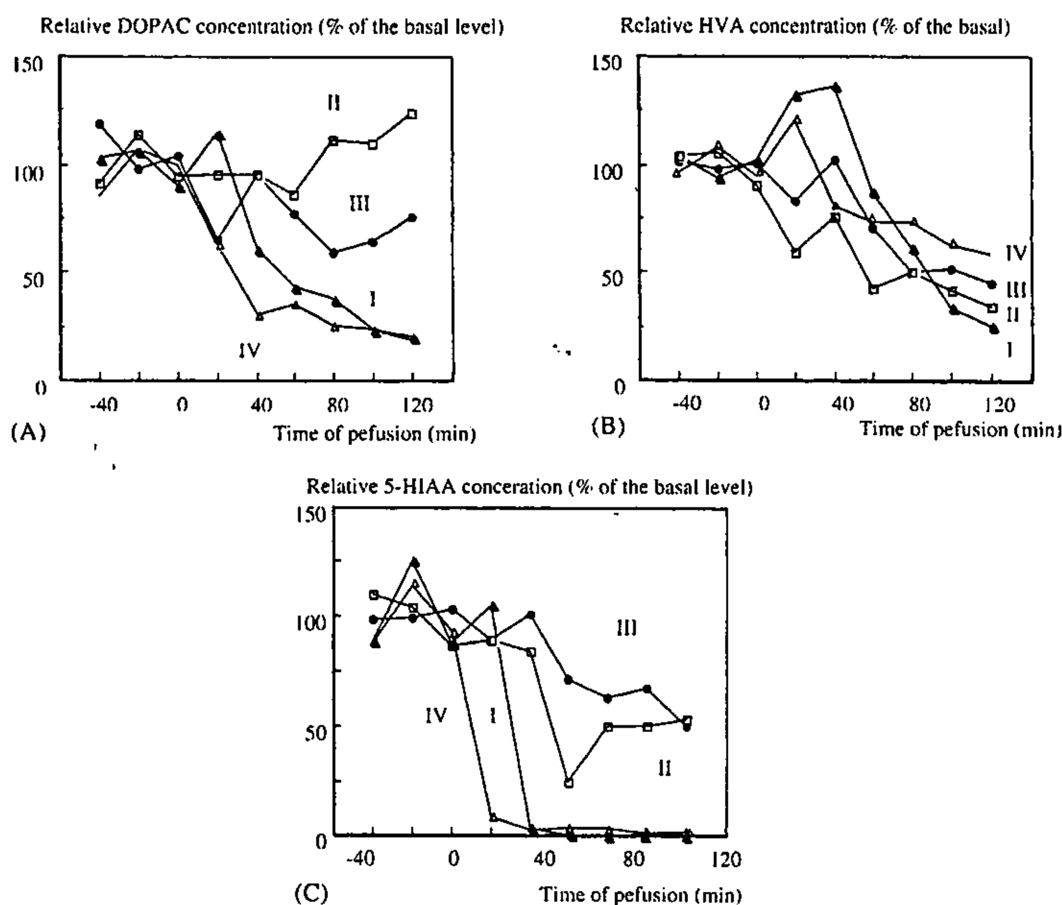

Fig. 5. In vivo effects of (*R*)salsolinol derivatives on the metabolism of catechol- and indoleamines in the rat striatum; in vivo microdialysis studies. Rat striatum was injected with isoquinoline solution in saline and collected samples were analyzed by HPLC with multi-electrochemical detectors: (A) the levels of 3,4-dihydroxyphenylacetic acid (DOPAC); (B) homovanillic acid (HVA); and (C) 5-hydroxyindolacetic acid (5-HIAA), expressed as percent of the basal level. I: (*R*)Sal; II: *N*M(*R*)Sal; III: norsalsolinol (6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline); IV: 2(*N*)-methylnorsalsolinol.

that Sal derivatives inhibit type A MAO more markedly than type B in the brain, also by in vivo experiments.

THE EFFECTS OF SALSOLINOL DERIVATIVES ON THE METABOLISM OF MONOAMINES

Sal and related TIQs inhibit the enzymes participating in the metabolism of monoamines. Sal inhibits a rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2, TH), prepared from rat brain. The inhibitor constant, K_i, value for Sal was 14 μM and the inhibition was competitive to a cofactor, 6,7-dimethyl-5,6,7,8-tetrahydropterin (Weiner and Collins, 1978). The naturally occurring cofactor of TH, L-*erythro*-5,6,7,8-tetrahydrobiopterin, induces allostery to the enzyme polymers and to change the affinity to the biopterin itself. The (*R*)enantiomer of Sal deletes the allostery of TH to the biopterin and reduces the activity more markedly than the (*S*)Sal (Minami et al., 1992). Norlaudanosolinecarboxylic acids and other TIQ carboxylic acids are potent inhibitors of TH, and the inhibition was non-competitive with respect to its substrate, tyrosine, and its cofactor, 6-methyltetrahydropterin (Coscia et al., 1980). These TIQs inhibited also dopamine-β-hydroxylase (3,4-dihydroxy-phenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1). One or more free hydroxyl groups of salsolinol and THP were methylated by catechol-*O*-methyltransferase (*S*-adenosyl-L-methionine: catechol *O*-methyltransferase, EC 2.1.1.6, COMT) (Collins et al., 1973). They are competitive inhibitors of dopamine *O*-methylation in vitro. COMT was inhibited by other TIQs, 1-benzyl and 1-methyl derivatives of 6,7-dihydroxy-3,4-dihydroisoquinolines, and the inhibition was non-competitive to substrate (Cheng et al., 1987).

Sal derivatives inhibit a rate-limiting enzyme in indoleamine synthesis, trytophan hydroxylase (L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14,16,2, TPH) (Matsubara et al., 1994). Sal derivatives inhibited markedly the activity of TPH, and $DMDHIQ^+$ was an extremely potent inhibitor. The inhibition was non-competitive in terms of either the biopterine cofactor or the substrate, L-tryptophan. On the other hand, the condensation products of indoleamines with acetaldehyde, β-carbolines, did not inhibit the TPH activity.

N-METHYL(*R*)SALSOLINOL AS AN ENDOGENOUS NEUROTOXIN TO DOPAMINE NEURONS: POSSIBLE INVOLVEMENT TO THE PATHOGENESIS OF PARKINSON'S DISEASE

Studies with 1-methyl-4-phenyl-1,2,3,6-tetrahydrpyridine (MPTP) (Langston et al., 1983) suggest that endogenous and xenobiotic neurotoxins may cause parkinsonism in humans. To find dopaminergic neurotoxins, the catechol isoquinolines were injected into the striatum of male Wistar rats, and the behavioral, biochemical and histo-pathological effects were examined. Among catechol isoquinolines, *N*M(*R*)Sal was the most cytotoxic to dopamine neurons, causing behavioral, histopathological and biochemical changes in the nigro-striatum, which were quite similar to those observed in parkinsonian patients (Naoi et al., 1996a, 1998a). After 1 week continuous injection of *N*M(*R*)Sal in the striatum, the number of dopamine neurons stained with anti-TH antibody reduced markedly in the substantia nigra, but necrotic reaction was not observed, suggesting that *N*M(*R*)Sal caused apoptotic cell death selectively in dopamine neurons of the rat model.

The involvement of *N*M(*R*)Sal in the pathogenesis of Parkinson's disease was proved by analyses of CSF from parkinsonian patients (Maruyama et al., 1996a). The *N*M(*R*)Sal levels in CSF from parkinsonian patients were significantly higher than in control, as summarized in Table 2. In parkinsonian CSF, the presence of Sal (Moser and Kämpf, 1992) and *N*-methylnorsalsolinol (2(*N*)-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) (Moser et al., 1995) was reported, but it was not confirmed whether they increased in Parkinson's disease (Muller et al., 1999).

