ELSEVIER

European Journal of Pharmacology 294 (1995) 577–583

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The vesicular monoamine transporter is not regulated by dopaminergic drug treatments

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Received 29 May 1995; revised 15 September 1995; accepted 19 September 1995

Abstract

The number of neuronal synaptic vesicular monoamine transporters (vesicular monoamine transporter type 2; VMAT2) has been recently proposed as an index of monoamine presynaptic terminal density. The present study investigated the possible regulation of the vesicular monoamine transporter. Rats were treated for 2 weeks with drugs known to influence dopaminergic neurotransmission, including those commonly used in the treatment of Parkinson's disease. Autoradiographic assays were performed using [^{3}H]methoxytetrabenazine, [^{3}H]raclopride, and [^{3}H]WIN 35,428 ([^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane) to measure vesicular monoamine transporter, dopamine D_2 receptor and synaptic plasma membrane dopamine re-uptake site bindings, respectively. None of the drug treatments significantly modified levels of vesicular monoamine transporter binding. In contrast, both dopamine D_2 receptors and dopamine re-uptake sites were altered by some of the treatment regimens. These data extend preliminary results that suggest the vesicular monoamine transporter is not easily regulated and confirm the plasticity of dopamine D_2 receptors and the dopamine re-uptake site. Measures of striatal vesicular monoamine transporter density may, thus, provide objective estimates of monoaminergic innervation in neurodegenerative diseases, unaffected by the use of symptomatic therapies.

Keywords: Vesicular monoamine transporter; VMAT2 (vesicular monoamine transporter type 2); Dopamine D_2 receptor; Presynaptic dopamine re-uptake site, presynaptic; Dopamine; Methoxytetrabenazine

1. Introduction

1.1. Biochemical markers of dopaminergic neurons

A variety of neurochemical markers have been proposed to quantify dopaminergic losses in neurodegenerative diseases in vivo using positron emission or single photon emission tomography. Prior studies employing [^{18}F]fluoroDOPA (6-[^{18}F]fluoro-L-3,4-dihydroxyphenylalanine) or radioligands which bind to the presynaptic plasma membrane dopamine re-uptake site including [^{11}C]nomifensine, [^{11}C]WIN 35,428 ([^{3}H]2β-carbomethoxy-3β-(4-fluoro-phenyl)tropane), or [^{123}I]β-CIT ([^{123}I]2β-carbomethoxy-3β-(4-iodophenyl)-tropane), have demonstrated decreases in Parkinson's disease (Leenders et al., 1986; Aquilonius et al., 1987; Frost et al., 1993; Innis et al., 1993). However, regulation of DOPA-decarboxylase activity (Zhu et al., 1992; Hadjiconstantinou et al., 1993; Gjedde et al., 1993) and of presynaptic dopamine re-uptake site expression (Ikegami and Prasad, 1990; Kilbourn et al., 1992; Sharpe et al., 1991; Wiener et al., 1989; Wilson et al., 1994) during drug treatment in experimental animals suggests that these measures may reflect both drug-induced plasticity as well as losses of dopaminergic neuron terminals. Discrimination between neuronal injury and regulatory changes is of particular importance in studying idiopathic neurodegenerative disorders such as Parkinson's disease, where objective assessments of presynaptic nigrostriatal integrity are needed to distinguish disease-modifying from symptomatic treatment strategies.

1.2. The neuronal vesicular monoamine transporter

The neuronal synaptic vesicular monoamine transporter (vesicular monoamine transporter type 2;

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SSDI 0014-2999(95)00594-3

VMAT2) has been proposed as a target for measuring presynaptic terminal density (DaSilva and Kilbourn, 1992). Evidence supporting this interpretation includes decreased radioligand binding to the vesicular monoamine transporter following 6-hydroxydopamine lesions in rat (Darchen et al., 1989; Masuo et al., 1990), MPTP (*N*-methyl-4-phenyl-1,2,5,6-tetrahydropyridine) lesions in monkey (DaSilva et al., 1993), and in post-mortem analyses of Parkinson's disease brain (Scherman et al., 1989). Further, vesicular monoamine transporter binding demonstrates linear correlation with substantia nigra pars compacta neuronal density in graded 6-hydroxydopamine lesions (Vander Borght et al., 1995a). Since a minority of total synaptic vesicles are actively involved in transmitter release (Greengard et al., 1993; Kelly, 1993), it has been hypothesized that the efficacy of synaptic neurotransmission may be up- or down-regulated by altering the relative populations of the cycling versus the reserve vesicle pools, rather than requiring synthesis or degradation of individual vesicles. This suggests that total vesicle number, and integral protein constituents including the vesicular monoamine transporter, need not be regulated by conditions which alter presynaptic dopamine turnover or release.

