JPET Fast Forward. Published on January 13, 2006 as DOI: 10.1124/jpet.105.097972
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Clozapine Modulates Aromatic L-Amino Acid Decarboxylase Activity in Mouse Striatum

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Running Title: AAAD Modulation by Clozapine

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Number of text pages: 36

Number of Tables: 3

Number of Figures: 6

Number of References: 40

Number of words in Abstract: 250

Number of words in Introduction: 632

Number of words in Discussion: 1500

Abbreviations used: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; AAAD, aromatic L-amino acid decarboxylase.

Recommended section assignment: Neuropharmacology

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Abstract

Clozapine is efficacious for treating dopaminergic psychosis in Parkinsons'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 of 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 (MPTP)-lesion. Chronically administered clozapine increased AAAD activity and protein and dopamine metabolism in striatum without affecting TH. 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-$HT1_A$ and 5-$HT2_A$, in addition to D1, D2 and D3, antagonists, augmented AAAD activity in striatum; while, 5-$HT2_C$, 5-HT3, muscarinic and alpha-1 and alpha-2 adrenergic antagonists were ineffective. These

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studies, for the first time, provide evidence that clozapine modulates AAAD activity in the brain and suggests that dopamine and serotonin receptors are involved.

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Introduction

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. Physiological stimuli (Hadjiconstantinou et al., 1988), neurotransmitter receptors (Rossetti et al., 1989, 1990; Zhu et al., 1992; Hadjiconstantinou et al., 1993, 1995; Cho et al., 1997; Fisher et al., 1998) and second messenger systems (Young et al., 1998) participate in the regulation of enzyme activity. In nigrostriatal neurons dopamine appears to regulate AAAD via D1-like and D2-like receptors. Indeed, acute blockade of dopamine D1-like receptors with SCH23390 and of dopamine D2-like receptors with haloperidol, sulpiride or spiperone augments AAAD activity *in vivo*. In both cases AAAD responds with a biphasic change; an early transient rise and a late protracted increase. Existing experimental evidence supports a scheme where the early change in activity is due to enzyme activation, while the late is the result of enzyme induction. The increase of AAAD activity by dopaminergic antagonists is apparently functional as it is accompanied by enhanced exogenous L-DOPA decarboxylation and dopamine synthesis and release (Cumming et al., 1997; Neff et al., 2000; Fisher et al., 2000).

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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 as 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 extrapyramidal signs liability (Tarsy et al., 2002), and reportedly, improve L-DOPA-induced dyskinesias and motor fluctuations (Durrif, 1999). They are multireceptor drugs and display varying affinities for dopamine D1, D2, D3 and D4 receptors, serotonin $5HT1_A$, $5\text{-}HT2_A$, $5\text{-}HT2_C$, 5-HT6 and 5-HT7 receptors, muscarinic M1 receptors, adrenergic alpha-1 and alpha-2 receptors, as well as histamine H1 receptors. The lack of extrapyramidal 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 $5\text{-}HT2_A$ 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 (Zivkovic et al., 1975; Hetey et al., 1985) and enhances

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dopamine metabolism (Zivkovic et al., 1975; Burki et al., 1975, Invernizzi et al., 1990; Karum 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.

Methods

1. Animals and Treatments

Male Swiss-Webster mice (Harlan, Indianapolis, IN), 25-30 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: clozapine, atypical antipsychotic drug, 20 mg/kg, ip; L-745,870 (3-([4-(4-chlorophenyl)piperazin-1-yl]methyl)-1H- pyrollo[2,3-b] pyridine), selective D4 receptor antagonist, 1 mg/kg, ip; Way 100635 (N-[2-[4-(2-[O-methyl]methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexane carboxamide trihydrochloride), selective 5-$HT1_A$ receptor antagonist, 1 mg/kg, sc; metergoline 5-$HT1_A$/5-$HT2_A$ receptor antagonist, 3 mg/kg, ip; ketanserin, 5-$HT2_A/2_C$

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antagonist, 20 mg/kg, ip; SB-242084 (6-Chloro-5-methyl-1-[(2-[2-methylpyrid-3-yloxy]pyrid-5yl)carbamoyl]indoline), 5-HT2$_C$ receptor antagonist, 1 mg/kg, ip; atropine, muscarinic receptor antagonist, 20 mg/kg, sc; prazocin, alpha-1 adrenergic receptor antagonist, 2 mg/kg, ip; yohimbine, alpha-2 adrenergic receptor antagonist; 10 mg/kg, ip, tropisetron, 5-HT3 antagonist, 1 mg/kg, ip; 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 ip. Some animals were treated with the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 30 mg/kg, ip, daily for 7 days and 24 h later injected clozapine, 20 mg/kg, ip, 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, ip, alone or together with clozapine, 20 mg/kg, ip, and killed 1 h later. In some studies the AAAD inhibitor NSD 1015 (m-hydroxybenzylhydrazine), 100 mg/kg, ip, 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, ip, 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 dissected and used for the various assays as indicated. One striatum was used for the assay of AAAD, dopamine and metabolites, and western blots; while, the other was used for the assay of tyrosine hydroxylase. Whole brains were immediately frozen on dry ice for *in situ* hybridization studies.

