Clozapine Modulates Aromatic L-Amino Acid Decarboxylase Activity in Mouse Striatum

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ABSTRACT

Clozapine is efficacious for treating dopaminergic psychosis in Parkinson’s disease and ameliorates L-DOPA-induced motor complications. Based on its pharmacology and reported enhancing effects on dopamine metabolism and tyrosine hydroxylase activity, we investigated whether it could modulate the activity of aromatic L-amino acid decarboxylase (AAAD), the second enzyme for the biosynthesis of catecholamines and indoleamines. A single dose of clozapine increased AAAD activity in striatum in a dose- and time-dependent manner. At 1 h, enhanced enzyme activity was characterized by an increased Vmax for substrate and cofactor and was accompanied by elevated levels of protein in striatum and mRNA in substantia nigra, ventral tegmental area, locus coeruleus, and raphe nuclei. Acute clozapine increased tyrosine hydroxylase activity in striatum but with differing temporal patterns from AAAD and heightened dopamine metabolism. Interestingly, the response of the dopaminergic markers to clozapine was greater following a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPP) lesion. Chronically administered clozapine increased AAAD activity and protein and dopamine metabolism in striatum without affecting tyrosine hydroxylase. Exogenous L-DOPA decarboxylation was accelerated in the striatum of intact and MPTP-lesioned mice following acute clozapine, and the effect was exaggerated in the MPTP mice. To identify receptors involved, antagonists of receptors occupied by clozapine were employed. D4, 5-HT1A, and 5-HT2A, in addition to D1, D2, and D3, antagonists were ineffective. For the first time, these studies provide evidence that clozapine modulates AAAD activity in the brain and suggests that dopamine and serotonin receptors are involved.

Aromatic L-amino acid decarboxylase (AAAD) is a ubiquitous enzyme essential for the formation of catecholamines, indoleamines, and trace amines (Berry et al., 1996). AAAD is not considered to be the rate-limiting enzyme for catecholamine or indoleamine synthesis; however, it is the rate-limiting step for the synthesis of trace amines and becomes rate-limiting for dopamine formation in Parkinson’s disease patients treated with L-DOPA. AAAD is a regulated enzyme, and accumulated evidence suggests that its activity in the rodent brain is tuned by short- and long-term mechanisms that apparently involve enzyme activation and induction.

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ABBREVIATIONS: AAAD, aromatic L-amino acid decarboxylase; SCH23390, R(-)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; 5-HT, 5-hydroxytryptamine; L-745,870, 3-[4-(4-chlorophenyl)piperazin-1-yl]methyl-1H-pyrrolo[2,3-b]pyridine; Way 106355, N-(2-[4-(2-[2-methylthiazol-4-yl]methoxy)phenyl]-1-piperazinyl)ethyl-N-(2-pyridyl)cyclohexane carboxamide trihydrochloride; SB-242084, 6-chloro-5-methyl-1-[2-[2-methylpyrid-3-yl]pyrid-5-yl]carbamoyl]indoline; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NSD 1015, m-hydroxybenzylhydrazine; HPLC, high-performance liquid chromatography; ED, electrochemical detector; DOPAC, 3,4-dihydroxyphenylacetic acid; DA, dopamine; TH, tyrosine hydroxylase; HVA, homovanillic acid.

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dopamine synthesis and release (Cumming et al., 1997; Fisher et al., 2000; Neff et al., 2000).