These results suggest that the metabolism of *N*M(*R*)Sal in the brain may be altered in Parkinson's disease. To confirm this point, the activities of enzymes related to the metabolism of *N*M(*R*)Sal were examined in lymphocytes prepared from parkinsonian patients (Naoi et al., 1998c). Among enzymes, only the activity of a neutral (*R*)Sal *N*-methyltransferase was significantly higher in parkinsonian lymphocytes. Recently the correlation was confirmed between the neutral (*R*)Sal *N*-methyltransferase activity in lymphocytes and the *N*M(*R*)Sal concentration in CSF from untreated parkinsonian patients. The results support our view that the activity of a neutral (*R*)Sal *N*-methyltransferase in the brain may determine the neurotoxin level in the substantia nigra of parkinsonian brains.

Table 5
Cytotoxicity of salsolinol derivatives to SH-SY5Y cells

Salsolinol derivatives	Apoptotic cells (percent of the total cells)	Necrotic cells
Control	4.86 ± 4.34	1.21 ± 1.68
(*R*)Salsolinol	9.55 ± 2.26	1.70 ± 1.41
(*S*)Salsolinol	10.71 ± 4.54	1.33 ± 0.68
N-Methyl(*R*)salsolinol	100[a]	0
N-Methyl(*R*)salsolinol[b]	26.73 ± 4.57[a]	0.85 ± 0.68
N-Methyl(*S*)salsolinol	10.9 ± 3.04	0.28 ± 0.44
DMDHIQ$^+$	28.02 ± 9.09[a]	1.58 ± 1.8
(*R*)-1-Carboxy-Sal	3.73 ± 1.75	1.08 ± 1.10
(*S*)-1-Carboxy-Sal	5.72 ± 2.41	1.01 ± 0.78
Norsalsolinol	34.06 ± 5.05[a]	1.63 ± 1.68
N-Methylnorsalsolinol	8.87 ± 0.73	0.13 ± 0.32
(*R*)-THP	0.52 ± 0.62	40.0 ± 6.71[a]
(*S*)-THP	0.07 ± 0.28	41.1 ± 8.10[a]
N-Methyl-THP	13.72 ± 4.48	0.94 ± 1.35
Tetrahydropapaverine	10.13 ± 4.57	1.79 ± 0.50
Papaveroline	32.30 ± 12.37[a]	67.88 ± 12.37[a]
N-Methylpapaveroline	100[a]	0
N-Methylpapaveroline[b]	30.82 ± 3.06[a]	3.93 ± 1.24

The apoptotic and necrotic cells were assessed by morphological observation of four microscopic fields containing about 200 cells, after staining with Hoechst 33258.

[a] The concentrations used were 500 μM, except 250 μM. The number represents mean ± S.D. of three independent experiments.

[b] Difference from control is statistically significant, $P < 0.01$.

Recently, apoptosis of dopamine neurons was confirmed in the substantia nigra of parkinsonian brains: activation of caspase 3, increase in Bax, and translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) into nuclei (Hartmann et al., 2000; Tatton, 2000). *N*M(*R*)Sal induced apoptosis in dopamine neurons, as shown in the rat model, and the mechanism of cell death was studied in human dopaminergic neuroblastoma SH-SY5Y cells (Akao et al., 1999, 2002; Maruyama et al., 1997b, 2001a,b; Naoi et al., 2002). Among catechol isoquinolines, *N*M(*R*)Sal was the most potent to induce DNA damage, whereas *N*M(*S*)Sal, Sal and 1-carboxy-Sal were less cytotoxic, as summarized in Table 5. Apoptosis was initiated by mitochondrial permeability transition (PT) as shown by the collapse in membrane potential, $\Delta\Psi_m$, followed by release of cytochrome *C*, activation of caspase 3, nuclear translocation of GAPDH and final fragmentation of nucleosomal DNA. Only the (*R*)enantiomer of *N*MSal induced $\Delta\Psi_m$ collapse in SH-SY5Y cells, and the (*S*)enantiomer did not (Maruyama et al., 2001a,b). The enantio-specificity to induce apoptosis was confirmed also in isolated mitochondria (Akao et al., 2002). These results suggest the presence of a binding site of *N*M(*R*)Sal in mitochondria outer membrane, which recognizes the enantiomeric structure and initiates apoptotic signal transduction. In the case of THP, the oxidized papaverolines induced apoptosis at the lower concentrations, whereas the reduced tetrahydropapaverolines induced necrosis by the potent inhibition of ATP synthesis in mitochondria (Maruyama et al., 2000).

Racemic Sal (Storch et al., 2000) and 1,2,3,4-tetrahydro-2-methyl-4,6,7-isoquinolinetriol (Willets et al., 1996) were also cytotoxic to dopamine neurons, but a quite different mechanism seems to function in the induction of cell death. These isoquinolines produce reactive oxygen species (ROS) by autoxidation, resulting in metabolic compromise, and necrotic, rather than apoptotic, cell death was induced.

FUNCTION OF SALSOLINOL IN THE BRAIN

The synthesis of Sal from dopamine and acetaldehyde, a product of ethanol, suggested its involvement in alcohol addiction (Cohen and Collins, 1970). The hypothesis that opiates may be biosynthesized from THP-related complex isoquinolines initiated the studies to find the involvement of THP in alcohol addicts possibly mediated by opiates. However, this hypothesis has not been proved after so many contradicting publications concerning the role of Sal and THP in alcoholism.

Recently we found a quite novel function of Sal in the brain. Dopamine as a neuro-hormone is delivered to the anterior lobe through the vascular connection between the hypothalamus and pituitary gland. It inhibits prolactin and maintains mammotropes in their tonically suppressed secretary state (Freeman et al., 2000). Suckling the nipples of lactating mothers elevated plasma prolactin, which may be caused by increased secretion of a prolactin-releasing hormone (PRH) from mammotropes of the anterior lobe of the pituitary galnd. Toth et al. (2001) detected Sal in the neuro-intermediate lobe and median eminence in rats and the levels increased by suckling in lactating rats, as shown in Table 6. The increase was in parallel with plasma prolactin levels. More recently, selective binding of Sal was confirmed in the rat striatum, anterior lobe and neuro-intermediate lobe of the pituitary gland, in addition to the cortex, median eminence and hypothalamus (Homicsko et al., 2002). Their results suggest that vesicular monoamine transporter in dopaminergic terminals may be a target of Sal. The values of the binding constant, K_D, were in the nanomolar ranges. These results suggest that Sal may

Table 6
Concentrations of (*R*)salsolinol in the neuro-intermediate lobe of the pituitary gland and the median eminence

Rats	(*R*)Salsolinol concentration (pmol/mg wet weight)	
	Neuro-intermediate lobe	Median eminence
Control male	$1.67 \pm 1.27^{*}$	$3.86 \pm 2.19^{*}$
Female (ovariectomized)	1.38 ± 0.39	11.96 ± 4.69
Female (ovariectomized + E_2)	$6.38 \pm 2.20^{*}$	$6.25 \pm 2.63^{*}$
Female (sham operated)	30.90 ± 2.6	ND[a]
Female (neuro-intermediate lobe denervated)	$2.60 \pm 0.6^{**}$	ND[a]

Adult male, ovariectomized, overirectomized rats administered with17β-oestradiol (E_2) or primiparous lactating female rats were used (Toth et al., 2001). Sal was measured by HPLC with electrochemical detection (Maruyama et al., 1997a).

[a] Not determined.

* $P < 0.05$ vs. female (ovariectomized).

** $P < 0.05$ vs. female (sham operated).

regulate the function of dopamine neurons as a neurotransmitter and may be a mediator in neuro-endocrine system, though its specified binding sites and intervention in dopamine system.

CONCLUSION

After the discovery of Sal by M. Sandler in 1973, the biosynthesis, metabolism and function of catechol isoquinoline derivatives have been clarified in human brains, as reviewed here. The stereochemical characters of the (*R*)enentiomers induce their selective occurrence and physiological function in human brain. The metabolism in situ, *N*-methylation and oxidation, gives arise novel biological activities, which may be involved in neurodegenerative disorders, such as Parkinson's disease. The recent results on the binding site of Sal suggest further new advances in finding the role of Sal as a mediator of dopamine neurons in the brain. The studies on salsolinol for these near 30 years show that the finding of a new compound in humans can trigger so-wide development of a series of new theories and findings, which should be attributed to M. Sandler.

