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Reduced striatal vesicular monoamine transporters after neurotoxic but not after behaviorally-sensitizing doses of methamphetamine

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Abstract

Prior studies indicate long-term reductions of striatal dopaminergic markers after sustained, high dose methamphetamine exposures in vivo, suggesting a neurotoxic effect. We have reported lack of regulation of vesicular monoamine transporter type-2 expression, as opposed to other markers of striatal dopaminergic terminals, under conditions that alter dopaminergic transmission without synaptic terminal losses. In the present study, we evaluated the vesicular monoamine transporter and the neuronal membrane dopamine transporter in rat striata after in vivo exposure to neurotoxic or to intermittent, low dose (behaviorally-sensitizing, non-neurotoxic) methamphetamine administrations. Vesicular monoamine transporter binding was measured by autoradiography of (+)-[^{3}H]dihydrotetrabenazine, the active isomer of (±)-[^{3}H]dihydrotetrabenazine. (+)-Dihydrotetrabenazine bound to a homogeneous population of striatal sites in controls with a K_d of 1.5 nM and a B_{max} of 3.8 fmol/μg protein. Neurotoxic methamphetamine treatment reduced both striatal vesicular monoamine transporter (−26%) and dopamine transporter (−39%) bindings. There were no changes after the non-neurotoxic treatment regimen. The vesicular monoamine transporter may thus be a valuable marker in the further clinical study of psychostimulant drug neurotoxicity.

Keywords: Vesicular monoamine transporter type-2 (VMAT2); Dopamine transporter, neuronal membrane; Methamphetamine; Amphetamine; Dihydrotetrabenazine; WIN 35,428; Dopamine

1. Introduction

1.1. Markers of dopaminergic terminal integrity and activity

A variety of clinical experimental evidence implicates altered striatal dopaminergic neurotransmission in disease. Human neurological and psychiatric disorders including Parkinson's disease (Scatton et al., 1984), multiple systems atrophy (Polinski, 1984), diffuse Lewy body disease (Perry et al., 1990), schizophrenia (Losonczy et al., 1987), Tourette's syndrome (Singer et al., 1991), and psychostimulant drug use (Ritz et al., 1987) may have dopaminergic involvement. Prior studies on chemical markers of presynaptic striatal dopamine nerve terminals indicate that many enzymes, transporters, and receptors are subject to activity- and drug treatment-related regulatory changes. For example, activities of the dopamine synthetic enzymes tyrosine hydroxylase and aromatic amino acid decarboxylase are subject to negative feedback regulation through dopamine D_2 receptors (see Vander Borght et al., 1995a). Furthermore, the binding sites associated with the neuronal membrane dopamine transporter (DAT, also designated the dopamine reuptake site) and with dopamine D_2 receptors undergo regulation of expression in response to changes in synaptic dopamine levels and turnover (Weiner et al., 1989; Sharpe et al., 1991; Wilson et al., 1994; Vander Borght et al., 1995a). We (Vander Borght et al., 1995a) and others (Naudon et al., 1994) have recently obtained evidence that the striatal vesicular monoamine transporter type-2 (VMAT2) binding site is not regulated after alterations in dopaminergic function. The density of striatal vesicular monoamine transporter binding sites is linearly related to the integrity of nigrostriatal projection neurons (Vander Borght et al., 1995b), indicating that measures of

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this transporter may provide estimates of neuronal and nerve terminal numbers. This is in contrast to altered activities per neuron or per terminal potentially reflected in other neurochemical assays.