Decarboxylation of exogenous L-DOPA by AAAD is the controlling step for the formation of dopamine in parkinsonian patients, and altered enzyme regulation might contribute to the appearance of motor complications and to decreasing therapeutic response. Although the site for the conversion of L-DOPA to dopamine in the parkinsonian brain is still debated (Hefti et al., 1980, 1981; Mura et al., 1995), boosting or stabilizing the activity of AAAD might be of therapeutic importance because it would reduce the required dose of L-DOPA, ameliorate adverse motor symptoms associated with high or fluctuating levels of L-DOPA, and perhaps prolong the responsiveness of dopaminergic neurons to L-DOPA. Thus, drugs that modulate AAAD activity theoretically could be used as an adjuvant when developing strategies for L-DOPA treatment augmentation. The atypical antipsychotics, clozapine and quetiapine, have been prescribed successfully to parkinsonian patients for treatment of psychosis (Friedman and Factor, 2000). They have low-extra-pyramidal sign liability (Tarsy et al., 2002) and, reportedly, improve L-DOPA-induced dyskinesias and motor fluctuations (Durif, 1999). They are multireceptor drugs and display varying affinities for dopamine D1, D2, D3, and D4 receptors, serotonin 5-HT1A, 5-HT2A, 5-HT2C, 5-HT6, and 5-HT7 receptors, and histamine H1 receptors. The lack of extra-pyramidal signs has been attributed to their propensity to partially block striatal dopamine D2 receptors, their fast dissociation and lower occupancy of D2 receptors, and their inherent antimuscarinic action and 5-HT2A antagonism (Kapur and Remington, 2001; Meltzer et al., 2003). Based on its dopamine receptor antagonistic profile and reports that it increases the activity of tyrosine hydroxylase (Zivkov et al., 1975; Hetey et al., 1985) and enhances dopamine metabolism (Burki et al., 1975; Zivkov et al., 1975; Invernizzi et al., 1990; Karoum and Egan, 1992; Broderick and Piercey, 1998), we hypothesized that clozapine enhances AAAD activity as well. Toward this goal, we investigated the effect of clozapine on AAAD activity, protein, and kinetics in the striatum of mice, characterized the pharmacology of the response, examined the effect of a dopaminergic lesion on the clozapine-induced modulation of AAAD, and determined whether clozapine affects exogenous L-DOPA decarboxylation. Moreover, the effect of the drug on tyrosine hydroxylase and dopamine metabolism in striatum was evaluated in parallel and contrasted to that seen for AAAD.

Materials and Methods

Animals and Treatments

Male Swiss-Webster mice (Harlan, Indianapolis, IN), 25 to 30 g, were used for the studies, which were approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health. Mice received a single injection of the following drugs: 20 mg/kg i.p. clozapine, atypical antipsychotic drug; 10 mg/kg i.p. prazosin, α-1 adrenergic receptor antagonist; 10 mg/kg i.p. yohimbine, α-2 adrenergic receptor antagonist; tropisopin, 1 mg/kg i.p. 5-HT3 antagonist; or vehicle and killed at various times as indicated in the tables and figures. Drug doses were based on literature and our experience, and for dose-response studies, clozapine, 1–20 mg, was administered i.p. Some animals were treated with the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 30 mg/kg i.p., daily for 7 days and 24 h later injected with 20 mg/kg i.p. clozapine and killed 30 or 60 min thereafter. For the exogenous L-DOPA decarboxylation studies, intact and MPTP-lesioned mice were administered L-DOPA, 100 mg/kg i.p., alone or together with 20 mg/kg i.p. clozapine, and killed 1 h later. In some studies, the AAAD inhibitor NSD 1015 (100 mg/kg i.p.) was administered to intact mice 1 h prior to L-DOPA or L-DOPA/clozapine treatment. For chronic studies, intact and MPTP-lesioned animals were treated with clozapine (20 mg/kg i.p.) or vehicle twice daily, 8 h apart, for 7 days and studied at 1 and 6 h after the last injection. Animals were killed by decapitation, and striata were dissected and used for the various assays as indicated. One striatum was used for the assay of AAAD, dopamine and metabolites, and Western blots, whereas the other was used for the assay of tyrosine hydroxylase. Whole brains were immediately frozen on dry ice for in situ hybridization studies.

Procedures

AAAD Activity Assay. AAAD activity was assayed as described previously (Hadjiconstantinou et al., 1993). In brief, tissue was homogenized in ice-cold 0.25 M sucrose. The reaction was started by incubating an aliquot of the homogenate (10 μg of protein) with assay buffer containing 50 mM sodium phosphate buffer, pH 7.2, 0.1 mM EDTA, 0.17 mM ascorbic acid, 1 mM β-mercaptoethanol, 0.1 mM pargyline, 10 μM pyridoxal-5’-phosphate, and 500 μM L-DOPA for 20 min at 37°C. The reaction was stopped by adding ice-cold 0.525 M HClO4 containing 3,4-dihydroxybenzylamine as an internal standard. Dopamine was extracted using alumina, resolved by high-performance liquid chromatography (HPLC), and monitored with an electrochemical detector (ED).