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N-Propargyl-1 (*R*)-aminoindan, rasagiline, increases glial cell line-derived neurotrophic factor (GDNF) in neuroblastoma SH-SY5Y cells through activation of NF-κB transcription factor

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Abstract

N-Propargyl-1(*R*)-aminoindan, rasagiline, an anti-Parkinson drug, was found to increase the protein and mRNA levels of glial cell line-derived neurotrophic factor (GDNF) in human neuroblastoma SH-SY5Y cells, whereas an analogue without a propargyl residue, aminoindan, did not. GDNF is known to protect dopaminergic neurons in animal and cellular models of Parkinson's disease, and the supplement has been tried for the treatment of degenerating dopamine neurons in Parkinsonian patients. In this paper, intracellular mechanism underlying the induction of GDNF was studied. Rasagiline induced phosphorylation of inhibitory subunit (IκB) of nuclear factor-κB (NF-κB), and translocation of active p65 subunit from cytoplasm into nuclei. Activation of NF-κB was also quantitatively determined by NF-κB p65 transcription assay. Sulfasalazine, an inhibitor of IκB kinase, suppressed the activation of NF-κB and the increase of GDNF by rasagiline simultaneously, further indicating the involvement of the IκB kinase-NF-κB pathway. The results on the activation of the transcription factor by rasagiline are discussed in relation to its possible application as a neuroprotective drug to halt declining of neurons in neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases.
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Keywords: Rasagiline; GDNF; NF-κB; Neuroprotection; Anti-Parkinson drugs; Neurotrophic factors

1. Introduction

In neurodegenerative disorders including Parkinson's disease (PD), hitherto treatments are intended to replace deficient neurotransmitters, such as dopamine. Recently, a new therapeutic strategy has been proposed to protect neurons from cell death by attenuating apoptotic signal transduction (Thompson, 1995). Apoptosis is proposed to be a major type of neuronal death in PD, as shown by the activated apoptotic cascade in the nigro-striatum of Parkinsonian brains (Tatton, 2000).

Recently, (-)deprenyl (selegiline) and related propargylamines were reported to prevent the cell death in neurons caused by various stimuli in animal and cellular models of neurodegenerative disorders. We reported that a series of propargylamines with β-phenethylamine, aminoindan and aliphatic structure protected human dopaminergic neuroblastoma SH-SY5Y cells from apoptosis induced by *N*-methyl(*R*)salsolinol [*NM*(*R*)Sal], an endogenous neurotoxin selective to dopamine neurons, 6-hydroxydopamine,

Abbreviations: BDNF, brain-derived neurotrophic factor; ΔΨm, mitochondrial membrane potential; DTT, dithiothreitol; ECF, enhanced chemifluorescence; EIA, enzyme immunoassay; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotrophic factor; HRP, horse radish peroxidase; IκB, inhibitor subunit of NF-κB; MAP, mitogen-activated protein; MEM, minimum essential medium; NF-κB, nuclear transcription factor kappa B; NGF, neurotrophic growth factor; *NM*(*R*)Sal, *N*-methyl(*R*)salsolinol; PBS, phosphate-buffered saline; PD, Parkinson's disease; SOD, superoxide dismutase; TNF-α, tumor necrosis factor α

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and peroxynitrite (Maruyama et al., 2000, 2001a,b, 2002). Among propargylamines, *N*-propargyl-1(*R*)-aminoindan (rasagiline) was found to be the most potent to suppress apoptosis (Maruyama and Naoi, 1999). Rasagiline is a propargylamine with a cyclic benzylamine, and not metabolized into amphetamine-like compounds as in the case with selegiline (Finberg et al., 1996), and it is now under the phase III trials to treat PD (Parkinson Study Group, 2002).

The mechanism of neuroprotection by propargylamines was studied using SH-SY5Y cells, and rasagiline was found to increase mRNA and protein levels of anti-apoptotic bcl-2 and bcl-xL in SH-SY5Y cells (Akao et al., 2002b). In addition, rasagiline treatment increased the activities of superoxide dismutase (SOD) and catalase in the rat brain regions containing dopamine neurons (Carrillo et al., 2000). These results suggest that rasagiline and related propargylamines may induce anti-apoptotic, pro-survival genes by activation of common intracellular signals, such as transcription factors. Nuclear factor-κB (NF-κB) is one of the most important transcriptional factors and its activation was reported to be essential for neuronal survival mediated by tumor necrosis factor-α (TNF-α) and delayed preconditioning (Blondeau et al., 2001; Kaltschmidt et al., 1999; Mattson et al., 2000). The activation of NF-κB was reported to induce pro-survival Bcl-2 and Bcl-xL, SOD, brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), a selective neurotrophic factor for dopamine neurons (Tomac et al., 1995; Tamatani et al., 1999). NF-κB is a redox-sensitive transcription factor and activated by various pathogenic stimuli, including oxidative stress, ischemic insult and β-amyloid deposition. Commonly, NF-κB consists of two 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. However, it has never been reported whether activated NF-κB mediates the anti-apoptotic function of neuroprotective agents, such as propargylamines.

This paper reports the increase of GDNF level by rasagiline in SH-SY5Y cells, in addition to previously reported Bcl-2. The mechanism underlying the gene induction of GDNF was studied in concern to the activation of NF-κB. The possibility to develop a new neuroprotective therapy for PD and other neurodegenerative diseases is discussed.

2. Materials and methods

2.1. Materials

(*R*)(+)-*N*-Propargyl-1-aminoindan (rasagiline) prepared as reported previously (Youdim et al., 1995) was kindly donated by Teva Pharmaceutical Co. (Netanya, Israel). Lactacystin and sulfasalazine were purchased from Sigma (Missouri, St. Louis, USA); NF-κB p65 transcription assay kit (Trans-AM NF-κB) from Active Motif (Carlsbad, CA, USA); enhanced chemifluorescence (ECF) Western blotting kit from Amersham Life Science (Buckinghamshire, UK). The lysis buffer for immunoblotting, RIPA buffer kit (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors), was obtained from Boehringer Mannheim (Mannheim, Germany). Anti-NF-κB p65 antibody (#100-4165) was purchased from Rockland (Gilbertsville, PA, USA); anti-IκB and anti-phosphorylated IκB (p-IκB) (Ser32/36) antibodies were from Cell Signaling (Beverly, MA, USA). Dulbecco's modified Eagle minimum essential medium (MEM) and other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Cell culture

SH-SY5Y cells were cultured in 75 cm^2 flasks at 37 °C in 95% atmosphere and 5% CO_2 and the culture medium used was Cosmedium-001 (CosmoBio, Tokyo, Japan) supplemented with 5% newborn calf serum.

2.3. Quantitative measurement of the protein and mRNA levels of GDNF

SH-SY5Y cells were cultured in 96-well poly-L-lysine coated plates (2×10^4 cells per well) in Cosmedium-001 with 5% calf serum for 24 h, then the medium was changed to MEM with or without rasagiline or aminoindan (the final concentration, 1 μM–10 nM) for 3 h, or with 100 nM of rasagiline for 3, 6, 18 and 24 h. The effect of sulfasalazine (100 μM), an inhibitor of IκB kinase (Wahl et al., 1998), was examined by adding the inhibitor 30 min before the treatment with rasagiline. After the treatment, the cells and the medium were frozen immediately and stored at −20 °C until analysis. The protein amount of GDNF was quantified as reported previously using the enzyme immunoassay (EIA) (Nitta et al., 2002). GDNF used as a standard was donated by Amgen (Munich, Germany). Anti-GDNF antibody was prepared and purified from rabbit serum (Nitta et al., 1999). The 96-well plates (Falcon 3910, NJ, USA) were incubated with 5 μl of anti-GDNF antibody in 500 μl of 0.1 M Tris–HCl buffer, pH 9.0, per well overnight, washed with the washing buffer (0.1 M Tris–HCl buffer, pH 7.4, containing 0.4 M NaCl, 0.02% Na_3N, 0.1% bovine serum albumin and 1 mM $MgCl_2$), and then blocked with the washing buffer containing 1% skim milk. Samples or standard in the washing buffer were added to GDNF antibody-coated wells, and the plate was incubated for 12–18 h at 4 °C. The biotinylated secondary antibody was reacted in avidin-conjugated β-galactoside (Boebringer Mannheim) for 1 h. After washing with the washing buffer, the enzyme activity retained in each well was measured by incubation with a fluorescent

substrate, 4-methylumbelliferyl-β-D-galactoside (100 μM) in the washing buffer. The fluorescence intensity of a product, 4-methylumbelliferone, was measured at 360 nm with excitation at 448 nm. Standard curve of human recombinant GDNF from 5.08 pg/ml to 3.3 ng/ml was used.