1.2. Effects of methamphetamine on striatal dopamine terminals

Multiple lines of evidence link the pharmacological effects of the psychostimulant methamphetamine to central nervous system monoaminergic terminals (Robinson and Becker, 1986; Robinson and Berridge, 1993; Seiden and Sabol, 1993). Apparent monoaminergic neurotoxicity of amphetamines, and particularly of methamphetamine, has been studied extensively in experimental animals (Seiden et al., 1976; Hotchkiss and Gibb, 1980; see Seiden and Ricaurte, 1987 for review). Both the behaviorally-reinforcing effects of methamphetamine as well as its potential neurotoxic effects are proposed to involve mobilization of presynaptic dopamine through interactions with the neuronal membrane dopamine transporter and with the vesicular monoamine transporter type-2 (Pifl et al., 1995). Recently, human postmortem studies of chronic methamphetamine users have demonstrated an apparent preservation of radioligand binding to the striatal vesicular monoamine transporter type-2 despite clear reductions in other nigrostriatal markers including dopamine levels, tyrosine hydroxylase activity, and both immunochemical and radioligand binding measures of neuronal membrane dopamine transporter expression (Wilson et al., 1996a). These observations suggest that chronic methamphetamine users may not self-administer sufficiently high dosages frequently enough to induce neurotoxicity in nigrostriatal dopaminergic nerve terminals. However, the alternative possibility that radioligand binding to the vesicular monoamine transporter type-2 might not reflect the neurotoxic effects of methamphetamine exposure can not be discounted. In the present study, we have addressed this issue by examining the effects of both intermittent, low dose (behaviorally-sensitizing) and acute, sustained high dose (neurotoxic) methamphetamine administration protocols on levels of radioligand binding to the striatal vesicular monoamine transporter type-2 and to the neuronal membrane dopamine transporter in the rat.

2. Materials and methods

2.1. Materials

(+)-[^{3}H]Dihydrotetrabenazine (specific activity 81 Ci/mmol) was custom synthesized by Amersham (Arlington Heights, IL) by [^{3}H]methylation of the desmethyl precursor as previously described (Jewett et al., 1997). Tritiated WIN 35,428 (Specific Activity 84.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Tetrabenazine was obtained from Fluka Chemical (Ronkonkoma, NY), nomifensine was obtained from Research Biochemicals (Natick, MA) and methamphetamine was purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

2.2. Methamphetamine administration

Male Sprague Dawley rats weighing approximately 350 g were purchased from Charles River (Portage, MI) and were assigned to one of three groups. Methamphetamine neurotoxicity is largely dependent on both dosage and duration of exposure (Seiden and Ricaurte, 1987). For example, if given frequently, even relatively small individual doses can be neurotoxic (Ellison et al., 1978), but if given intermittently at longer intervals, much higher individual doses are required (Robinson and Becker, 1986; Seiden and Ricaurte, 1987). Therefore, in the present study one group of rats received 5 mg/kg methamphetamine i.p. once every two hours for a total of 5 injections on day 1 (sustained, high dose, neurotoxic treatment protocol). This regimen was chosen because it does not result in the lethality associated with very high dosage treatment regimens, but is similar to a neurotoxic regimen producing a persistent 40–50% depletion of striatal dopamine and a comparable reduction in [^{3}H]mazindol binding to the neuronal membrane dopamine transporter (O'Dell et al., 1991; Eisch et al., 1992). A second group of animals received 2 mg/kg methamphetamine i.p. once daily on days 1–5 (intermittent, low dose, behaviorally-sensitizing protocol). This treatment regimen is not neurotoxic, but results in behavioral sensitization (Robinson and Becker, 1986). Control animals were injected i.p. with saline daily on days 1–5. Animals were killed by decapitation on day 7, 6 days after completion of neurotoxic treatment or 2 days after the last sensitizing methamphetamine treatment. The investigators performing radioligand binding assays were blinded to the treatment conditions.

2.3. Ligand binding assays

Forebrains were dissected and frozen for cryostat sectioning as described previously (Vander Borght et al., 1995a). Coronal, 20 μm thick tissue sections through the striatum were thaw-mounted in pairs on poly-L-lysine subbed microscope slides and allowed to air-dry. Slides were then stored at -70°C until use in binding assays.

Binding of [^{3}H]dihydrotetrabenazine to the vesicular monoamine transporter was conducted by modification of the method described previously for the radioligand [^{3}H]methoxytetrabenazine (Vander Borght et al., 1995b). Slides were pre-incubated for 5 min in potassium phosphate buffer with EDTA: 137 mM KCl; 3 mM NaCl; 8 mM K_2HPO_4; 1.5 mM $Na_2H_2PO_4$; 1 mM EDTA; pH 8.0 at 25°C. Slides were next incubated in buffer containing 10 nM [^{3}H]dihydrotetrabenazine for 30 min, followed by rins-

ing in fresh buffer at 4°C twice for 2 min each. Sections were then wiped from slides with glass fiber filters for liquid scintillation spectrometry in exploratory assays, or were dipped briefly in distilled water at 4°C to remove buffer salts and air-dried. Slides were apposed to tritium-sensitive film (Hyperfilm, Amersham) for 4 weeks in autoradiographic studies. Nonspecific binding was determined in the presence of 10 μM unlabeled tetrabenazine.