Tyrosine Hydroxylase Activity Assay. Tissue was homogenized in 10 mM Tris acetate buffer, pH 7.0, containing 0.2% Triton X-100 and 1 mM β-mercaptoethanol. Samples were centrifuged, and an aliquot of supernatant (30 μl) was added to an incubation mixture containing 40 mM sodium acetate, pH 6.0, 200 μM unsaturated conditions, or 1 mM (saturated conditions) 6-methyl-5,6,7,8-tetrahydroberdine, 10 μg/100 μl catalase, 1 mM ferrous ammonium sulfate, and 200 μM L-tyrosine, with 1 μCi of [3H]-L-tyrosine (48 Ci/mmol; Ameresham Bioscience, Piscataway, NJ). After 20 min of incubation at 37°C, the reaction was terminated by adding a mixture of charcoal in 0.01 M HCl. Following centrifugation, [3H]O in the supernatant was counted in a scintillation counter (Hadjiconstantinou et al., 1995).

Dopamine and Metabolites Estimation. Dopamine, DOPAC, and HVA were analyzed by HPLC-ED. To 200 μl of the homogenate used for assaying AAAD activity, 200 μl of 0.2 M HClO4 containing 0.05 mM sodium bisulfite was added and centrifuged. An aliquot of supernatant, 100 μl, was injected into the HPLC-ED system (Cho et al., 1997).

Western Blots. Tissue lysates of equal protein quantity, 20 μg, were separated by SDS-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membrane. Blots were incubated overnight in Tris-buffered saline-Tween (10 mM Tris, 150 mM NaCl, 0.1% Tween 20) containing 5% dry milk as a blocking agent. They were then sequentially incubated for 1 h at room temperature with rabbit anti-AAAD and anti-tyrosine hydroxylase antibody (Ab1569 and Ab152; Chemicon, Temecula, CA) and horseradish per-oxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), with extensive washing in Tris-buffered saline-Tween after incubation with each antibody. Bound antibody was visualized with enhanced chemiluminescence (Amersham Bio-
HCl, 1 mM EDTA, 0.1 M dithiothreitol, and 0.3 dextran sulfate, 1/100
ment of the mouse brain AAAD cDNA (Eaton et al., 1993). Sections
sense and antisense riboprobes were prepared from a 286-bp frag-
fixed in 4% paraformaldehyde followed by 0.25% acetic anhydride in
Materials and Methods.

Results

Administration of a single dose of clozapine increased the
activity of AAAD in the striatum in a dose- and time-depen-
dent manner (Fig. 1, A and B). Increased activity was ob-
served between 5 and 20 mg/kg i.p. Striatum was assayed for AAAD and TH as described under Materials and Methods. Mice were treated with increasing doses of clozapine or vehicle and killed at the time intervals indicated. For the time study, the dose of clozapine was 20 mg/kg i.p. Striatum was assayed for AAAD and TH as described under Materials and Methods. *p < 0.05 compared with control (0 time or 0 dose). n = 6 to 8 animals/group.

Fig. 1. Acute administration of clozapine increases AAAD and TH activity in striatum in a time- (A) and dose-dependent (B) manner. Mice were treated with increasing doses of clozapine or vehicle and killed at the time intervals indicated. For the time study, the dose of clozapine was 20 mg/kg i.p. Striatum was assayed for AAAD and TH as described under Materials and Methods. *p < 0.05 compared with control (0 time or 0 dose). n = 6 to 8 animals/group.

Fig. 2. Clozapine increases AAAD but not TH protein after acute administration. Mice were treated with 20 mg/kg i.p. clozapine or vehicle and killed at the time intervals indicated. AAAD and TH protein were estimated in striatum by Western blot and quantified as under Materials and Methods. A, graphic presentation of AAAD (open bars) or TH (closed bars), protein content expressed as percentage of control (vehicle). B, representative Western blot. *p < 0.05 compared with control. n = 6 to 8 animals/group.
Because of the complexity of the pharmacology of clozapine, to identify possible receptors involved with the AAAD response-selective antagonists for receptor subtypes, known to be occupied by clozapine, were administered to mice, and AAAD activity was assayed in striatum. Because we have already shown that blockade of dopamine D1, D2, and D3 receptors increases the activity of AAAD in striatum (Hadjiconstantinou et al., 1993; Cho et al., 1997), studies with antagonist drugs for these receptors were not repeated. A single dose of the selective D4 antagonist L-745,870 increased AAAD activity in striatum (Fig. 4). Enzyme activity was increased between 5 and 30 min, returned to normal levels by 1 h, and then increased again by 3 h and remained elevated for over 6 h, a temporal pattern similar to that observed for D1, D2, and D3 antagonists (Zhu et al., 1992; Hadjiconstantinou et al., 1993; Cho et al., 1997). To determine whether receptors other than dopamine contribute to the clozapine effect, in a series of studies, mice were injected with a single dose of antagonist drugs to block serotonin, muscarinic, or adrenergic receptors, and AAAD activity in striatum was followed over time from 5 min to 6 h post-treatment. Blocking muscarinic receptors with atropine, α-1 adrenergic receptors with prazosin, α-2 adrenergic receptors with yohimbine, 5-HT2C receptors with SB-242084, and 5-HT3 receptors with tropisetron had no effect on AAAD activity in striatum at any of the times studied, 5 min to 6 h (data not shown). The 5-HT2A/5-HT2C antagonist ketanserin, the 5-HT1A/5-HT2A antagonist metergoline, and the 5-HT1A selective antagonist Way 100635 all increased AAAD activity in striatum (Fig. 4). The MPTP mouse model of parkinsonism was used to investigate whether clozapine could augment AAAD activity in the dopaminergically denervated striatum and whether the lesioned dopaminergic neurons retain the ability to respond