For the measurement of mRNA of GDNF by reverse transcription-polymerase chain reaction (RT-PCR), the cells were treated with 1 μM–10 nM of rasagiline for 3 h and the total RNA was isolated using a TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). To avoid DNA contamination, the samples were subjected to DNase I (Invitrogen). Single strand cDNA was synthesized from 1 μg total RNA in a volume of 20 μl containing 0.5 μg oligo(dT)12–18 primer (Invitrogen), 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 units of RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 10 mM DTT and 200 units of MMLV reverse transcriptase (SuperScript™ II, Invitrogen). The reaction mixture was incubated for 50 min at 42 °C, terminated by heating for 15 min at 70 °C with 37 cycles. Under this condition, the linearity of mRNA increment to the number of PCR cycles was confirmed. To remove RNA complementary to the cDNA, 2 units of RNase H were added and incubated for 20 min at 37 °C. PCR was performed at an annealing temperature of 57 °C using the following primers: GDNF, 5′ CGGGACTCTAAGATGAAGTTATGGGATGTCGTG-3′ and 5′-GGGTCAGATACATCCACACCGTTTAGCGGAATGC-3′, giving a 651-bp product; GAPDH, 5′-GGAGATTGTTGCCATCAACGAC-3′ and 5′-ATGAGCCCTTCCACAATGCCAAAG-3′, giving a 441-bp product.

2.4. Western blot analysis of NF-κB, IκB and phosphorylated IκB

NF-κB p65 translocated into nuclei was identified by Western blotting of the subcellular fractions using ECF Western blotting kit. SH-SY5Y cells were cultured in 75 cm^2 flasks with Cosmedium-001 and 5% calf serum overnight. The medium was changed to MEM with rasagiline (the final concentrations, 1 and 0.1 μM) and incubated at 37 °C. The cells were harvested and washed twice by PBS, suspended in 10 mM sodium phosphate buffer, pH 7.4, then allowed to stand at 4 °C for 30 min. To the cell suspension, the equal volume of 0.64 M sucrose in 10 mM sodium phosphate buffer, pH 7.4, was added. The sample was homogenized and centrifuged at 800 × *g* for 15 min. The pellet and the supernatant were used as nuclear and cytoplasmic fraction, respectively. The nuclear sample was prepared from pellet dissolved in the RIPA buffer by sonication and centrifugation. The purity of the nuclear fraction was confirmed by detection of the nuclear and cytoplasmic markers, using anti-C23 and anti-14-3-3β antibodies (Santa Crutz Biotechnology, Santa Cruz, CA, USA), respectively. β-Actin and glyceraldehyde-3-phosphate dehydrogenase were used as controls to confirm protein amounts loaded to SDS-polyacrylamide gel electrophoresis.

Since immediate degradation of phosphorylated IκB by proteasome hinders its immunoblot detection, SH-SY5Y cells were pre-treated with 10 μM of lactacystin, a proteasome inhibitor, for 4 h, and then treated with 100 nM of rasagiline for 0, 5, 15, and 30 min according to Casciati et al. (2002). The cells were washed twice with PBS and lysed in the RIPA buffer. β-Actin was used as a loading control. The protein concentration was quantified according to Bradford (1976). The lysate (40 μg protein per well) was subjected to SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gel, and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were treated with Block Ace (Yukijirushi, Sapporo, Japan) for 1 h, and incubated with the first antibody overnight at 4 °C. The membranes were incubated with the second antibody conjugated with fluorescein, then with the anti-fluorescein antibody conjugated with alkaline phosphatase, and finally with an ECF substrate. The fluorescence at 540 nm was measured with excitation at 488 nm using a Fluoroimage analyzer (Amersham Pharmacia, Pyscataway, NJ, USA).

2.5. 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 according to Kretz-Remy et al. (2001), who reported the validity of this method to measure activated NF-κB. The principle of this assay is to measure the binding of activated NF-κB p65 to an oligonucleotide containing the NF-κB consensus-binding site. SH-SY5Y cells were cultured in six-well flasks with Cosmedium-001 containing 5% calf serum for 24 h. After the medium was changed to MEM, the cells were treated with 100 nM of rasagiline for 30–120 min or with 10 μM–10 nM of rasagiline for 60 min. The effect of sulfasalazine, an inhibitor of IκB kinase, was examined by incubation of the cells with the inhibitor (100 μM) for 30 min before rasagiline treatment. The cells were mechanically harvested, washed with PBS twice, lysed in 100 μl of the Lysis buffer [20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Igapal CA-630 (Sigma), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 50 mM DTT and a protease inhibitor cocktail]. Twenty micrograms of protein in the Binding buffer (4 mM HEPES buffer, pH 7.5, containing 120 mM KCl, 8% glycerol, 1% bovine serum albumin, 10 μg/ml Herring sperm DNA, 2 mM DTT) was applied to each well coated with oligonucleotide (5′-GGGACTTTCC-3′) corresponding to the NF-κB binding site, and incubated for 60 min at the room temperature. The wells were washed three times with 10 mM phosphate buffer, pH 7.5, containing 50 mM NaCl and 0.1% Tween 20, then incubated with p65 NF-κB antibody for 60 min, washed again three times, and incubated with horse radish peroxidase (HRP)-conjugated antibody for 60 min. Using a substrate, 3,3′,5,5′-tetramethylbenzidine, the activity of HRP was measured spectrophotometrically at 450 nm with reference at 655 min. Five micrograms 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 percentage of the positive control. To confirm the specificity of the NF-κB binding to the oligonucleotide, 20 pmol of wild or mutated NF-κB oligonucleotide was added in the reaction mixture and the binding activity of NF-κB was compared with that without addition of the oligonucleotide.

2.6. Statistics

Experiments were repeated four times and the data were shown as the mean ± S.D. Difference was statistically evaluated by analysis of variance (ANOVA) followed by Sheffe's F-test. A P-value <0.05 was considered to be statistically significant.

3. Results

3.1. Rasagiline increased protein and mRNA level of GDNF

The effect of rasagiline on GDNF protein level was examined by the EIA. The assay system of GDNF was specific for GDNF, and no cross-reactivity was observed with other TGF-β super family proteins (Nitta et al., 2002). Rasagiline markedly increased GDNF protein level in SH-SY5Y cells, whereas the level in the culture medium was lower than the detection limit, 0.5 pg/ml. The increase was observed most markedly with 100 nM of rasagiline, but aminoindan did not affect GDNF level at any concentrations used in the experiments (Fig. 1A). Rasagiline at 100 nM increased GDNF protein to six folds of the basal level after 3 h incubation and the level was sustained for 24 h (Fig. 1B). Rasagiline induced GDNF mRNA significantly at 100 nM after 3 h incubation, as shown in Fig. 1C. The concentration increasing the mRNA level corresponded to that of the most optimal to increase GDNF protein level. Rasagiline at higher than 1 μM or lower than 1 nM did not affect the protein and mRNA levels of GDNF, showing an inverted U-shaped curve of concentration-activity relationship. On the other hand, in control and the cells treated with rasagiline at other concentrations, mRNA level of GDNF was lower than the detection limit.