As there is currently limited data on the possible regulation of the vesicular monoamine transporter, the present study was designed to investigate its level of expression employing drug treatment regimens known to influence dopaminergic transmission with particular emphasis on agents used commonly in patients with Parkinson's disease.

2. Materials and methods

2.1. Materials

High specific activity [^{3}H]MTBZ (2-α-[*O-methyl*-^{3}H]-3-isobutyl-1,2,3,4,5,6,7-hexahydro-11b*H*-benzo[*a*]-quinolizine; [^{3}H]methoxytetrabenazine; 82 Ci/mmol) was synthesized by custom O-methylation of the normethyl precursor (Amersham Corp., Arlington Heights, IL, USA). [^{3}H]Raclopride (*methoxy*-^{3}H; 70.7 Ci/mmol) and [^{3}H]WIN 35,428 ([^{3}H]2β-carbomethoxy-3β-(4-fluoro-phenyl)tropane; *N-methyl*-^{3}H; 84 Ci/mmol) were obtained from DuPont NEN (Wilmington, DE, USA). Benserazide HCl, clorgyline HCl, L-deprenyl HCl, mazindol and nomifensine were purchased from Research Biochemicals (Natick, MA, USA); apomorphine, ascorbic acid, benztropine, L-3,4-dihydroxyphenylanaline HCl (L-DOPA), L-3,4-dihydroxyphenylanaline methyl ester (L-DOPA methyl ester), EDTA, haloperidol, propylene glycol and tartaric acid from Sigma (St. Louis, MO, USA); and tetrabenazine from Fluka Chemical (Ronkonkoma, NY, USA).

2.2. Animals and drug treatment regimens

Male Sprague-Dawley rats (Charles River Laboratories, Portage, MI, USA) weighing approximately 180 g were housed in a temperature-controlled vivarium on a 12 h light-dark cycle with free access to food and water. Eight rats in each experimental group received daily i.p. injections of either 1.75 mg/kg/day benztropine, 5 mg/kg/day haloperidol or 0.5 mg/kg/day mazindol using 0.3% tartaric acid as vehicle. Subtype-selective monoamine oxidase inhibition was achieved by injections of 2.0 mg/kg clorgyline or of 0.5 mg/kg L-deprenyl every other day. All drug injections were made in a volume of 1 ml/kg body weight, and the doses were adjusted to individual animal weight every 2 days. Continuous administrations of approximately 18 mg/kg/day apomorphine, 100 mg/kg/d L-DOPA methyl ester with 25 mg/kg/day benserazide (Cooper et al., 1984) or 10 mg/kg/day tetrabenazine were provided for 2 weeks using osmotic pumps (Alzet model 2ML2, Alza Corporation, Palo Alto, CA, USA) implanted subcutaneously under light diethyl ether anesthesia. L-DOPA methyl ester/benserazide treatment was studied in two separate experiments ($n = 8$ treated and control subjects in each experiment). An assumed body weight of 280 g was employed when calculating infused doses to approximate delivery of the specified doses at the end of the continuous treatment period. L-DOPA methyl ester/benserazide and apomorphine were dissolved in 0.1% ascorbic acid, whereas tetrabenazine was dissolved in propylene glycol. Due to the poor water solubility of apomorphine, 2 osmotic pumps per animal were implanted to deliver the necessary drug dose. Twenty-four rats were used as controls: 8 were injected intraperitoneally with the vehicle alone (0.3% tartaric acid), 8 received propylene glycol by osmotic pump and 8 had a sham operation. All animals were treated for a 2-week period, then killed by decapitation between the 13th and the 14th days for the osmotic pump groups and between 1 h and 5 h after the last injection on the 14th day for the i.p. injection groups. The protocols were approved by The University of Michigan Committee on Use and Care of Animals.

2.3. Ligand binding autoradiography

Brains were rapidly removed and divided in the coronal plane. The forebrain was frozen in crushed dry ice, coated with embedding medium (Lipshaw, Detroit, MI, USA) to prevent desiccation and stored at −70°C for subsequent cryostat sectioning and autoradiography. 20-μm thick sequential coronal brain sections through the striatum, rostral to the decussation of the anterior commissure, were thaw-mounted in pairs on

polylysine-coated microscope slides and allowed to air-dry before storage at −70°C until use.