As expected, acute administration of clozapine enhanced the synthesis and metabolism of dopamine in striatum as evidenced by increases in the tissue content of the amine and its metabolites DOPAC and HVA (Fig. 5). Dopamine and metabolite content rose as early as 10 min after drug administration and were elevated for more than 3 h. The maximal increase of dopamine was modest, approximately 35% over control, whereas the content of the metabolites doubled. The onset of the change coincided with the activation of tyrosine hydroxylase (Fig. 1), whereas the duration of the change followed the temporal pattern of AAAD induction more closely. Clozapine also increased AAAD activity after chronic administration. Indeed, after daily administration of clozapine for 7 days, there was a rise in AAAD activity, 44% over control value (Fig. 6A), as well as enzyme protein, and 37% over control value (percentage of control ± S.E.M., 137 ± 6; p < 0.05, n = 6–8) (Fig. 6B) in striatum. This was evident at 1 h post-treatment, where enzyme activity and protein appeared to decline over time, and by 6 h, where a small increase was still present. The increase of AAAD activity coincided with an enhancement of dopamine metabolism, as indicated by elevated tissue dopamine, DOPAC, and HVA content (Fig. 6A). In contrast to AAAD, neither tyrosine hydroxylase activity (data not shown), measured with saturated cofactor conditions, nor tyrosine hydroxylase protein changed (Fig. 6B) after chronic clozapine.

The MPTP mouse model of parkinsonism was used to investigate whether clozapine could augment AAAD activity in the dopaminergically denervated striatum and whether the lesioned dopaminergic neurons retain the ability to respond

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**Fig. 3.** Clozapine increases AAAD mRNA in the mouse brain. Mice were treated with 20 mg/kg i.p. clozapine or vehicle and killed 1 h later. AAAD mRNA was evaluated with in situ hybridization as described under Materials and Methods. Representative images from five animals/group. A, SNc, substantia nigra pars compacta. VTA, ventral tegmental area. B, LC, locus coeruleus. C, MR, median raphe nucleus; DR, dorsal raphe nucleus.

**Fig. 4.** Serotonin 5-HT2A and 5-HT1A as well as dopamine D4 receptor antagonists increase AAAD activity in striatum. A single dose of 20 mg/kg i.p. ketanserin (A, open bar), 3 mg/kg i.p. metergoline (A, closed bar), 1 mg/kg i.p. L-745,870 (B, open bar), or 1 mg/kg s.c. Way 100635 (B, closed bar) was administered to mice, and they were killed at the time intervals indicated. AAAD activity was assayed as described under Materials and Methods. *, p < 0.05 compared with control (0 time). n = 6 to 8 animals/group.
to clozapine. Twenty four hours after a MPTP regimen that causes loss of approximately 50% of the dopaminergic neurons, AAAD and tyrosine hydroxylase activity in striatum was decreased by approximately 60%, suggesting that the majority of the AAAD activity in the nucleus is contained in dopaminergic neurons (Table 1). A single dose of clozapine enhanced the activity of AAAD and tyrosine hydroxylase in the striatum of both intact and lesioned mice when estimated at 30 and 60 min post-treatment, and there was a trend for larger percentage of enzyme activity increase in the MPTP-treated mice. In addition to the activity of the dopamine synthetic enzymes, the clozapine-stimulated dopamine metabolism was greater in the dopaminergically denervated striatum as demonstrated by significant increases, 2- to 3-fold, of dopamine, DOPAC, and HVA content (Table 2).