Binding of [^{3}H]WIN 35,428 to the neuronal membrane dopamine transporter was performed as described previously (Vander Borght et al., 1995a) with minor modification. Briefly, sections were washed for 30 min in phosphate-buffered saline with EDTA: 137 mM NaCl; 3 mM KCl; 8 mM Na_2HPO_4; 1.5 mM $K_2H_2PO_4$; 1 mM EDTA; pH 7.4. Slides were incubated in buffer containing 3 nM [^{3}H]WIN 35,428 for 60 min at 4°C, rinsed twice for 1 min each in fresh buffer, and dipped briefly in distilled water. After air-drying, autoradiographs were exposed for 6 weeks. Nonspecific binding was assessed in the presence of 10 μM unlabeled nomifensine.

2.4. Autoradiographic analyses and statistics

Autoradiograms were analyzed with the use of a CCD-video computer densitometry system (MCID model M2, Imaging Research, St. Catherines, Ontario). Autoradiographic optical density was converted to apparent tissue radioligand concentration on the basis of calibrated standards co-exposed with the tissue on each film (Pan et al., 1983).

Saturation isotherms were analyzed with the use of a computer-assisted curve-fitting procedure designed for ligand binding analyses (LIGAND Program, Elsevier-Biosoft, Cambridge; Munson and Rodbard, 1980). Both one- and two-site models were evaluated, and the model of least complexity selected unless significant improvement in the runs test or in residual error were detected. In addition to individual experiment fitting analyses, the entire group of experiments was analyzed simultaneously for added statistical power.

Analyses of regional methamphetamine effects were conducted by one-way analysis of variance (ANOVA), followed by pairwise Student's *t*-tests with Bonferroni correction for multiple comparisons. A significance threshold of $P < 0.05$ was employed throughout.

3. Results

3.1. Binding of (+)-[^{3}H]dihydrotetrabenazine to tissue sections

In initial studies we explored the temporal properties of (+)-[^{3}H]dihydrotetrabenazine binding to intact tissue sections by liquid scintillation spectrometry. Specific binding reached equilibrium with a 1 nM radioligand concentration between 30 and 60 min of incubation. Post-incubation washing of sections for up to 16 min reduced nonspecific binding by approximately 10-fold, reaching a near-minimum without detectable loss of specific binding after 4 min (Fig. 1). Total (+)-[^{3}H]dihydrotetrabenazine binding was 97% attributable to specific vesicular monoamine transporter type-2 binding under these conditions.

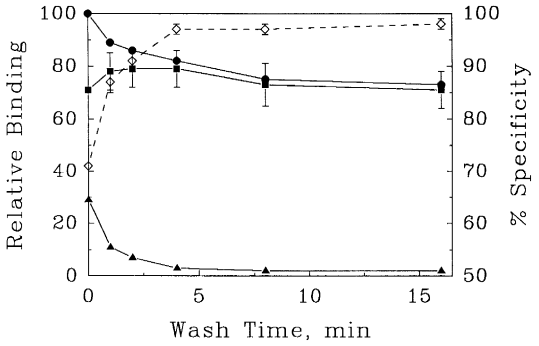

Fig. 1. Effect of post-incubation washing of slide-mounted tissue sections on nonspecific and total bindings of (+)-[^{3}H]dihydrotetrabenazine. Total binding (filled circles, solid line), nonspecific binding (filled triangles, solid line), and specific binding (filled squares, solid line) in adjacent sections through the rat striatum are depicted at varying wash times following incubation in 1 nM (+)-[^{3}H]dihydrotetrabenazine. Data are expressed relative to initial (no post-incubation washing) total binding within each series of brain sections, and represent the mean ± SD of 3 independent experiments. The specificity of total binding, expressed as a percentage, is also shown (open diamonds, dashed line). Near-maximal specificity of total binding is achieved with a 4 min post-incubation wash time, without loss of initial specific binding.