Preliminary in vitro studies were performed to determine direct interactions between study drugs and radioligands, and the lengths of pre-incubation required to dissociate the study drugs from the assayed binding sites. Competition experiments were carried out in triplicate by incubating brain sections in the presence of each radioligand and in additional presence or absence of 1 μM concentrations of the unlabeled study drugs. L-DOPA, rather than its methyl ester derivative, was employed in the in vitro assays. After incubation and rinsing, sections were wiped from slides with glass fiber filters (GF/C filters, Whatman, Hillsboro, OR, USA) and assayed by liquid scintillation spectrometry. Drugs with significant competitive inhibitory effects on radioligand binding were further investigated to determine the necessary pre-incubation washing conditions to minimize or eliminate residual drug. Serial sections were preincubated with the unlabeled drug and subsequently transferred to fresh buffer to allow dissociation prior to binding assays. The occupancy of receptors by residual cold drug was assessed by comparison with parallel sections, omitting the initial cold drug incubation.

2.3.1. [^{3}H]Methoxytetrabenazine binding

The binding of [^{3}H]methoxytetrabenazine was assayed under conditions previously established in our laboratory (Vander Borght et al., 1995a). Sections were prewashed for 5 min (except where otherwise noted) at 25°C in 300 mM sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 (sucrose buffer). Subsequently, sections were incubated in sucrose buffer containing 10 nM [^{3}H]methoxytetrabenazine at 25°C for 60 min. Following incubation, slices were washed for 3 × 3 min, briefly dipped in distilled water at 4°C to remove excess buffer and allowed to air dry before autoradiography. Under these conditions, specific binding represents more than 95% of total binding, thus, only total binding was assessed in the present experiments.

2.3.2. [^{3}H]Raclopride binding

Dopamine D_2 receptor binding was assayed as previously described by Köhler and Radesäter (1986). Briefly, brain sections were rinsed for 5 min (except where otherwise noted) in 0.17 M Tris-HCl (pH 7.6 at 25°C), then incubated at 25°C in the same buffer containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.001% ascorbic acid in the presence of 4 nM [^{3}H]raclopride for 15 min. Following a 15 min wash in 0.17 M Tris-HCl at 4°C, slices were briefly dipped in distilled water and dried overnight before autoradiography.

2.3.3. [^{3}H]WIN 35,428 binding

The presynaptic dopamine re-uptake site was assayed by the binding of [^{3}H]WIN 35,428 according to a modification of the method of Canfield et al. (1990). Sections were prewashed for 20 min (except where otherwise noted) at 4°C in 50 mM Tris-HCl containing 120 mM NaCl, then incubated with 3 nM [^{3}H]WIN 35,428 for 1 h. The incubation was terminated by a 2 × 1 min rinse in the buffer at 4°C followed by a brief dip in distilled water. Non-specific binding was estimated in the presence of 10 μM nomifensine maleate.

2.4. Densitometry and data analysis

Autoradiographs were generated by apposition of dried radiolabeled sections to tritium-sensitive X-ray film (Hyperfilm-^{3}H, Amersham Corp., Arlington Heights, IL, USA) for 2 weeks for [^{3}H]MTBZ, and 6 weeks for [^{3}H]raclopride and [^{3}H]WIN 35,428 binding assays. Calibrated plastic radioactive standards were included with each exposure to correct for variation in exposure and development technique as described previously (Pan et al., 1983). After development, autoradiograms were analyzed by computer-assisted video densitometry (MCID, Imaging Research, St. Catherines, Ontario, Canada). Autoradiographic film densities were converted to apparent tissue ligand concentration on the basis of film densities overlying the radioactive standards and the specific activity of the radioligand. Striatal binding of each ligand was expressed relative to that in parallel untreated controls from the same experiment. Treatment differences were assessed in comparison to the appropriate controls by 2-tailed Student's *t*-test.

3. Results

3.1. Evaluation of unlabeled drug competition effects

Competition assays identified significant interactions of [^{3}H]MTBZ with tetrabenazine, of [^{3}H]-raclopride with apomorphine and haloperidol, and of [^{3}H]WIN 35,428 with mazindol. There were no direct effects of the other study drugs on any of the markers assayed. A 120 min prewash at 25°C in sucrose buffer was required to dissociate residual tetrabenazine from the vesicular monoamine transporter, and a 4 h prewash in 0.17 M Tris-HCl was needed to dissociate haloperidol and apomorphine from the dopamine D_2 receptors. A 60 min prewash at 25°C in 50 mM Tris-HCl buffer preceding a 20 min prewash at 4°C was adequate to remove mazindol from the presynaptic dopamine re-uptake site. However, a net loss of dopamine re-uptake binding sites was observed with prolonged prewashing, accounting for decrease of mean

[^{3}H]WIN 35,428 binding between the first and second control groups (Table 1).