2. Procedures

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AAAD Activity Assay: AAAD activity was assayed as described previously (Hadjiconstantinou et al., 1993). Briefly, 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 $HClO_4$, containing 3,4-dihydroxybenzylamine as an internal standard. Dopamine was extracted using alumina, resolved by high-performance liquid chromatography and monitored with an electrochemical detector (HPLC-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, of 6-methyl-5,6,7,8-tetrahydropteridine, 10 μg/100 μl catalase, 1 mM ferrous ammonium sulfate, and 200 μM L-tyrosine with 1 μCi [^{3}H]-L-tyrosine (48 Ci/mmol; Amersham 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_2O in the supernatant was counted in a scintillation counter (Hadjiconstantinou et al., 1994).

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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 $HClO_4$ containing 0.05 mM sodium bisulfite were added and centrifuged. An aliquot of supernatant, 100 µl, was injected into the HPLC-ED system (Cho et al., 1996).

Western Blots: Tissue lysates of equal protein quantity, 20µg, were separated by SDS-PAGE and proteins transferred to nitrocellulose membrane. Blots were incubated overnight in TBS-Tween (10 mM Tris, 150 mM NaCl, 0.1% Tween-20) containing 5 % dry milk as a blocking agent. Then, they were 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 peroxidase (HRP) conjugated secondary antibody (Santa Cruz, Santa Cruz, CA) with extensive washing in TBS-Tween after incubation with each antibody. Bound antibody was visualized with Enhanced Chemiluminescence (Amersham Bioscience, Piscataway, NJ). Band intensity was determined by image analysis (MetaMorph, Sunnyvale, CA) and data expressed as percent of control for each blot (Cho et al., 1996).

In situ Hybridization: For the AAAD *in situ* hybridization studies, frozen brain sections, 12 µm, were thaw-mounted on Superfrost/Plus (Erie Scientific Co, Portsmouth, NH) microscope slides, and fixed in 4% paraformaldehyde followed by 0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0. ^{35}S-labelled (Amersham Bioscience, Piscataway, NJ) sense and anti-sense riboprobes were prepared from a 286 bp fragment

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of the mouse brain AAAD cDNA (Eaton et al, 1993). Sections were incubated with the probe for 20 h in 50% formamide, 10% dextran sulfate, 1 X Denhardt's solution, 0.3 M NaCl, 20 mM Tris HCl, 1 mM EDTA, 0.1M dithiothreitol and 0.3 μg tRNA. Sections were then washed and treated with RNAseA, and exposed to X-ray film. Quantitative analysis of the autoradiographs was performed by image analysis (MetaMorph) using [^{14}C] standards (Amersham Bioscience, Piscataway, NJ) and data presented as percent of control for each experiment.

Statistical Analysis: Statistical analysis for the enzyme activities and tissue content of dopamine and metabolites was performed by one- or two-way analysis of variance followed by a Newman-Keuls test for group comparisons or a t-test. Data expressed as percentages were evaluated by the non-parametric Kruskal-Wallis analysis of variance followed by a Dunn's test for multiple comparisons or a Mann-Whitney test. Results were considered statistically significant when $p<0.05$.

Results

Administration of a single dose of clozapine increased the activity of AAAD in the striatum in a dose- and time-dependent manner (Figure 1, A and B). Increased activity was observed between 30 min- 3 h post-injection and by 6 h it was at control values. Maximal activity, 40-50% over control values, was observed between 5 and 20 mg/kg, ip, of clozapine and 20 mg was chosen as this dose has been used commonly in the literature. Clozapine also enhanced the activity of striatal tyrosine hydroxylase, estimated under

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cofactor non-saturating conditions, in a dose- and time-dependent fashion (Figure 1, A and B) with a maximal response of about 40% over control value. Increased tyrosine hydroxylase activity, however, was observed earlier than that of AAAD, at 10 min post treatment, lasted shorter, returned to control values by 60 min, and required smaller drug doses. Kinetic analysis of AAAD at 1 h showed an increase in the apparent Vmax for both the substrate L-DOPA (vehicle, 45 ± 2; clozapine, 70 ± 3* nmol/mg prot/20 min ± SEM, N=3) and the cofactor pyridoxal-5'-phosphate (vehicle, 46 ± 2; clozapine, 73 ± 3 nmol/mg prot/20 min ± SEM, N=3) with no changes for Km (data not shown).

AAAD protein was elevated between 1-3 h, about 50% over control values, after clozapine (Figure 2). No change in the content of tyrosine hydroxylase protein was observed. The finding that at 30 min the increase of AAAD activity was not accompanied by a concomitant significant increase of protein might be indicative of enzyme activation. *In situ* hybridization studies, at 30 and 60 min post-treatment, revealed that clozapine elevated AAAD mRNA not only in the dopaminergic midbrain nuclei, substantia nigra pars compacta and ventral tegmental area, but also in locus coeruleus, site of noradrenergic cell bodies, and in both dorsal and median raphe nuclei, sites of serotonergic cells bodies (Figure 3). The response (percent of control ± SEM) was greatest in dorsal raphe nuclei (160 ± 6*) and median raphe nuclei (150 ± 3*), followed by substantia nigra pars compacta (134 ± 4*), locus coeruleus (127 ± 3*) and ventral tegmental area (118 ± 3*), *$p<0.05$ compared with vehicle, N= 5.