Autoradiographic saturation analyses of (+)-[^{3}H]dihydrotetrabenazine binding to the striatum were conducted over a range of ligand concentrations between 0.25 and 25 nM. Incubation times were extended to 120 min in these studies to assure equilibration at the lower concentrations. A single, high-affinity, saturable interaction of (+)-[^{3}H]dihydrotetrabenazine was observed with an equilibrium dissociation affinity constant (K_d) of 1.54 ± 0.09 nM ($n = 3$ experiments), a capacity (B_{max}) of 3.75 ± 0.15 fmol/μg protein, and a Hill coefficient of 0.95 ± 0.03 (Fig. 2).

3.2. Effect of methamphetamine treatment on vesicular monoamine transporter type-2 and neuronal dopamine transporter binding sites

In preliminary studies, the competitive effects of 1 μM methamphetamine on radioligand binding were determined in vitro. No significant methamphetamine competition was detectable in (+)-[^{3}H]dihydrotetrabenazine assays at this concentration. Conversely, methamphetamine reduced specific [^{3}H]WIN 35,428 binding by approximately 50%, but could be largely removed by a 30 min preincubation washing step interposed between methamphetamine exposure and incubation in [^{3}H]WIN 35,428.

There were significant group differences in the striatial binding of (+)-[^{3}H]dihydrotetrabenazine (ANOVA: $F(12)$

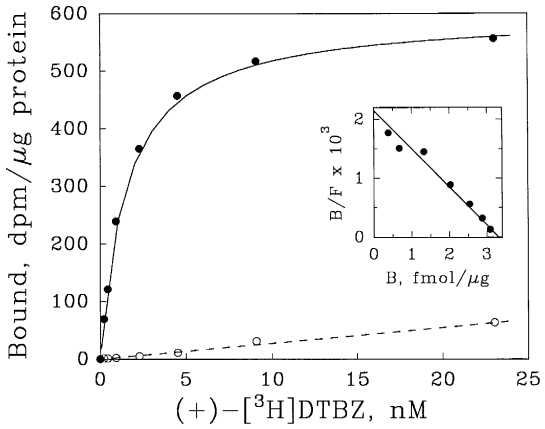

Fig. 2. Saturability of (+)-[^{3}H]dihydrotetrabenazine (DTBZ) binding in rat striatum. Specific (filled circles, solid line) and nonspecific (open circles, dashed line) binding as determined by quantitative autoradiography of a series of adjacent coronal sections incubated in varying concentrations of (+)-[^{3}H]dihydrotetrabenazine are depicted from a typical experiment. Inset: Rosenthal plot of specific binding. The data reveal a single saturable binding site with apparent K_d of 1.5 nM and B_{max} of 3.3 fmol/μg protein.

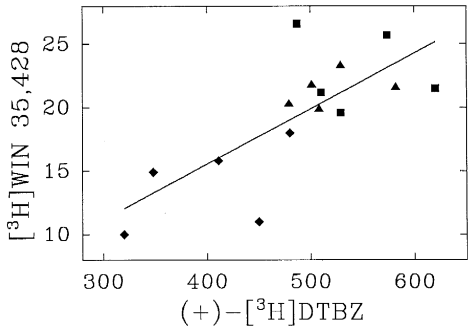

Fig. 3. Relationship between radioligand binding to the vesicular monoamine transporter type-2 and to the neuronal plasma membrane dopamine transporter in striata of control and amphetamine-treated animals. Specific striatal binding of (+)-[^{3}H]dihydrotetrabenazine (DTBZ) and [^{3}H]WIN 35,428 are depicted for individual control (squares), intermittent, low dose amphetamine-treated (triangles) and sustained, high dose amphetamine-treated (diamonds) animals. Both the vesicular monoamine transporter type-2 and neuronal membrane dopamine transporter are reduced by high dose amphetamine treatment. The line of regression is given by: WIN = −1.9 + 0.044(DTBZ); $R = 0.745$; $P = 0.001$.