3.2. Effects of in vivo drug treatments on dopaminergic marker bindings

[^{3}H]MTBZ binding was not substantially modified by any of the repeated drug treatment regimens (Table 1; Fig. 1). In contrast, [^{3}H]raclopride binding increased by 26% after treatment with haloperidol and decreased by 23% and 15% after clorgyline and apomorphine treatments, respectively. Chronic treatment with mazindol significantly increased the [^{3}H]WIN 35,428 binding (29%), whereas haloperidol and the monoamine oxidase type B-selective dose of L-deprenyl had the reverse effect (27% and 14% decreases, respectively). Control groups, including animals treated by sham operation, i.p. injection of 0.3% tartaric acid and implantation of osmotic pumps filled with propylene glycol, showed no statistically significant differences from one another in any of the ligand binding assays except for the previously noted effect of pre-incubation washing on [^{3}H]WIN 35,428 binding.

4. Discussion

4.1. Neuronal vesicular monoamine transporter binding

The present study demonstrates that the neuronal synaptic vesicular monoamine transporter (VMAT2), as depicted by the binding of [^{3}H]methoxytetrabenazine, is not readily regulated by drugs affecting dopaminergic neurotransmission, and in particular not by drugs used in the treatment of Parkinson's disease (L-DOPA, L-deprenyl, benztropine and apomorphine). This is consistent with the lack of change in [^{3}H]dihydrotetrabenazine binding previously reported following short-term (2-day) treatments with haloperidol or with bromocriptine (Naudon et al., 1994). Our data further demonstrate that the absence of synaptic vesicular monoamine transporter regulation cannot be at-