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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 assayed in stiatum. Since 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 (Figure 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 other than dopamine receptors 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, alpha-1 adrenergic receptors with prazocin, alpha-2 adrenergic receptors with yohimbine, 5-HT2_C receptors with SB-242084 and 5-HT3 receptors with tropisetron, had no effect on AAAD activity in striatum at any time studied, 5 min–6 h (data not shown). The 5HT2_A/5-HT2_C antagonist ketanserin, the 5-HT1_A/5-HT2_A antagonist metergoline and the 5HT1_A selective antagonist WAY 100635, all increased AAAD activity in the striatum (Figure 4, A and B). The time-course of the response was similar, but not identical, for all three serotonergic antagonist drugs, with an early transient increase in enzyme activity, between 5 and 30

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min, return to basal levels between 30-60 min, and late rise lasting over 6 h. The temporal pattern of AAAD response to D4 and 5-HT antagonists was different compared to clozapine, as enzyme activity rose later and lasted shorter following the atypical antipsychotic (Figure 1).

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 (Figure 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, about 35% over control, while the content of the metabolites doubled. The onset of the change coincided with the activation of tyrosine hydroxylase (Figure 1); while, the duration of the change followed more closely the temporal pattern of AAAD induction. Clozapine also increased AAAD activity after chronic administration. Indeed, following daily administration of clozapine for 7 days there was a rise in AAAD activity, 44 % over control value, (Figure 6, A) as well as enzyme protein, 37 % over control value (percent of control ± SEM, 137 ± 6; $*p<0.05$, N=6-8) (Figure 6, B) in striatum. This was evident at 1 h post-treatment, and enzyme activity and protein appeared to decline over time and by 6 h 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 (Figure 6, A). In contrast to AAAD, neither tyrosine hydroxylase activity (data not shown), measured with saturated

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cofactor conditions, nor tyrosine hydroxylase protein changed (Figure 6.B) 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 to clozapine. Twenty four h after a MPTP regimen that causes loss of about 50% of the dopaminergic neurons, AAAD and tyrosine hydroxylase activity in striatum was decreased by about 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 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: 1) clozapine and L-DOPA were administered concomitantly and animals killed 1 h later, and 2) clozapine was administered first followed by L-DOPA 2 h later and animals killed 1 h

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afterward. Accordingly, the animals were killed at 1 or 3 h post-clozapine, at time points where AAAD activity is elevated (Figure 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. In the MPTP- mice, however, L-DOPA elevated the content of dopamine by 2-fold, and there was a marked, 6-fold, rise 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, while both DOPAC and HVA were significantly elevated compared to either treatment, and the estimated magnitude of response was about 6-fold over control value for DOPAC and 3-fold over control value for HVA. A similar pattern was observed in the MPTP-treated mice also, but the response was exaggerated with a dramatic rise of DOPAC, about 17-fold, and of HVA, about 12-fold. Dopamine turnover, estimated as the ratio of DOPAC and HVA 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. Co-administration 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). NSD1015, 100 mg/kg, ip, abrogated AAAD activity across all

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treatment groups with a residual activity of about 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 (Zivkovic et al., 1975; Burkey et al., 1975; Invernizzi et al., 1990; Karum and Egan, 1992; Broderick and Piercey 1998). The novelty of our studies lies on 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 in striatum increased in a dose- and time-dependent manner for over 3 h. The elevation of AAAD protein and mRNA indicates enzyme induction, characterized by increased Vmax 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 which 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. It appears thus, that acute clozapine

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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, though 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 post-synaptic) requiring different receptor selectivity and occupation. An interesting finding of our studies is the number of receptors that modulate AAAD activity in striatum. Blockade of D1, D2, D3 and D4 as well as 5-HT2_A and 5-HT1_A receptors increases AAAD activity and could contribute to the clozapine effect. Ostensibly, D2-like autoreceptors are involved with the tyrosine hydroxylase activation by clozapine, while the role of serotonin receptors is still to be determined (Onali et al., 1992; Hetey et al., 1985).

The regulation of AAAD in extra-dopaminergic 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 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-regulated the expression of AAAD mRNA in substantia nigra pars compacta and ventral tegmental area, indicating that enzyme activity is modulated in

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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 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-HT1$_A$ and 5-HT2$_A$ and alpha-2 adrenergic receptors as putative candidates. The inability of piperoxan to increase AAAD activity in striatum does not exclude an effect in locus coeruleus, as both clozapine and alpha blockers increase norepinephrine turnover in regions with rich noradrenergic innervation but not striatum (McMillan 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 (Rosas et al., 1998) or glia proliferation (Mao et al., 2001). Clozapine-induced tyrosine hydroxylase activation and

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dopamine metabolism were also amplified in the striatum of the lesioned mice, indicating a heightened response by the remaining dopaminergic neurons.