$= 9.814$; $P < 0.003$) and of [^{3}H]WIN 35,428 (ANOVA: $F(12) = 15.58$; $P < 0.001$). Pairwise group comparisons revealed that sustained, high dose methamphetamine treatment significantly reduced specific (+)-[^{3}H]dihydrotetrabenazine binding from 544 ± 53 dpm/μg protein in controls to 402 ± 68 dpm/μg protein (−26%). The binding

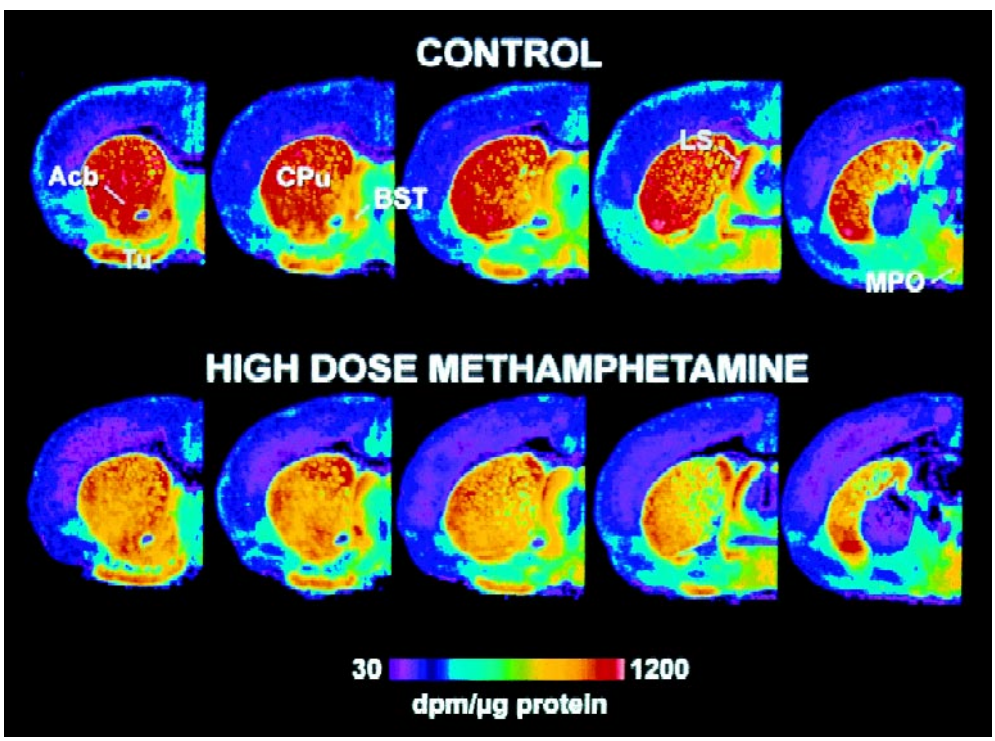

Fig. 4. Anatomic distribution of changes in vesicular monoamine transporter type-2 binding associated with methamphetamine neurotoxicity. Coronal autoradiographic sections from representative control and sustained, high dose methamphetamine treated animals are shown at 400 μm intervals from approximately 0.8 mm anterior to 0.8 mm posterior to the Bregma, according to the atlas of Paxinos and Watson (1982). Images are pseudocolor representations of total (+)-[^{3}H]dihydrotetrabenazine binding, according to the scale at the bottom. Vesicular monoamine transporter type-2 binding is reduced throughout the striatal complex (caudate-putamen, nucleus accumbens and olfactory tubercle), but appears relatively less affected in the septum and hypothalamus. Abbreviations: Acb – nucleus accumbens septi; BST – bed nucleus of the stria terminalis; CPu – caudate-putamen; LS – lateral septal nucleus; MPO – medial preoptic nucleus of the hypothalamus; Tu – olfactory tubercle.

in the intermittent, low dose amphetamine group (520 ± 39 dpm/μg protein) was not significantly different from control levels. Similar changes in specific striatal [^{3}H]WIN 35,428 binding were observed (Fig. 3), with a significant reduction from 23 ± 3 dpm/μg protein in controls to 14 ± 3 dpm/μg protein in the sustained, high dose amphetamine group (-39%). There was no significant effect of the intermittent, low dose amphetamine treatment on [^{3}H]WIN 35,428 binding (21 ± 1 dpm/μg protein).