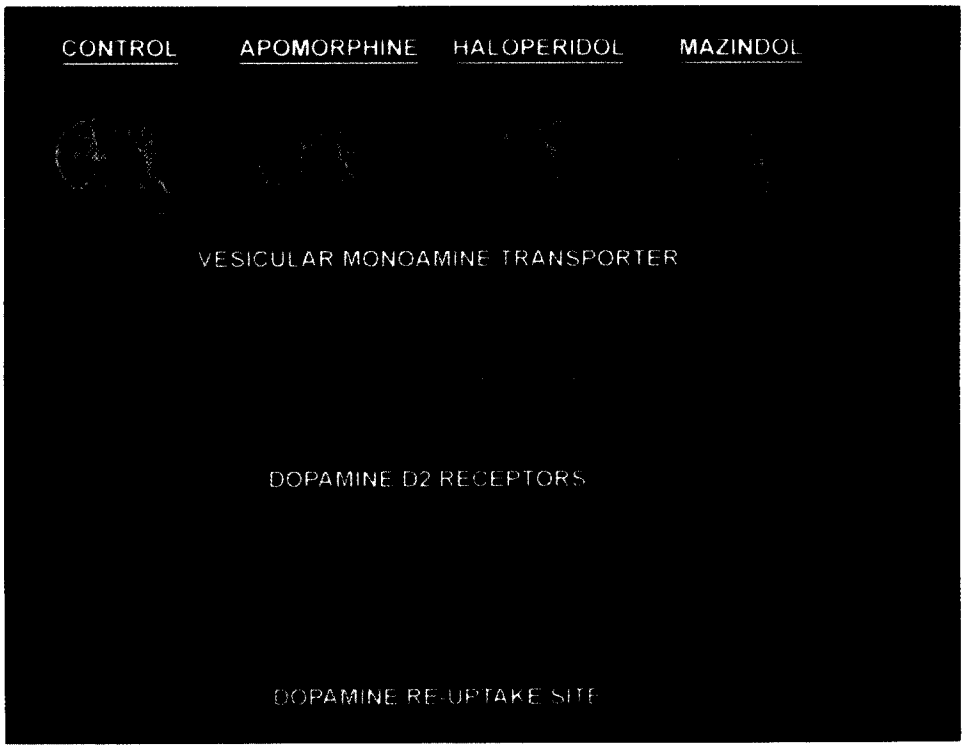

Fig. 1. Representative autoradiograms of ligand binding from control and apomorphine-, haloperidol-, or mazindol-treated animals (left to right columns). Coronal autoradiograms of rat brains at the level of the head of the caudate nucleus describe vesicular monoamine transporters ([^{3}H]methoxytetrabenazine binding, top row), dopamine D_2 receptors ([^{3}H]raclopride binding, middle row), or dopamine presynaptic re-uptake sites ([^{3}H]WIN 35,428 binding, bottom row). Images depict radioligand concentration in pseudocolor representation, according to the scale at the right with areas of highest binding represented in red and those of lowest binding in violet. Within each row, all images are scaled to the same levels for comparison. Each radioligand, however, is scaled differently to emphasize binding differences: [^{3}H]methoxytetrabenazine – 5 to 270 dpm/μg protein; [^{3}H]raclopride – 2 to 16 dpm/μg protein; [^{3}H]WIN 35,428 – 2 to 10 dpm/μg protein. There are no apparent differences in vesicular transporter binding sites between the treatment groups. Dopamine D_2 receptors are increased following haloperidol and decreased following apomorphine treatments. Dopamine re-uptake site binding is decreased following haloperidol and increased following mazindol treatments.

tributed to inefficacy of drug treatment protocols, since dopamine D_2 receptors and presynaptic dopamine re-uptake sites were significantly altered in parallel assays.

4.2. Dopamine D_2 receptor changes

Changes in the binding density of dopamine D_2 receptors are well-recognized as an indicator of short-term and chronic alterations in synaptic dopamine levels. The up-regulation of dopamine D_2 receptors by 26% achieved in our studies following receptor blockade by haloperidol is comparable to that described in prior studies (Burt et al., 1977; List and Seeman, 1979). In addition, as reproduced in our experiments with apomorphine, chronic agonist stimulation of dopamine receptors by bromocriptine decreases the number of striatal dopamine D_2 receptors (Mishra et al., 1978; Quik and Iversen, 1978). In agreement with the majority of previous studies (see Jenner et al., 1988 for review), administration of L-DOPA had no significant effect on dopamine D_2 receptors in the intact striatum. In contrast, clorgyline, a selective monoamine oxidase type-A inhibitor, may have induced down-regulation of the dopamine D_2 receptors indirectly by elevating dopamine concentration (Buu, 1989).

4.3. Dopamine presynaptic re-uptake site regulation

Our experiments reproduce the significant increase in [^{3}H]WIN 35,428 binding during chronic inhibition of presynaptic dopamine re-uptake with mazindol, while the less potent inhibitor benztropine produces a non-significant trend towards an increase (Ikegami and Prasad, 1990). Previous reports also demonstrate that chronic haloperidol treatment decreases the re-uptake transport activity (Meiergerd et al., 1993) and autoreceptor-mediated modulation of dopamine release (Nowak et al., 1983; Yamada et al., 1993). Therefore, our finding of reduced presynaptic dopamine re-uptake binding sites is not unanticipated. Allard et al. (1990) previously reported non-significant 15–19% decreases of [^{3}H]GBR 12935 binding after chronic treatment with haloperidol or raclopride and a non-significant 12% increase following bromocriptine treatments. The lack of statistical significance in this prior study may have resulted from the lower administered drug doses or from subtle distinctions between the [^{3}H]WIN 35,428 and [^{3}H]GBR 12935 binding sites (Wilson et al., 1994). Our present results additionally suggest that the apparent number of dopamine re-uptake sites, as assessed with [^{3}H]WIN 35,428 binding, may be sensitive to tissue incubation conditions. We found decreased bind-

Table 1
Effect of chronic drug treatments on vesicular monoamine transporter, dopamine D_2 receptor and presynaptic dopamine re-uptake site bindings in the rat striatum [a]