While 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 closely the AAAD induction. 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., pre-and post-synaptic receptors, feedback loops, neuronal firing rate, synthesis, release and re-uptake, which might be affected by clozapine. Notwithstanding, clozapine alters the metabolism of serotonin and norepinephrine as well (Burki et al., 1975; McMillan 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 being noticeably pronounced in the denervated striatum (Hefti et al., 1980, 1981; Nguyen and Angers, 1987; Kang et al. 1992; Kaakkola et al., 1993; Neff et al., 2000). The latter was apparently due to accelerated L-DOPA decarboxylation, as the percent increase of total decarboxylation product (DA+DOPAC+HVA) was larger in

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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). As clozapine elevates the AAAD mRNA in serotonergic and noradrenergic neurons also, accelerated L-DOPA decarboxylation could occur in extra-dopaminergic 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 5-6 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 parallel that of AAAD, in agreement with reports that NSD 1015 primarily inhibits the accumulation of dopamine and the formation of DOPAC in the cytostolic compartment

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(Neff et al., 2000), which is apparently the major metabolic route of exogenous L-DOPA when administered without a peripheral AAAD inhibitor (Miwa 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 point to a link between AAAD and L-DOPA-induced dopamine metabolism in striatum. Furthermore, the percent increase in enzyme activity after clozapine appears to be proportional to the basal or L-DOPA-stimulated total decarboxylation product, between 40-50%. 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; Neff et al., 2000; Fisher 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 as over 80% of dopaminergic neurons are lost in the parkinsonian brain and dopamine formation from exogenous L-DOPA takes place in non dopaminergic neurons. Finally, the finding that dopamine D4 and 5-$HT2_A$ and 5-$HT1_A$ antagonists also increase AAAD activity in striatum provides new clues for

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understanding the pharmacological mechanism(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 model to synthesize drugs that can be administered safely along with L-DOPA in parkinsonian patients.

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Footnotes

Research was supported, in part, by grant NS34571.

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Figure Legends

Figure 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, ip. Striatum was assayed for AAAD and TH as described in Methods. *p<0.05 compared with control (0 time or 0 dose). N=6-8 animals/group.

Figure 2. Clozapine increases AAAD but not TH protein after acute administration. Mice were treated with clozapine, 20 mg/kg, ip, or vehicle and killed at the time intervals indicated. AAAD and TH protein were estimated in striatum by western blot and quantified as in Methods. A. Graphic presentation of AAAD, open bars, or TH, closed bars, protein content expressed as percent of control (vehicle). B. Representative western blot. *p<0.05 compared with control. N=6-8 animals/group.

Figure 3. Clozapine increases AAAD mRNA in the mouse brain. Mice were treated with clozapine, 20 mg/kg, ip, or vehicle and killed 1 h later. AAAD mRNA was evaluated with *in situ hybridization* as described in Methods. Representative images from 5 animals/group. A. substantia nigra pars compacta, SNc, and ventral tegmental area, VTA; B. locus coerulelus, LC; C. median raphe nucleus, MR, and dorsal raphe nucleus, DR.

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Figure 4. Serotonin 5-HT_{2A} and 5-HT_{1A} as well as dopamine D4 receptor antagonists increase AAAD activity in striatum. A single dose of ketanserin, 20 mg/kg, ip (A open bar); metergoline, 3 mg/kg, ip (A closed bar); L745870, 1 mg/kg, ip (B open bar); or WAY100635, 1 mg/kg, sc (B closed bar), was administered to mice and they were killed at the time intervals indicated. AAAD activity was assayed as described in Methods. *$p<0.05$ compared with control (0 time). N=6-8 animals/group.

Figure 5. Acute clozapine enhances the metabolism of dopamine in striatum in a time – dependent manner. Animals were treated as Figure 1 and dopamine (A), DOPAC (B) and HVA (B) in the striatum were estimated as described in Methods. *$p<0.05$ compared with control (0 time). N=6-8 animals/group.

Figure 6. Chronic administration of clozapine increases AAAD activity and dopamine metabolism in the striatum. Mice were treated with clozapine, 20 mg/kg, ip, or vehicle, twice daily for 7 days, and AAAD and dopamine and metabolite content (A) as well as AAAD and TH protein (B) in striatum estimated 1 or 6 h after the last injection. Assays were performed as described in Methods. *$p<0.05$ compared with vehicle. N=6-8 animals/group.

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Table 1

Clozapine Increases the Activity of TH and AAAD in the Striatum of the MPTP-Mouse

	TH	AAAD
Treatment	(nmol/mg prot/20 min ± SEM)	
Saline	0.95 ± 0.03	37 ± 2
Clozapine 30 min	1.3 ± 0.7*	51 ± 5*
Clozapine 60 min	1.3 ± 0.9*	54 ± 3*
MPTP	0.32 ± 0.02	16 ± 1*
Clozapine 30 min	0.60 ± 0.03†	24 ± 4†
Clozapine 60 min	0.50 ± 0.02†	25 ± 3†

Mice were treated with MPTP, 30 mg/kg ip, or saline daily for 7 days and 24 h later with a single dose of clozapine, 20 mg/kg, ip, or vehicle and killed at the indicated times. TH and AAAD activities were estimated in striatum as described in Methods. *$p<0.05$ compared with the saline-treated group. †$p<0.05$ compared with the MPTP- treated group. N=8.