To evaluate whether the clozapine-induced increase of AAAD is functional, we administered L-DOPA to intact and MPTP-lesioned animals treated with clozapine. Based on the clozapine response studies, two treatment regimens were investigated; clozapine and L-DOPA were administered concomitantly (animals were killed 1 h later), and clozapine was administered first followed by L-DOPA 2 h later (animals were killed 1 h afterward). Accordingly, the animals were killed at 1 or 3 h postclozapine at time points where AAAD activity is elevated (Fig. 1). Both treatments produced similar results, and Table 2 shows results from the concomitant drug administration. As we have reported (Neff et al., 2000), exogenous L-DOPA had no significant effect on the steady-state levels of dopamine in the striatum of the intact mice, but it doubled the levels of DOPAC and HVA. However, in the MPTP mice, L-DOPA elevated the content of dopamine by 2-fold, and there was a marked rise (6-fold) of DOPAC and HVA. After combined clozapine/L-DOPA administration to intact mice, the dopamine content in striatum was not different from that of clozapine or L-DOPA alone, whereas both DOPAC and HVA were significantly elevated compared with either treatment, and the estimated magnitude of response was approximately 6-fold over control value for DOPAC and 3-fold over control value for HVA. A similar pattern also was observed in the MPTP-treated mice, but the response was exaggerated with a dramatic rise of DOPAC (~17-fold) and of HVA (~12-fold). Dopamine turnover, estimated as the ratio of DOPAC to dopamine, was enhanced in the striatum of both intact and lesioned mice following L-DOPA, and the rate appeared greater in the MPTP-treated mice. Coad-

TABLE 1
Clozapine increases the activity of TH and AAAD in the striatum of the MPTP mouse

Mice were treated with 30 mg/kg i.p. MPTP or saline daily for 7 days and 24 h later with a single dose of 20 mg/kg i.p. clozapine or vehicle and killed at the indicated times. TH and AAAD activities were estimated in striatum as described under Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TH</th>
<th>AAAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.95 ± 0.03</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Clozapine, 30 min</td>
<td>1.3 ± 0.07**</td>
<td>51 ± 5*</td>
</tr>
<tr>
<td>Clozapine, 60 min</td>
<td>1.3 ± 0.09**</td>
<td>54 ± 3*</td>
</tr>
<tr>
<td>MPTP</td>
<td>0.32 ± 0.02</td>
<td>16 ± 1*</td>
</tr>
<tr>
<td>Clozapine, 30 min</td>
<td>0.80 ± 0.03**</td>
<td>24 ± 4**</td>
</tr>
<tr>
<td>Clozapine, 60 min</td>
<td>0.50 ± 0.02**</td>
<td>25 ± 3**</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with the saline-treated group.
** p < 0.05 compared with the MPTP-treated group, n = 8.
ministration of clozapine and l-DOPA further accelerated the
turnover of dopamine.

To ascertain the contribution of AAAD in the observed changes of dopamine metabolism following l-DOPA and/or clozapine, a group of intact animals was treated with the AAAD inhibitor NSD 1015, and then l-DOPA or clozapine alone or in combination was administered (Table 3). NSD 1015 (100 mg/kg i.p.) abrogated AAAD activity across all treatment groups with residual activity of ~5 pmol/mg protein remaining (data not shown). AAAD inhibition had minimal effect on the content of dopamine on the various treatment groups. In contrast, the intracellular metabolite DOPAC was barely detectable, and the extracellular metabolite HVA was significantly decreased in all treatment groups (Table 3).