Qualitative visual autoradiographic analyses of other forebrain regions indicated that the sustained, high dose amphetamine effect involved (+)-[^{3}H]dihydrotetrabenazine binding throughout the entire striatal complex, including the caudate-putamen, the nucleus accumbens septi, and the olfactory tubercle (Fig. 4). Septal and hypothalamic radioligand binding appeared less affected than striatal binding. Smaller reductions in the neocortex (from 39 ± 7 dpm/μg protein to 34 ± 5 for (+)-[^{3}H]dihydrotetrabenazine and from 2.8 ± 0.4 dpm/μg protein to 1.8 ± 03 for [^{3}H]WIN 35,428 in the control and sustained, high dose amphetamine groups, respectively) were not statistically significant.

4. Discussion

4.1. (+)-[^{3}H]dihydrotetrabenazine binding to the vesicular monoamine transporter type-2

The present studies confirm and extend prior reports of the interaction between benzo[*a*]quinolizine radioligands and the brain vesicular monoamine transporter binding site. In comparison with racemic radioligands such as (±)-[^{3}H]methoxytetrabenazine (Vander Borght et al., 1995a,b) and (±)-[^{3}H]dihydrotetrabenazine (Darchen et al., 1989), the (+)-[^{3}H]dihydrotetrabenazine employed in the current studies demonstrates reduced nonspecific binding as predicted by competition binding assays of the unlabeled isomers (Kilbourn et al., 1995); the (+)-isomer demonstrates a 1,000-fold lower IC_{50} than the (−)-isomer. The estimated K_d for (+)-[^{3}H]dihdyrotetrabenazine binding, 1.5 nM, is in good agreement with previous competition assays predicting an affinity of approximately 1 nM. Future use of the resolved radioligand will permit more accurate assays of the vesicular monoamine transporter type-2 in tissues with low levels of expression such as the cerebral cortex, where almost 70% of its total binding at 10 nM is specific (total binding 56 ± 5 dpm/μg protein, nonspecific binding 18 ± 5 dpm/μg protein).

4.2. Effect of methamphetamine on vesicular monoamine transporter type-2 and neuronal membrane dopamine transporter bindings

Prior studies in experimental animals have demonstrated regulation of the expressed level of the striatal neuronal membrane dopamine transporter by drugs affecting dopaminergic neurotransmission (Weiner et al., 1989; Sharpe et al., 1991; Wilson et al., 1994; Vander Borght et al., 1995a). Conversely, only lesions of nigrostriatal neurons have resulted in altered striatal vesicular monoamine transporter type-2 levels, suggesting utility of this marker as in index of presynaptic terminal integrity (Vander Borght et al., 1995a,b). In experimental animals, lasting depletions of several presynaptic striatal dopaminergic indices are observed after presumably neurotoxic treatment regimens, including the one used here (O'Dell et al., 1991; Eisch et al., 1992; for review, see Robinson and Becker, 1986; Seiden and Ricaurte, 1987). In these previous studies, however, the vesicular monoamine transporter was not examined. A recent report of striatal monoaminergic markers in autopsied brains of methamphetamine users revealed reductions in the neuronal membrane dopamine transporter, but not in the vesicular monoamine transporter type-2 (Wilson et al., 1996b), which suggests that recreational psychostimulant drug use may not be neurotoxic in humans. However, another possibility is that vesicular monoamine transporter type-2 levels are not a sensitive indicator of methamphetamine neurotoxicity.

The present studies demonstrate that administration of methamphetamine to experimental animals in a neurotoxic regimen does affect both vesicular monoamine transporter type-2 and neuronal membrane dopamine transporter expressions. These results establish that vesicular monoamine transporter type-2 binding assays are a sensitive indicator of methamphetamine neurotoxicity. Many of the human methamphetamine users studied by Wilson et al. (1996a) self-administered the drug regularly (daily to weekly frequency) for long periods of time (up to 23 years), but had normal levels of vesicular monoamine transporter binding on post-mortem examination. When taken together with the present results this suggests that in humans, long-term methamphetamine abuse is not necessarily accompanied by toxicity to nigrostriatal nerve terminals.