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Table 2

Clozapine Enhances the L-DOPA-stimulated DA Metabolism in Striatum

Treatment	DA	DOPAC	HVA	DOPAC+HVA/DA
	(pmol/mg prot ± SEM)			
Saline	464 ± 15	70 ± 9	50 ± 2	0.25
MPTP	116 ± 16*	23 ± 2*	21 ± 2*	0.38
Control +L-DOPA	544 ± 12†	160 ± 2†	108 ± 5†	0.49
MPTP+L-DOPA	228 ± 12†	145 ± 20†	123 ± 14†	1.17
Saline+Clozapine	580 ± 48†	147 ± 9†	78 ± 4†	0.39
MPTP+Clozapine	246 ± 21	62 ± 5†	45 ± 5†	0.43
Saline+Clozapine+L-DOPA	567 ± 39	432 ± 51††	172 ± 6††	1.06
MPTP+Clozapine+L-DOPA	279 ± 11	402 ± 60††	243 ± 31††	2.31

Twenty-four h after MPTP treatment (see Table 1), lesioned and intact (saline- treated) mice were administered a single dose of clozapine, 20 mg/kg ip, L-DOPA, 100 mg/kg ip, clozapine and L-DOPA or vehicle and killed 1 h later. DA and metabolites were estimated in striatum as described in Methods. *$p<0.05$ compared with saline-treated animals. †$p<0.05$ compared with saline- or MPTP- treated animals, respectively. ††$p<0.05$ compared with saline- and MPTP- treated animals not given L-DOPA. N=6-8.

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Table 3

NSD 1015 Inhibits the Decarboxylation of L-DOPA

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

NSD 1015 (NSD), 100 mg/kg ip, was administered 1 h prior to clozapine (CLZ), 20 mg/kg ip, L-DOPA, 100 mg/kg ip, or combined clozapine and L-DOPA, and animals killed 2 h later. DA and metabolites were estimated in the striatum as described in Methods.*p<0.05 compared with comparison group not receiving NSD 1015. N=6-8.

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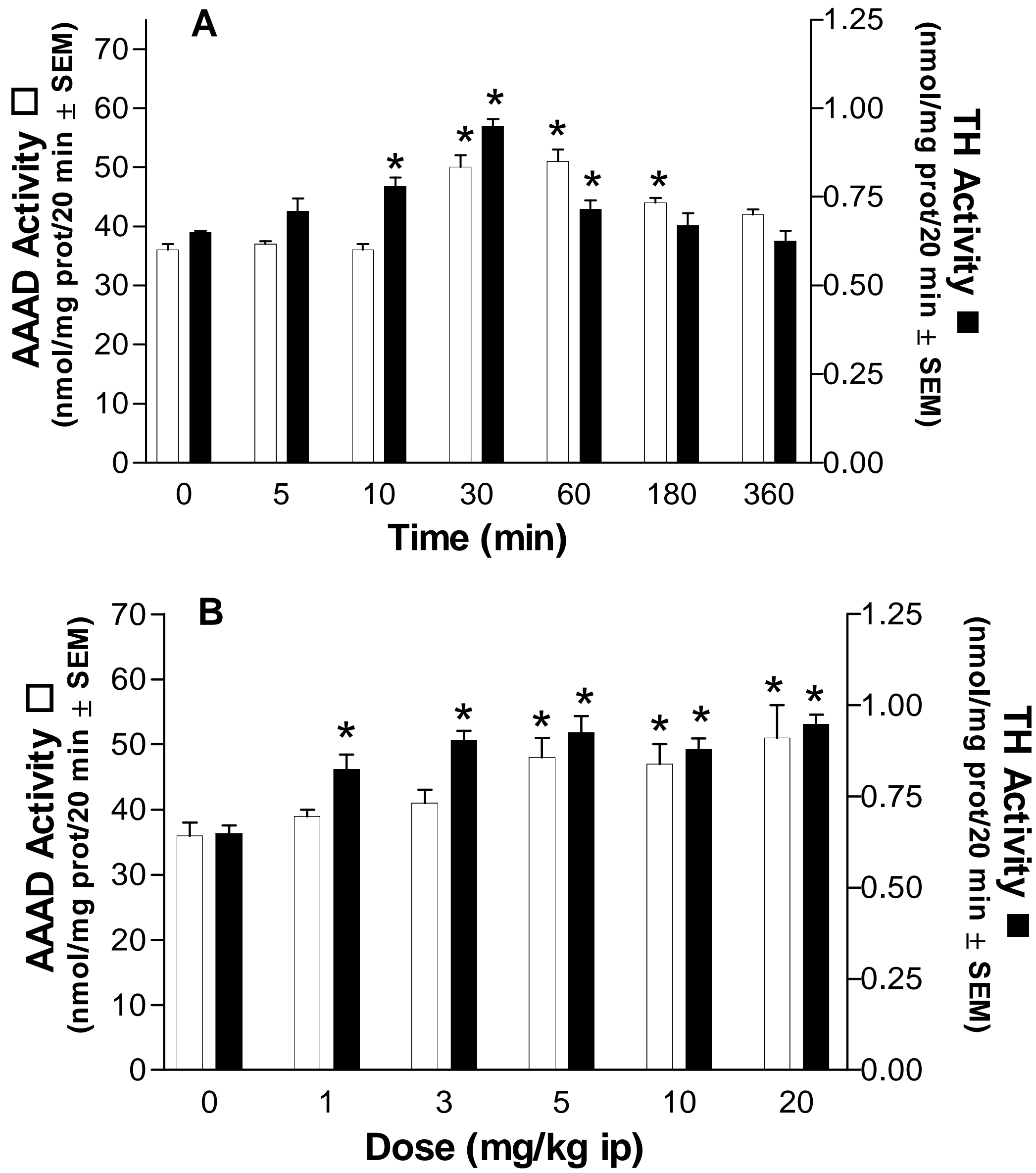