3.2. Activation of NF-κB by rasagiline

To examine the involvement of transcription factors in the induction of prosurvival genes, the effects of rasagiline

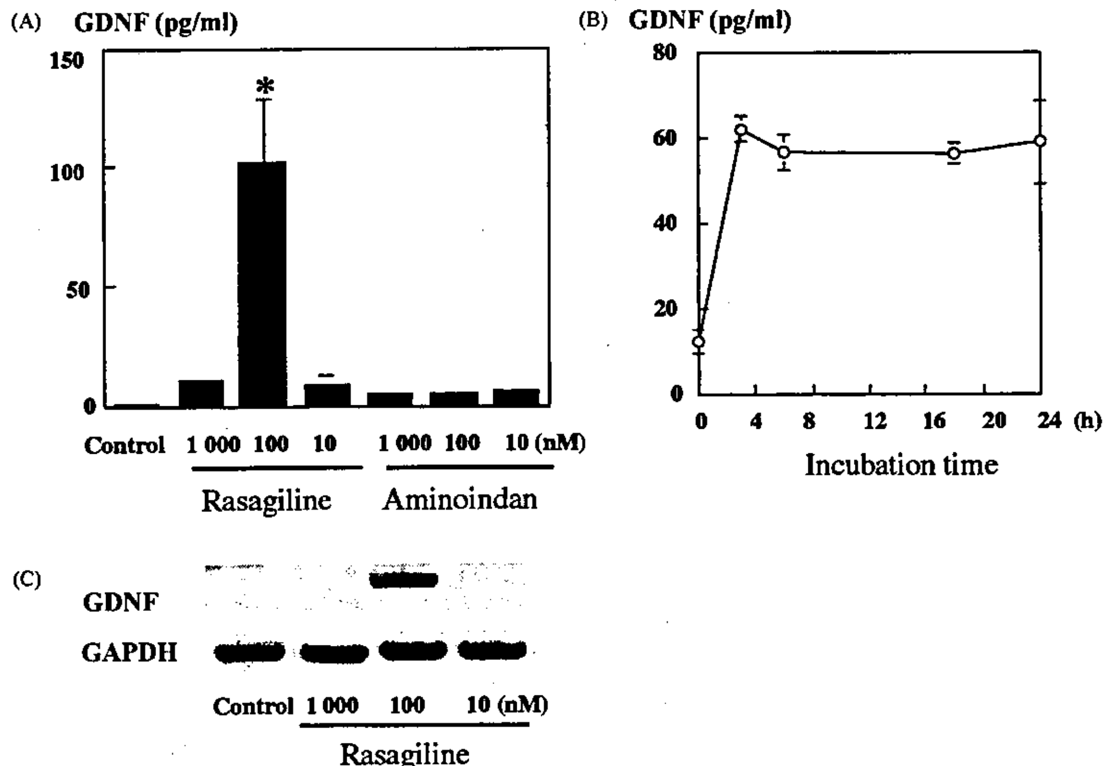

Fig. 1. Effects of rasagiline and aminoindan on the protein and mRNA level of GDNF in SH-SY5Y cells. (A) SH-SY5Y cells were treated with 1 μM–10 nM of rasagiline or aminoindan for 3 h, and GDNF protein level was analyzed by the EIA, as described in Section 2. Each column and bar represent the mean and S.D. of four experiments. (*) The difference from the control is statistically significant ($P < 0.01$) by ANOVA. (B) SH-SY5Y cells were treated with 100 nM of rasagiline for 3–24 h and the amount of GDNF protein was analyzed by the EIA. Each circle and bar represent the mean and S.D. of four experiments. (C) Effect of rasagiline on mRNA level of GDNF. SH-SY5Y cells were treated with 1 μM–10 nM of rasagiline for 3 h. Total RNA was extracted and subjected to RT-PCR for GDNF as described in Section 2. GAPDH was used as control.

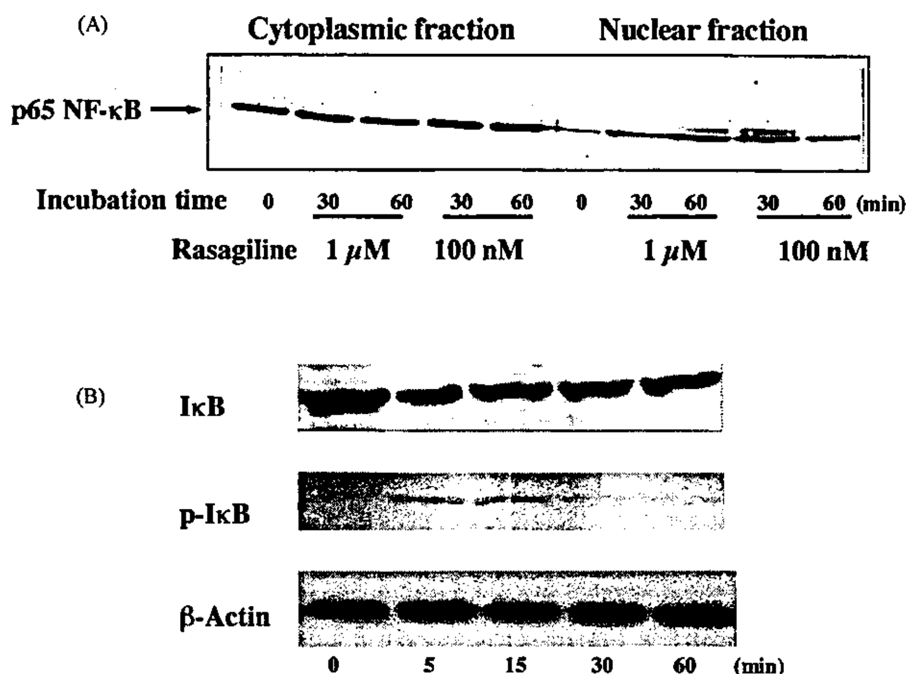

Fig. 2. Rasagiline induced nuclear translocation of p65 NF-κB and phosphorylation of IκB. (A) SH-SY5Y cells were treated with 1 M–100 μM of rasagiline for 30 and 60 min and then the cytoplasmic and nuclear fraction were isolated as described in Section 2. The samples were subjected to Western blotting analysis using anti-p65 NF-κB antibody. (B) SH-SY5Y cells were treated with a proteasome inhibitor, lactacystin, for 4 h and then with 100 nM of rasagiline. The total cell lysate was analyzed by immunoblotting using antibodies against IκB (IκB), phosphorylated IκB (p-κB), and β-actin (β-actin) as control.

were studied on the activation of NF-κB, a common transcription factor for GDNF, BDNF, Bcl-2 and SOD, whose levels were increased by propargylamines. After treatment of SH-SY5Y cells with rasagiline at 1 μM and 100 nM, the p65 subunit of NF-κB in the cytoplasmic and nuclear fraction was analyzed by immunoblotting, as shown in Fig. 2A. The p65 subunit increased significantly in the nuclear fraction after 30 and 60 min incubation with rasagiline. Phosphrylation of IκB was detected in the cells after 5–15 min treatment with 100 nM of rasagiline (Fig. 2B).

The activation of p65 NF-κB was also shown by the increase in its binding activity to oligonucleotide containing the NF-κB consensus-binding site, as shown in Fig. 3A. The binding capacity increased in the cells treated with rasagiline at 1 μM–10 nM for 60 min (Fig. 3B), and the highest binding activity was detected at 100 nM rasagiline. The effects of rasagiline concentration on the binding capacity also followed an inverted U-shaped dose-response curve. The specificity of the binding was examined by competition studies with the wild and mutated oligonucleotide, and the binding was inhibited by the wild, but not by the mutated, oligonucleotide (Fig. 3C).

The role of NF-κB in increasing GDNF was studied further by use of an inhibitor of IκB kinase, sulfasalazine. Sulfasalazine at 100 μM suppressed the increase in GDNF protein level by rasagiline to the basal level, as shown in Fig. 4. Sulfasalazine inhibited also rasagiline- induced increase in the binding capacity of NF-κB to the oligonucleotide containing the binding site, as shown in Fig. 3D.