Discussion

Following a single administration of clozapine, tyrosine hydroxylase activity and dopamine synthesis, release, and metabolism are enhanced in striatum (Burki et al., 1975; Zivkovic et al., 1975; Invernizzi et al., 1990; Karoum and Egan, 1992; Broderick and Piercey, 1998). The novelty of our studies lies in the finding that clozapine also increases the activity of the second enzyme in the biosynthetic pathway for dopamine, AAAD. Indeed, after acute clozapine, AAAD activity is increased, characterized by increased V_{max} for l-DOPA and pyridoxal-5’-phosphate. In contrast to the late time points, AAAD protein did not change concurrently with the early rise of enzyme activity, suggesting activation. This effect is reminiscent of that of D1- and D2-like antagonists that cause a biphasic change of AAAD activity in striatum, an early and transient activation and a late and prolonged induction (Zhu et al., 1992; Hadjiconstantinou et al., 1993; Cho et al., 1997). Contrary to AAAD, clozapine caused a relatively early and short-lasting activation of tyrosine hydroxylase (Zivkovic et al., 1975), which partially overlapped with the AAAD response. Thus, it appears that acute clozapine modulates the activity of dopamine synthetic enzymes, with activation being the sole regulatory mode for tyrosine hydroxylase and induction being the predominant regulatory mode for AAAD, although activation is possible. Notably, the clozapine dose required for maximal tyrosine hydroxylase activation was lower (<5 mg/kg) than that for maximal AAAD induction (>5 mg/kg). Similar observations have been made with D1- and D2-like antagonists, with the AAAD maximal response generally seen with doses at the higher end of the range used in the literature (Hadjiconstantinou et al., 1993). Perhaps clozapine exerts its action on tyrosine hydroxylase and AAAD via distinct mechanisms and sites (pre- or postsynaptic) requiring different receptor selectivity and occupation. An interesting finding of our studies is the number of receptors that modulates AAAD activity in striatum. Blockade of D1, D2, D3, and D4, as well as 5-HT2A and 5-HT1A receptors, increases AAAD activity and could contribute to the clozapine effect. Ostensibly, D2-like auto-receptors are involved with the tyrosine hydroxylase activation by clozapine, whereas the role of serotonin receptors is still to be determined (Hetey et al., 1985; Onali et al., 1992).

The regulation of AAAD in extrapiramidal sites might contribute to the observed tyrosine hydroxylase versus AAAD response discrepancy. Eighty percent of the striatal AAAD is located in dopaminergic neurons, and the remainder is in noradrenergic, serotonergic, and intrinsic AAAD-positive neurons (Melamed et al., 1981; Eaton et al., 1993; Mura et al., 1995), all of which add to the AAAD activity assayed in striatum homogenates. Clozapine up-regulates the expression of AAAD mRNA in substantia nigra pars compacta and ventral tegmental area, indicating that enzyme activity is modulated in dopaminergic neurons. The finding that clozapine increases the AAAD mRNA levels in locus coeruleus and dorsal and median raphe nuclei is the first indication that AAAD is modulated in noradrenergic and serotonergic neurons and implies that both neuronal

### Table 2

**Clozapine enhances the l-DOPA-stimulated DA metabolism in striatum**

Twenty-four hours after MPTP treatment (see Table 1), lesioned and intact (saline-treated) mice were administered a single dose of 20 mg/kg i.p. clozapine, 100 mg/kg i.p. l-DOPA, clozapine, and l-DOPA or vehicle and killed 1 h later. DA and metabolites were estimated in the striatum as described under Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DA (pmol/mg protein)</th>
<th>DOPAC (pmol/mg protein)</th>
<th>HVA (pmol/mg protein)</th>
<th>DOPAC + HVA/DA (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>464 ± 15</td>
<td>70 ± 9</td>
<td>50 ± 2</td>
<td>0.25</td>
</tr>
<tr>
<td>MPTP</td>
<td>116 ± 16*</td>
<td>23 ± 2*</td>
<td>21 ± 2*</td>
<td>0.38</td>
</tr>
<tr>
<td>Control + l-DOPA</td>
<td>544 ± 12**</td>
<td>160 ± 2**</td>
<td>105 ± 5**</td>
<td>0.49</td>
</tr>
<tr>
<td>MPTP + l-DOPA</td>
<td>228 ± 12**</td>
<td>145 ± 20**</td>
<td>123 ± 14**</td>
<td>1.17</td>
</tr>
<tr>
<td>Saline + clozapine</td>
<td>580 ± 48**</td>
<td>147 ± 9**</td>
<td>78 ± 4**</td>
<td>0.39</td>
</tr>
<tr>
<td>MPTP + clozapine</td>
<td>246 ± 21</td>
<td>62 ± 5**</td>
<td>45 ± 5**</td>
<td>0.43</td>
</tr>
<tr>
<td>Saline + clozapine + l-DOPA</td>
<td>567 ± 39</td>
<td>432 ± 51***</td>
<td>172 ± 6***</td>
<td>1.06</td>
</tr>
<tr>
<td>MPTP + clozapine + l-DOPA</td>
<td>279 ± 11</td>
<td>402 ± 60***</td>
<td>243 ± 31***</td>
<td>2.31</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with saline-treated animals.