Figure 1

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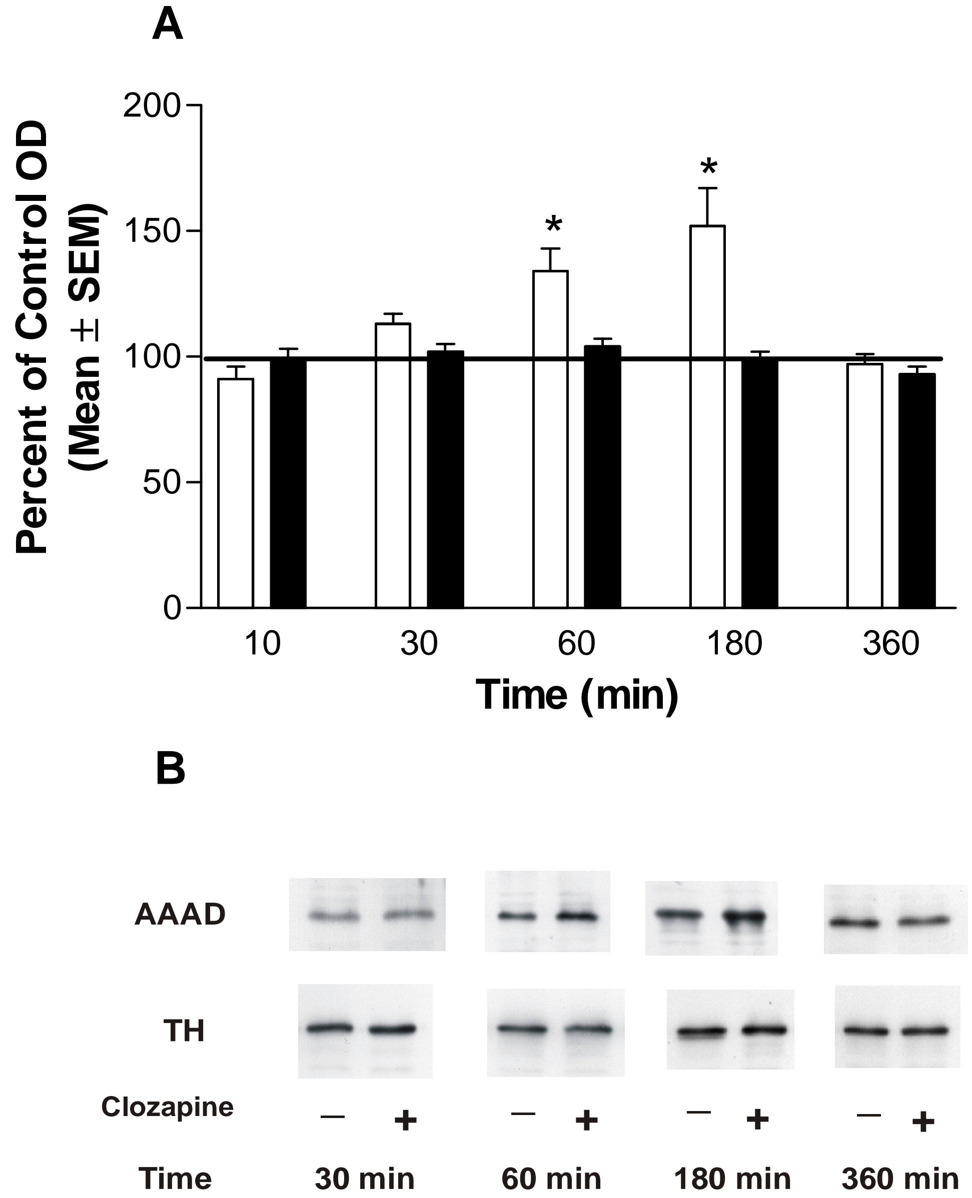