4. Discussion

In this paper, rasagiline was proved to increase the mRNA and protein levels of GDNF, in addition to those of anti-apoptotic bcl-2 and bcl-xL as previously reported (Akao et al., 2002b). Selegiline and desmethylselegiline, propargylamines structurally related to rasagiline, were reported to increase mRNA level of neuroprotective proteins, bcl-2, SOD, glutathione peroxidase and GDNF in PC12 cells (Tatton et al., 2002). The results reported in this paper clearly show that the activation of NF-κB by rasagiline mediates the increase in the transcription of pro-survival genes. In our previous papers, rasagiline protected SH-SY5Y cells from apoptosis induced by a neurotoxin, *N*M(*R*)Sal, through the stabilization of mitochondrial membrane potential ($\Delta\Psi m$) (Akao et al., 2002a; Maruyama et al., 2000), and the increased expression of Bcl-2 and Bcl-xL (Akao et al., 2002b). It may be interesting enough, since Bcl-2 was confirmed to prevent apoptosis in SH-SY5Y cells (Maruyama et al., 2001a) and also permeability transition in isolated mitochondria induced by the neurotoxin (Akao et al., 2002a). In neurodegenerative disorders, such as PD and Alzheimer's diseases, particular neurons deteriorate in slow and continuous process, where not only apoptogenic,

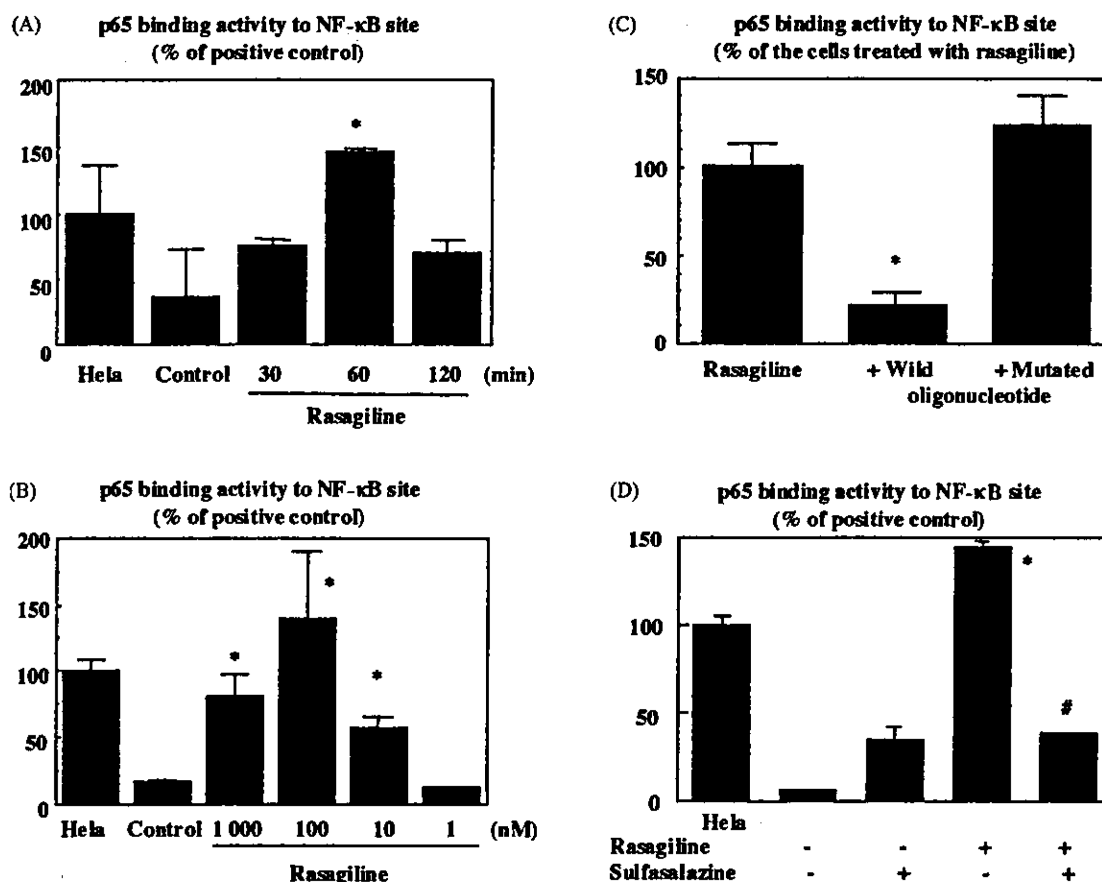

Fig. 3. Increase in NF-κB binding activity by rasagiline and the suppression by NF-κB oligonucleotide and sulfasalazine The lysate of the Hela cells treated with TNF-α: (Hela) was used as a positive control and the amounts of activated NF-κB were expressed as percentage of Hela (A, B, D) or the lysate of the cells treated with 100 nM of rasagiline alone for 60 min (C). (A) The effects of the incubation time were examined in SH-SY5Y cells treated with 100 nM of rasagiline for 30, 60 and 120 min. The activation of NF-κB in the cell lysate was quantified as the p65 binding activity to the NF-κB binding oligonucleotide by use of the NF-κB p65 transcription assay kit as described in Section 2. (*) The difference from the control is statistically significant ($P < 0.01$) by ANOVA. (B) The effects of rasagiline concentration were examined in SH-SY5Y cells treated with 1 μM–1 nM of rasagiline for 60 min and the amounts of activated NF-κB was measured. Each column and bar represent the mean and S.D. of four experiments. (*) The difference from the control (C) is statistically significant ($P < 0.01$) by ANOVA. (C) The specificity of NF-κB binding to the binding site was examined. SH-SY5Y cells were treated with 100 nM of rasagiline for 60 mm, and the p65 binding activity was measured in the presence or absence of wild (+Wild) or the mutated (+Mutated) oligonucleotide containing the NF-κB binding site. Each column and bar represent the mean and S.D. of four experiments. (*) The difference from the binding activity of the cell lysate without oligonucleotide is statistically significant ($P < 0.01$) by ANOVA. (D) Effects of an inhibitor of IκB kinase, sulfasalazine, were examined in SH-SY5Y cells treated with 100 μM of sulfasalazine for 30 min, then, 100 nM of rasagiline. Activated NF-κB in the cells was quantified by the NF-κB p65 transcription assay kit. (*) The difference from the control is statistically significant ($P < 0.01$) by ANOVA. (#) The difference from the cells treated with rasagiline alone is statistically significant ($P < 0.01$) by ANOVA.

but also pro-survival factors should be activated. NF-κB may be a common regulator collecting the information of upstream signal events to decide the survival and death of the cells (Grilli and Memo, 1999). Increased levels of NF-κB were detected in the brain of Alzheimer's disease (Kaltschmidt et al., 1997) and PD (Hunot et al., 1997). In apoptosis induced by excitotoxicity and oxidative stress, NF-κB was activated (Qin et al., 1998), and the cytotoxicity was mediated by the activation of pro-apoptotic members of Bcl-2 family (Shou et al., 2002). On the contrary, NF-κB was reported to be involved in cytoprotection against apoptosis induced by oxidative stress and excitotoxic insults (Goodman and Mattson, 1996). These controversial 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 induces cell death.