**Table 3**

**NSD 1015 inhibits the decarboxylation of l-DOPA**

NSD 1015 (NSD 1015, 100 mg/kg i.p.) was administered 1 h prior to 20 mg/kg i.p. clozapine (CLZ), 100 mg/kg i.p. l-DOPA, or combined clozapine and l-DOPA, and animals were killed 2 h later. DA and metabolites were estimated in the striatum as described under Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DA (pmol/mg protein)</th>
<th>DOPAC (pmol/mg protein)</th>
<th>HVA (pmol/mg protein)</th>
<th>DOPAC + HVA/DA (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>464 ± 8</td>
<td>74 ± 2</td>
<td>50 ± 3*</td>
<td>0.26</td>
</tr>
<tr>
<td>+NSD</td>
<td>389 ± 10</td>
<td>11 ± 1*</td>
<td>21 ± 1*</td>
<td>0.08</td>
</tr>
<tr>
<td>+CLZ</td>
<td>580 ± 48</td>
<td>194 ± 30</td>
<td>86 ± 4</td>
<td>0.48</td>
</tr>
<tr>
<td>+NSD + CLZ</td>
<td>430 ± 10</td>
<td>12 ± 1*</td>
<td>22 ± 3*</td>
<td>0.08</td>
</tr>
<tr>
<td>l-DOPA</td>
<td>550 ± 31</td>
<td>160 ± 2*</td>
<td>108 ± 13</td>
<td>0.49</td>
</tr>
<tr>
<td>+NSD</td>
<td>546 ± 23</td>
<td>11 ± 2*</td>
<td>18 ± 3*</td>
<td>0.05</td>
</tr>
<tr>
<td>+CLZ</td>
<td>567 ± 39</td>
<td>432 ± 57</td>
<td>177 ± 13</td>
<td>1.07</td>
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<tr>
<td>+NSD + CLZ</td>
<td>635 ± 54</td>
<td>22 ± 3*</td>
<td>20 ± 2*</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with comparison group not receiving NSD 1015. n = 6 to 8.
populations could contribute to the changes of enzyme activity and protein in striatum, albeit to a lesser degree than the dopaminergic afferents. The receptors involved are presently unknown, but in analogy with the regulation of AAAD by presynaptic dopaminergic receptors, we have identified 5-HT1A and 5-HT2A and α-2 adrenergic receptors as putative candidates. The inability of piperoxan to increase AAAD activity in striatum does not exclude an effect in locus coeruleus, because both clozapine and α blockers increase noradepinephrine turnover in regions with rich noradrenergic innervation but not striatum (McMillen and Shore, 1978).

The regulation of AAAD by clozapine was evident in the striatum of mice bearing a 50% lesion of dopaminergic neurons with MPTP, and the enzyme responded more robustly. Similar observations have been made with dopamine antagonists and forskolin, with enzyme activity increases being greater and occurring at earlier times and with lower drug doses (Hadjiconstantinou et al., 1993; Young et al., 1998). The exaggerated AAAD response in the partially dopamine-depleted striatum occurs within 24 h after MPTP, at a time when dopamine receptor supersensitivity has not developed (Weihmuller et al., 1990), and there is no serotonergic neuronal sprouting (Rozas et al., 1998) or glia proliferation (Mao et al., 2001). Clozapine-induced tyrosine hydroxylase activation and dopamine metabolism were also amplified in the striatum of the lesioned mice, indicating a heightened response by the remaining dopaminergic neurons.

Although undoubtedly AAAD is a regulated enzyme, there is no evidence supporting a role for the synthesis of dopamine under normal conditions (Cho et al., 1997, 1999). Following acute clozapine, the onset of dopamine metabolism enhancement coincided with the tyrosine hydroxylase activation, whereas its duration followed the AAAD induction closely. After repeated clozapine, there was a temporal association between AAAD activity and dopamine metabolism, with no concurrent tyrosine hydroxylase changes. The significance of these observations for dopamine synthesis is unclear. Dopamine metabolism is the reflection of multiple control mechanisms, e.g., presynaptic and postsynaptic receptors, feedback loops, neuronal firing rate, synthesis, release, and reuptake, which might be affected by clozapine. Notwithstanding, clozapine alters the metabolism of serotonin and noradrenergic as well (Burki et al., 1975; McMillen and Shore, 1978), and AAAD is involved with the synthesis of trace amines (Berry et al., 1996).