Figure 2

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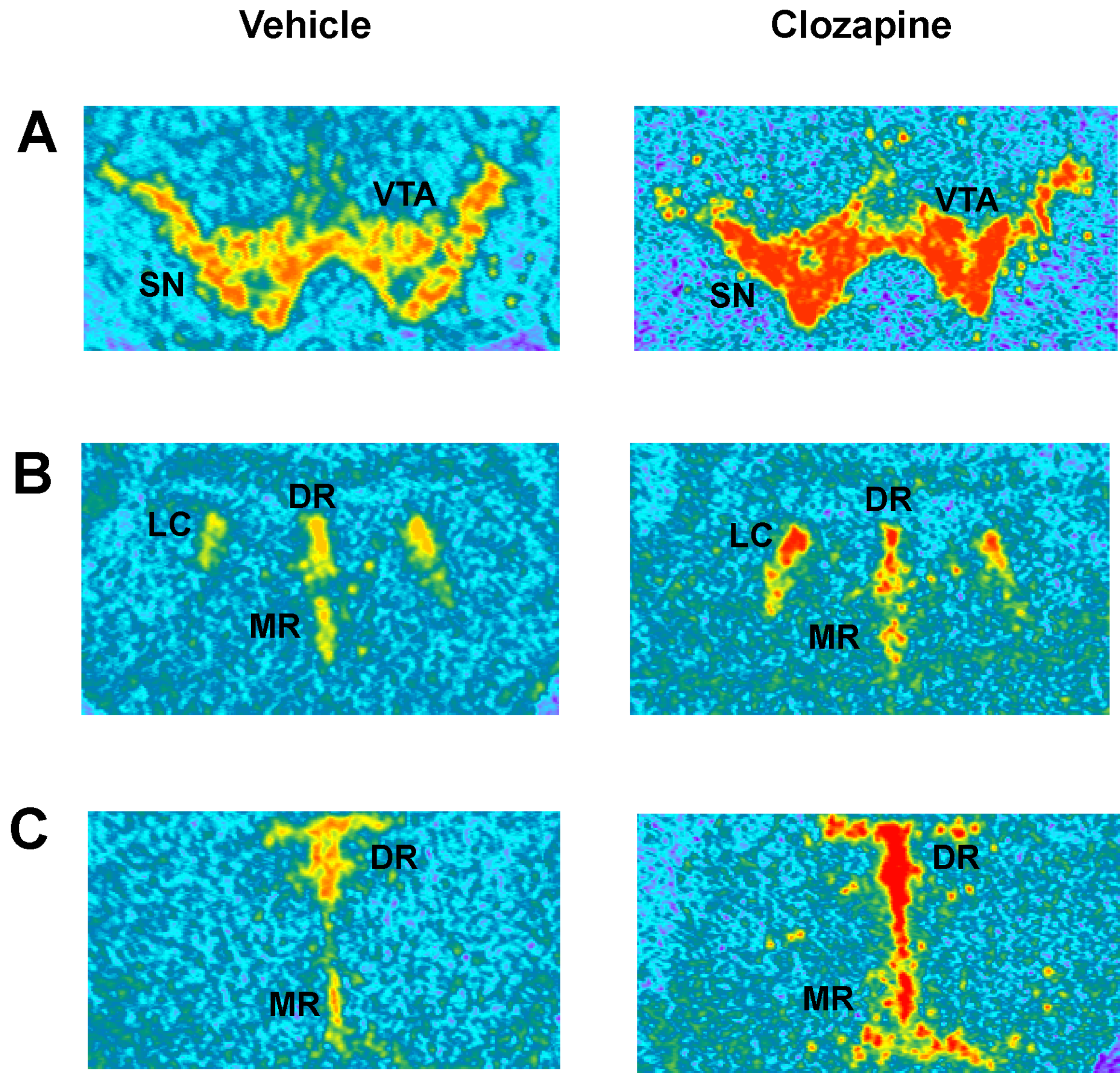

Figure 3

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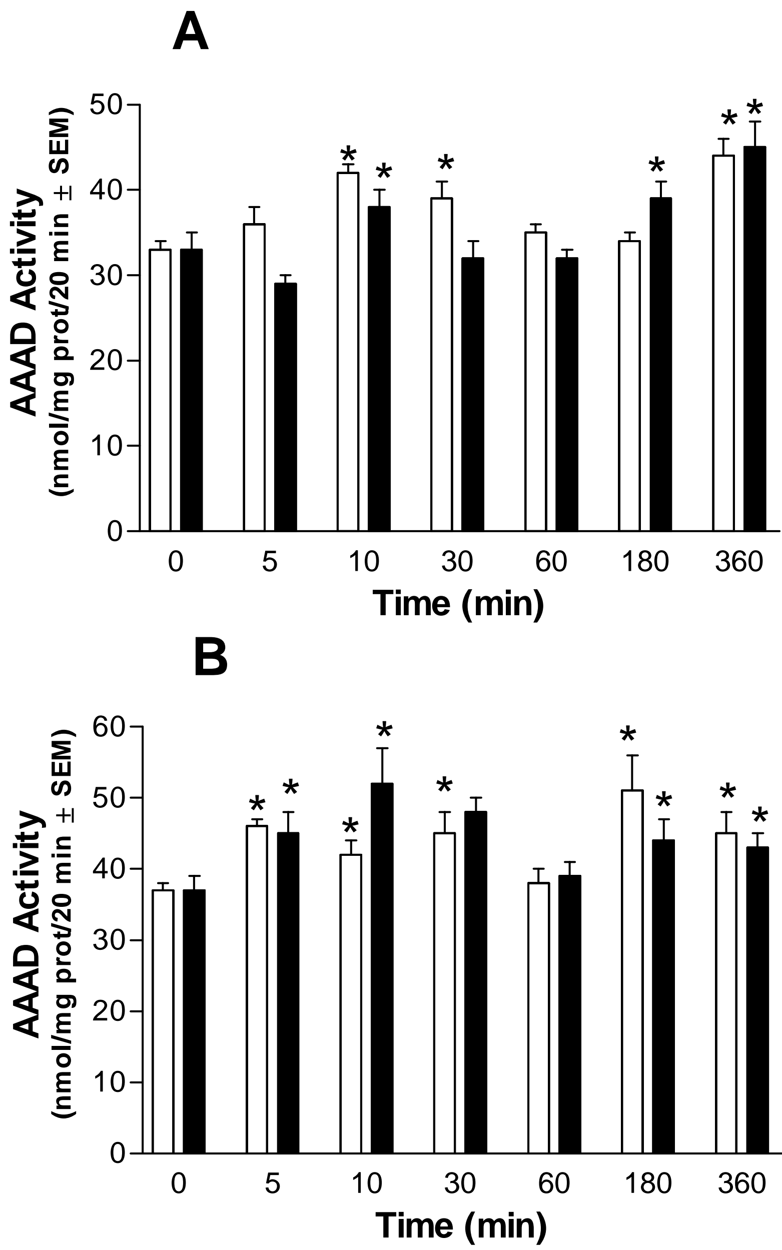