The molecular mechanisms underlying the activation of NF-κB is now on the way for elucidation (Karin and Ben-Nariah, 2000) NF-κB is activated by IκB phosphorylation by kinase complex (IκB kinase, IKK) composed with IKK-α, -β and -γ. When the IKK complex is phosphorylated, IκBα and IκBβ were cleaved at two serine residues in the N-terminal and dissociated, resulting in the activation of NF-κB. Sulfasalazine, an inhibitor of IKK-α and -β (Weber et al., 2000), inhibited the NF-κB activation and the

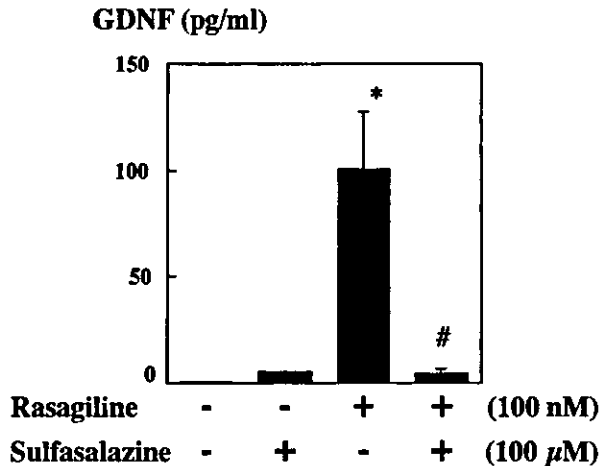

Fig. 4. Effects of sulfasalazine on the increase in GDNF protein by rasagiline. SH-SY5Y cells were incubated with or without 100 μM of sulfasalazine for 30 min, and then treated with 100 nM of rasagiline for 3 h. GDNF protein level was measured by the EIA. Each column and bar represent the mean and S.D. of four experiments. (*) The difference from control is statistically significant ($P < 0.01$) by ANOVA. (#) The difference from the cells treated with rasagiline alone is statistically significant ($P<0.01$) by ANOVA.

induction of GDNF protein, simultaneously. These results suggest that rasagiline may activate NF-κB through the IKK pathway to induce GDNF. It requires further studies to clarify whether rasagiline may affect directly the IKK components or indirectly through activation of upstream kinases in the cells. Recently, rasagiline and TV3324, a rasagiline analogue with a carbamyl moiety, were reported to activate protein kinase C and Erk1/2 mitogen-activated protein (MAP) kinase (Yogev-Falach et al., 2002). These results suggest that the propargylamines may target protein regulating signal transduction in MAP kinase system.

The potency of rasagiline to activate NF-κB and increase GDNF did not linearly depend on the concentration, but followed an inverted U-shaped dose-response curve. We observed also that rasagiline showed such an inverted U-shaped relationship in the anti-apoptotic function (Maruyama et al., 2001b) and in the induction of Bcl-2 (Akao et al., 2002b). It was reported that TNF-α, reactive oxygen species (H_2O_2) and also β-amyloid activated NF-κB according to an inverted U-shaped dose-response curve. At the low concentration, TNF-α was neuroprotective in correlation with NF-κB activation, whereas at the high concentration it was neurotoxic (Kaltschmidt et al., 1999). The mechanism behind inverted U-shape type of the concentration-activity relationship of NF-κB remains to be fully elucidated.

GDNF is now proposed as an agent to rescue declining dopamine neurons in PD as shown by the effectiveness in animal and cellular models (Wang et al., 2002). Lentivirally-delivered GDNF was reported to rescue dying dopamine neurons in a monkey model of PD prepared with l-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (Palfi et al., 2002). Infusion of GDNF protein by an implanted intracerebroventricular catheter failed to improve the symptoms in Parkinsonian patients, (Nutt et al., 2003). However, recently it was reported that the direct infusion of GDNF into the putamen of Parkinsonian patients improved clinical symptoms and fluorodopa uptake to the dopamine terminal (Gill et al., 2003). These results indicate that GDNF supplement therapy requires further technical improvement for the administration. If rasagiline, which is now under phase III clinical trials for Parkinsonian patients (Parkinson Study Group, 2002), can also increase endogenous GDNF in the human brain, as shown here in SH-SY5Y cells, it may be a more practical therapy to prevent cell death of dopamine neurons.

In conclusion, rasagiline and related propargylamines are possible neuroprotective agents and a part of their pharmacological action may be due to the induction of pro-survival genes through NF-κB activation. The activation of transcription factors related to anti-apoptotic proteins, GDNF and bcl-2 (Maruyama et al., in preparation), by propargylamines might enable us to suppress the neuronal apoptosis in neurodegenerative disorders in general. Clinical trials with propargylamines are waiting for the final evaluation of the efficacy to protect specified neurons from degeneration.

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Neuroprotective function of *R*-(−)-1-(benzofuran-2-yl)-2-propylaminopentane, [*R*-(−)-BPAP], against apoptosis induced by *N*-methyl(*R*)salsolinol, an endogenous dopaminergic neurotoxin, in human dopaminergic neuroblastoma SH-SY5Y cells

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Abstract

R-(−)-1-(Benzofuran-2-yl)-2-propylaminopentane HCl [*R*-(−)-BPAP] is one of "catecholaminergic and serotonergic enhancers", which were proposed to improve symptoms through increase in impulse-evoked release of monoamine neurotransmitters for Parkinson's disease. It was reported that (−)-BPAP up-regulated the synthesis of neurotrophic factors in mouse astrocytes, suggesting the neuroprotective potency of (−)-BPAP. In this paper, the neuroprotective function of (−)-BPAP and the related compounds was examined against apoptosis induced by an endogenous neurotoxin, *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal], a possible pathogenic toxin in Parkinson's disease, in human dopaminergic neuroblastoma SH-SY5Y cells. The anti-apoptotic activity was confirmed with some of (−)-BPAP analogues, and the mechanism was found to be due to the direct stabilization of mitochondrial membrane potential and the induction of anti-apoptotic Bcl-2. The studies on structure-activity relationship demonstrated that the potency to stabilize the mitochondrial membrane potential depended on the absolute stereo-chemical structure of BPAP derivatives. The compounds with dextrorotation prevented the mitochondrial permeability transition, whereas those with levorotation did not. The presence of a propargyl or

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propyl group at the amino residue of *R*-(−)-1-(benzofuran-2-yl)-2-propylamine increased potency to stabilize the membrane potential and prevent apoptosis. *R*-FPFS-1169 and *R*-FPFS-1180 had more potent to induce Bcl-2 and prevent apoptosis than the corresponding *S*-enantiomers. These results are discussed with the possible application of BPAP derivatives as neuroprotective agents in Parkinson's disease and other neurodegenerative disorders.
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Keywords: *R*-(−)-1-(Benzylfuran-2-yl)-2-propylaminopentane [*R*-(−)-BPAP]; Apoptosis; Bcl-2; Mitochondrial membrane potential; Parkinson's disease; Neuroprotection

Introduction

For neurodegenerative diseases, Parkinson's disease (PD) and Alzheimer's disease (AD), hitherto available therapy intends to replace or increase deficient neurotransmitters, using the metabolic precursors, such as L-DOPA, or the inhibitors of metabolizing enzymes, monoamine oxidase, catechol-O-methyltransferase and cholinesterase. As another strategy of the therapy, some compounds including (−)-enantiomers of deprenyl, amphetamine and methamphetamine, and β-phenylethanolamine, were proposed to enhance the impulse-evoked release of catecholamine and serotonin (Knoll et al., 1996). Among catecholaminergic-serotonergic enhancers, (−)-1-phenyl-2-propylaminopentane [(−)-PPAP] and *R*-(−)-(benzofuran-2-yl)-2-propylaminopentane [*R*-(−)-BPAP, the development number; FPFS-1169] are the most promising agents (Knoll et al., 1999). They do not inhibit type B monoamine oxidase and are not metabolized to amphetamine, in contrast to (−)-deprenyl.

Recently neuroprotection to halt progressive cell death of neurons has been proposed as a future therapy for neurodegenerative disorders. In these disorders, such as PD and AD, apoptosis contributes to neuronal death in most cases (Tatton, 2000) and the well regulated and relatively slow apoptotic process was proposed as a target of neuroprotection (Thompson, 1995; Naoi and Maruyama, 2001). Apoptosis is induced in neurons by various insults; oxidative stress, metabolic compromise, excitotoxicity and neurotoxins. Apoptotic signaling is a multi-step pathway induced by opening a mitochondrial megachannel called permeability transition (PT) pore, followed by decline in membrane potential, $\Delta\Psi m$, release of apoptosis-inducing factors, activation of caspases and fragmentation of nuclear DNA. Mitochondrial PT pore is regulated by Bcl-2 protein family, preventively by Bcl-2 and Bcl-xL and promotively by BAX and BAD.