As expected, administration of a single dose of L-DOPA to intact or MPTP-lesioned mice, not treated with a peripheral AAAD inhibitor, enhanced the metabolism of dopamine in the striatum, with the response noticeably pronounced in the denervated striatum (Hefti et al., 1980, 1981; Nguyen and Angers, 1987; Kang et al., 1992; Kaakkola and Wurtman, 1993; Neff et al., 2000). The latter was apparently due to accelerated L-DOPA decarboxylation, because the percentage increase of total decarboxylation product (DA + DOPAC + HVA) was larger in the MPTP mice, suggesting enhanced L-DOPA decarboxylation after a partial dopaminergic lesion. Dopamine from exogenous L-DOPA is largely, but not exclusively, formed in dopaminergic neurons (Hefti et al., 1980, 1981). Because clozapine also elevates the AAAD mRNA in serotonergic and noradrenergic neurons, accelerated L-DOPA decarboxylation could occur in extradopaminergic sites to a lesser degree.

Despite the striking increases in DOPAC and HVA content in intact and lesioned mice, the change of dopamine was modest, in line with the notion that dopamine formed from exogenous L-DOPA is not stored in vesicles and is rapidly metabolized (Melamed, 1990). Clozapine accelerated the exogenous L-DOPA decarboxylation in striatum, especially after a dopaminergic lesion. Indeed, it increased the L-DOPA-stimulated dopamine metabolism in striatum, and the effect was most dramatic in the MPTP mice, with a 5- to 6-fold increase in metabolite formation and turnover rate and doubling of total decarboxylation product. In addition to AAAD activity, substrate availability and neuronal firing may contribute to this response. However, following L-DOPA, DOPA content increases similarly in the striatum and midbrain of intact and MPTP-lesioned mice (Neff et al., 2000), and neuronal firing has no effect on the synthesis and release of dopamine formed from exogenous L-DOPA (Melamed, 1990).

The AAAD inhibitor NSD 1015 had no appreciable effect on dopamine but attenuated the L-DOPA-stimulated increase of DOPAC and HVA. The decrease of DOPAC was dramatic and paralleled that of AAAD, in agreement with reports that NSD 1015 primarily inhibits the accumulation of dopamine and the formation of DOPAC in the cytosolic compartment (Neff et al., 2000), which is apparently the major metabolic route of exogenous L-DOPA when administered without a peripheral AAAD inhibitor (Mowa et al., 1992). The observation that NSD 1015 inhibits the activity of AAAD, as well as the L-DOPA and clozapine/L-DOPA-stimulated DOPAC and HVA formation and dopamine turnover, points to a link between AAAD and L-DOPA-induced dopamine metabolism in striatum. Furthermore, the percentage increase in enzyme activity after clozapine appears to be proportional to the basal or L-DOPA-stimulated total decarboxylation product, between 40 and 50%. Thus, it is reasonable to assume that the accelerated L-DOPA decarboxylation by clozapine is, in part, due to AAAD induction. Supporting this notion are reports that AAAD plays a role in the dopamine receptor-mediated modulation of exogenous L-DOPA-stimulated dopamine metabolism (Cumming et al., 1997; Fisher et al., 2000; Neff et al., 2000).

In summary, we provide evidence that clozapine modulates AAAD activity in striatum and enhances exogenous L-DOPA decarboxylation in intact and MPTP-lesioned mice. The potential to modulate AAAD might be, in part, responsible for the reported effects of clozapine on L-DOPA-induced dyskinesias and motor fluctuations. That, in addition to dopaminergic neurons, AAAD induction by clozapine occurs in serotonergic and noradrenergic neurons is of clinical importance because over 80% of dopaminergic neurons are lost in the Parkinsonian brain and dopamine formation from exogenous L-DOPA takes place in nondopaminergic neurons. Finally, the finding that dopamine D4 and 5-HT2A and 5-HT1A antagonists also increase AAAD activity in striatum provides new clues for understanding the pharmacological mechanisms(s) for the regulation of the enzyme. Amplification of AAAD activity might be a novel strategy to augment L-DOPA response while decreasing unwanted side effects. Clozapine can serve as a model to synthesize drugs that can be administered safely along with L-DOPA in Parkinsonian patients.

References


Young EA, Duchemin A-M, Neff NH, and Hadjiconstantinou M (1998) Parallel modulation by Clozapine 487