There are, however, apparent discrepancies between the observations of Wilson et al. (1996a) in human brain and the present rodent data on methamphetamine exposure effects on the neuronal membrane dopamine transporter. In the human methamphetamine abusers, levels of dopamine and of the neuronal membrane dopamine transporter were reduced by 30% to 50% in the putamen, while vesicular monoamine transporter type-2 radioligand binding was not altered (95% of control values). However, our results indicate that intermittent, non-neurotoxic methamphetamine treatment has no effect on radioligand binding to the neuronal membrane dopamine transporter ([^{3}H]WIN 35,428 levels $> 90\%$ of control specific binding). There could be a variety of co-morbid conditions or exposures in the human methamphetamine users to account for the apparent reduction in dopamine transporter levels. In particular, the presence of both methamphetamine and amphetamine in the assayed tissues may be important distinc-

tions. Methamphetamine levels in the human brains examined were in excess of 1 μM, and as high as 300 μM in some individuals (see Table 1 in Wilson et al., 1996a). Under our assay conditions, these levels would have reduced neuronal membrane dopamine transporter binding by over 50%, unless the competing drug were eliminated from the tissue during the assay procedure. It is possible, therefore, that the apparent decreases in dopamine transporter binding in the human study might be due to residually bound amphetamines.

Immunological quantification of the dopamine transporter was also performed by Wilson et al. (1996a) in the human methamphetamine users and revealed reductions similar to radioligand binding assays. This complimentary experimental approach should control for the confounding effects of unlabeled methamphetamine or amphetamine in the radioligand binding assays. However, the relationships between actual dopamine transporter protein expression and the radioligand binding or immunological assays of its level are potentially complex. Studies reported previously employing both binding and immunological assays together reveal apparently greater decreases of immunoreactivity than of radioligand binding in Parkinson's disease (Wilson et al., 1996b), spinocerebellar ataxia (Kish et al., 1997) and in the methamphetamine users (Wilson et al., 1996a). The antibodies employed in these studies are directed against the N-terminus of the dopamine transporter, and it is possible that antibody recognition might be affected by post-translational modifications such as phosphorylation in this region (Vandenbergh et al., 1992; Amara and Kuhar, 1993) or by proteolysis. If antibody interaction with the transporter were altered by nerve terminal activity or in disease, differential effects might be obtained in radioligand binding versus immunological assays (Wilson et al., 1996b). Alternatively, a non-dopamine transporter component of saturable WIN 35,428 binding could be present, diminishing radioligand binding estimates of its losses.

A final distinction between the human and rat methamphetamine studies relates to the drug dosages and proximity of the last exposure to the experimental assays. In the human study of Wilson et al. (1996a), presence of methamphetamine in blood and tissue samples indicates very recent drug use. The dopamine transporter and other presynaptic marker changes observed may thus represent transient responses to acute drug exposure (Fleckenstein et al., 1997), rather than long-term changes associated with chronic methamphetamine exposure such as those potentially accompanying behavioral sensitization or neurotoxicity. Conversely, our present studies explored only two treatment regimens, but allowed post-exposure intervals of several days prior to the neurochemical assays. It remains possible that intermediate dosages and administration frequencies could differentially affect the neuronal dopamine transporter versus the vesicular monoamine transporter in the rat, as reported in the human study.

4.3. Summary

In conclusion, our studies demonstrate significant reductions in radioligand binding to the vesicular monoamine transporter type-2 associated with methamphetamine neurotoxicity in the rat. Measurements of the vesicular monoamine transporter type-2 in human psychostimulant drug users may thus be expected to provide information relevant to irreversible neurotoxic sequellae. This is of particular importance since even moderate presynaptic injury, as demonstrated in our present studies, could lead to increased risk for dopamine deficiency syndromes such as parkinsonism in later life. The prior postmortem human data indicating preserved striatal vesicular monoamine transporter binding suggest that most contemporary methamphetamine users may not have irreversible losses of nigrostriatal terminals. Nevertheless, it is possible that higher individual methamphetamine doses or more frequent exposures than usually encountered could be neurotoxic in humans. In particular, the possibility that methamphetamine doses sufficiently high to produce psychosis may be neurotoxic should be further investigated.

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