Treatment	Dose	Route	[^{3}H]Methoxytetrabenazine		[^{3}H]Raclopride		[^{3}H]WIN 35,428	
Controls [b]			100 ± 13		100 ± 6 [c]		100 ± 14 [d]	
Apomorphine	18 mg/kg/day	s.c. pump	117 ± 16	(0.03)	85 ± 7 [c]	(< 0.001)	114 ± 18 [d]	(0.12)
Haloperidol	5 mg/kg/day	i.p.	103 ± 19	(0.68)	126 ± 11 [c]	(< 0.001)	73 ± 15 [d]	(< 0.001)
Benztropine	1.75 mg/kg/day	i.p.	113 ± 21	(0.17)	105 ± 11 [c]	(0.30)	112 ± 28 [d]	(0.29)
Mazindol	0.5 mg/kg/day	i.p.	112 ± 17	(0.21)	101 ± 7 [c]	(0.72)	129 ± 23 [d]	(0.01)
Controls [e]			100 ± 18		100 ± 6		100 ± 8	
L-Deprenyl	0.5 mg/kg/2 day	i.p.	82 ± 18	(0.07)	92 ± 7	(0.03)	86 ± 10	(0.01)
Clorgyline	2.0 mg/kg/2 day	i.p.	100 ± 20	(0.96)	77 ± 3	(< 0.001)	90 ± 12	(0.08)
Controls [f]			100 ± 12		100 ± 6		100 ± 11	
L-DOPA + benserazide [f]	100 + 25 mg/kg/day	s.c. pump	98 ± 23	(0.79)	95 ± 9	(0.06)	104 ± 19	(0.50)
Controls [g]			100 ± 20 [h]		100 ± 5		100 ± 11	
Tetrabenazine	10 mg/kg/day	s.c. pump	92 ± 22 [h]	(0.47)	105 ± 5	(0.07)	90 ± 10	(0.06)

[a] Data (mean ± S.D.) are expressed as a percentage of the appropriate control group mean. Groups of 8 rats per condition, except where otherwise noted, were studied with [^{3}H]methoxytetrabenazine, [^{3}H]raclopride and [^{3}H]WIN 35,428 binding to striatal vesicular monoamine transporter, dopamine D_2 receptor and presynaptic dopamine re-uptake sites, respectively, following 2-week drug treatments. Statistical significance between treated and matched controls was assessed by ANOVA followed by two-tailed Student's t-test (P values are shown in parentheses). [b]Control values: [^{3}H]methoxytetrabenazine: 956; [^{3}H]raclopride: 61; and [^{3}H]WIN 35,428: 24 fmol/mg protein. The lower absolute value of WIN 35,428 in these groups (compare with control values in notes [e] and [f], below) is attributable to the prolonged pre-incubation washing. [c]Prewashing for 240 min was employed to remove competing apomorphine and haloperidol from the dopamine D_2 receptor binding sites. [d]Pre-prewashing for 60 min at 25°C was employed to remove competing mazindol from presynaptic dopamine re-uptake sites. [e]Control values: [^{3}H]methoxytetrabenazine: 974; [^{3}H]raclopride: 82; and [^{3}H]WIN 35,428: 52 fmol/mg protein. [f]$n = 16$. [g]Control values: [^{3}H]methoxytetrabenazine: 1117; [^{3}H]raclopride: 72; and [^{3}H]WIN 35,428: 46 fmol/mg protein. [h]Prewashing for 120 min was employed to remove competing tetrabenazine from the vesicular monoamine transporter binding sites.

ing in sections incubated at 25°C for only 60 min, while bindings of [^{3}H]methoxytetrabenazine and of [^{3}H]-raclopride were relatively unaffected by pre-incubations of 2 and 4 h duration, respectively. The apparent loss of [^{3}H]WIN 35,428 binding sites may relate to conformational instability or degradation of the ligand recognition site, or to loss of an essential cofactor during the pre-incubation procedure. Additional studies will be required to further clarify this initial observation.

4.4. Distinction of monoamine nerve terminal integrity from altered synaptic activity

In conclusion, our data suggest that the neuronal synaptic vesicular monoamine transporter is not readily regulated by manipulations of dopamine receptor stimulation or of presynaptic dopamine turnover. In contrast, other biochemical aspects of the dopaminergic system including dopamine D_2 receptors, the dopamine presynaptic membrane re-uptake transporter, tyrosine hydroxylase (Joh et al., 1986) and DOPA-decarboxylase (Zhu et al., 1992; Hadjiconstantinou et al., 1993; Gjedde et al., 1993) undergo alterations following comparable interventions. Vesicular monoamine transporter binding may, thus, permit discrimination of neuronal injury and losses from compensatory or treatment-related regulatory changes. We have recently developed positron-emitter labeled ligands (Kilbourn et al., 1993, 1995; Vander Borght et al., 1995b) to non-invasively measure the vesicular monoamine transporter density in vivo in human brain. Such measurements appear well-suited to analyses of neurodegenerative disorders, potentially providing measures of disease severity and treatment response.

Acknowledgements

This work was supported by grants designated MH47611, NS15655, and AG08671 from the National Institute of Mental Health, the National Institutes of Health, and the National Institute of Aging, respectively.

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