After the discovery of (−)-deprenyl as an agent preventing cell death in the animal and cellular models of neurodegenerative disorders (Finnegan et al., 1990; Heikkila et al., 1984), a series of propargylamines with β-phenylethylamine [(−)-deprenyl], cyclic benzylamine [*N*-propargyl-1(*R*)-aminoindan, rasagiline] and aliphatic structure [*N*-(2-heptyl)-*N*-methylpropargylamine] were confirmed to protect neurons against apoptosis induced by various insults (Maruyama and Naoi, 1999; Maruyama et al., 2000, 2001a, 2001b, 2002). However, the effectiveness of these compounds as neuroprotective agents has not been fully confirmed in clinical studies, maybe because of difficulty to evaluate neuroprotective effects in patients. Nevertheless, significant insights into the anti-apoptotic function of propargylamines have been well achieved, and mitochondria emerge as a key organelle playing a role in apoptosis. The regulation of mitochondrial PT was found to be critical for decision of cell survival and death (Naoi et al., 2002). Our studies show that the neuroprotection of propargylamines is ascribed to (1) the stabilization of mitochondria membrane potential, $\Delta\Psi m$, and prevention of PT (Maruyama et al.,

2001a, 2001b), (2) the induction of anti-apoptotic Bcl-2 family regulating PT (Akao et al., 2002a, 2002b) and (3) of neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF) selective to dopamine neurons (Maruyama et al., 2004), and (4) of anti-oxidant enzymes, such as superoxide dismutase and catalase (Carrillo et al., 2000). *R*-(–)-BPAP was found to increase the biosynthesis and secretion of neurotrophins; such as GDNF, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), in mouse asctrocytes, suggesting its neuroprotective potency (Ohta et al., 2002; Shimazu et al., 2003).

This paper describes that (–)-BPAP and related benzofuran derivatives suppressed apoptosis induced by a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol [*R*(1),*N*-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, *N*M(*R*)Sal] (Maruyama et al., 1996, 2001b; Naoi et al., 1998) in human dopaminergic neuroblastoma SH-SY5Y cells. The structure-activity relationship and the mechanism underlying the anti-apoptotic function were studied. The results are discussed in relation to the possible application of (–)-BPAP analogues to the neuroprotective therapy for PD and other neurodegenerative diseases.

Materials and methods

Materials

(–)-BPAP and other related benzofuran-2-yl derivatives were synthesized by Fujimoto Pharmaceutical Corp. (Osaka, Japan), and *N*M(*R*)Sal according to Teitel et al. (1972). Rhodamine 123, YO-PRO and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR, USA), Dulbecco's modified Eagle's medium (DMEM) and other drugs from Nacarai tesque (Kyoto, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in an atmosphere of 95% air – 5% CO_2.

Assessment of apoptosis induced by NM(R)Sal and the protection by BPAP derivatives

Apoptosis was quantitatively measured by fluorescence-augmented flow cytometry (FACS) with FACScan and CellQuest software (Becton Dickinson, San Jose, CA, USA). Cells cultured in a 6-well poly-L-lysine-coated culture flask were incubated with or without 1 μM – 1 nM (–)-BPAP analogues at 37°C for 30 min, and then for 24 h with 250 μM *N*M(*R*)Sal in Cosmedium-001 culture medium supplemented with fetal calf serum. The cells were treated with trypsin, gathered, washed with the culture medium and twice with phosphate-buffered saline (PBS). The cells were incubated with 100 nM YO-PRO and 1.5 μM PI solution in an ice-bath for 30 min, washed and suspended in PBS, then subjected to FACS analysis.

Measurement of changes in $\Delta\Psi m$

Decline in $\Delta\Psi$m induced by *N*M(*R*)Sal was quantified by measuring the reduction of Rhodamine 123 fluorescence pre-loaded in the cells (Patorino et al., 1996), as reported previously (Akao et al., 2002a). To examine the effects of (–)-BPAP analogues, the cells cultured in 6-well poly-L-lysine-coated tissue culture flasks were stained with 5 μM Rhodamine 123 in DMEM for 30 min at 37°C. After washed twice with PBS, the cells were suspended in DMEM, incubated with 1 μM – 1 nM BPAP derivatives for

30 min, then with 250 μM *N*M(*R*)Sal for 1 h. After washed and gathered by treatment with trypsin, the cells were suspended in PBS and the fluorescence at 535 nm was measured with excitation at 505 nm in a Shimadzu spectrofluorophotometer, RF-5000 (Kyoto, Japan).

Measurement of bcl-2 mRNA level in the cells treated with BPAP derivatives

SH-SY5Y cells were cultured in the presence of various concentrations (100 nM-10 pM) of (−)-BPAP analogues for 24 h, and mRNA levels of bcl-2 were quantitatively assessed by RT-PCR method (Akao et al., 2002a, 2002b). The cells were gathered and washed with PBS, and the total RNA was extracted by the phenol/guanidinium thiocyanate method. cDNA was generated by reverse transcription of 2 μg of the total RNA, and the cDNA fragments were amplified using the PCR primers. The linearity of the amount of PCR product to the time of PCR amplification was confirmed under the conditions used in this study. PCR products were analyzed by electrophoresis on 3% agarose gels, and β-actin was used as an internal standard. The amounts of mRNA were quantified using NIH imaging software (version 1.62, developed at the U.S. National Institute for Health).

Measurement of Bcl-2 levels in the cells treated with (–)-BPAP derivatives

SH-SY5Y cells treated with 1 μM-1 pM (−)-BPAP analogues for 24 h, and the cells were gathered, washed with PBS and suspended in RIPA buffer [10 mM Tris-HCl buffer, pH 7.5, containing 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl and 1 mM EDTA 2Na]. The lyzed protein (5 μg) was separated by SDS-PAGE using a 10 – 20% gradient polyacrylamide gel (Bio-Rad Lab., Hercules, CA, USA) and electroblotted onto PVDF membranes (Du Pont, Boston, MA, USA). After blockage with 5% nonfat milk in PBS containing 0.1% Tween 20, the membrane was incubated overnight

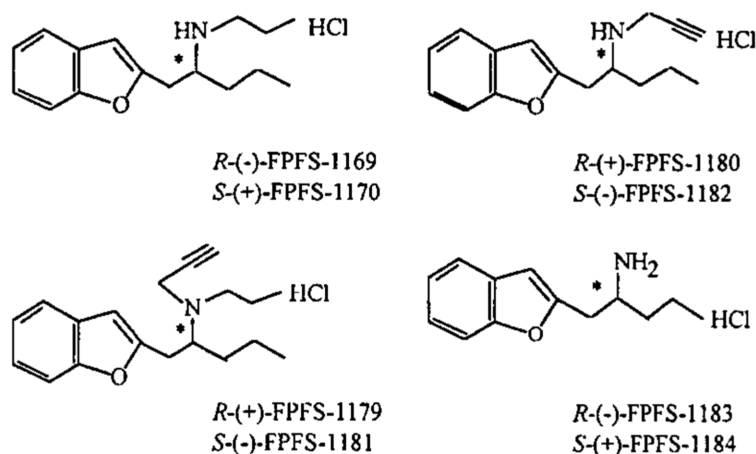

Fig. 1. Chemical structure and abbreviations of used BPAP derivatives. FPFS-1169 and FPFS-1170: *R*-(−)- and *S*-(+)-1-(benzofuran-2-yl)-2-propylamino-pentane hydrochloride, FPFS-1180 and FPFS-1182: *R*-(+)- and *S*-(−)- *N*-(2-propynyl)-1-(benzofuran-2-yl)-2-aminopentane hydrochloride, FPFS-1179 and FPFS-1181: *R*-(+)- and *S*-(−)-*N*-(2-propynyl)-1-(benzofuran-2-yl)-2-propylaminopentane hydrochloride, FPFS-1183 and FPFS-1184: *R*-(−)- and *S*-(+)-1-(benzofuran-2-yl)-2-aminopentane hydrochloride.