Figure 4

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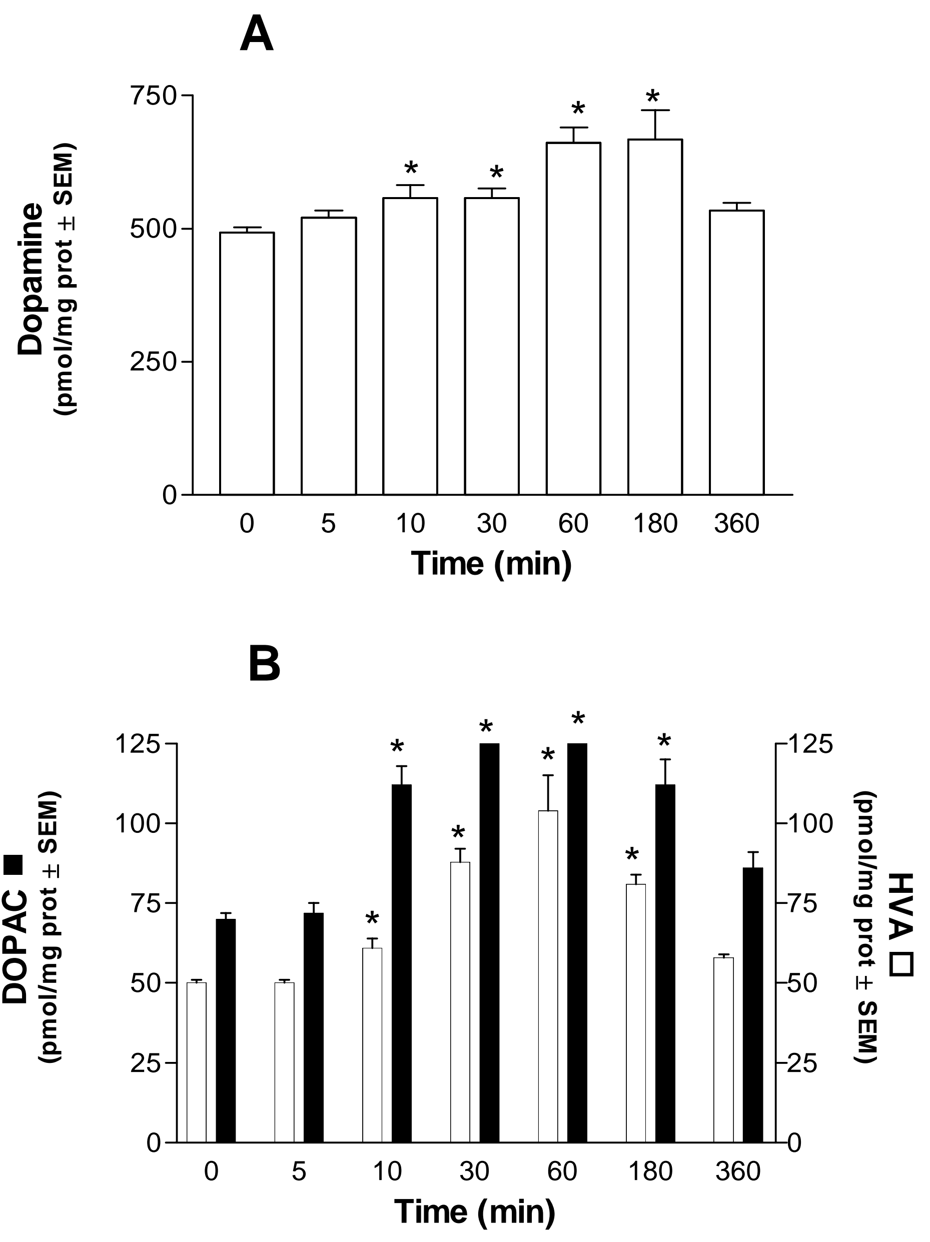

Figure 5

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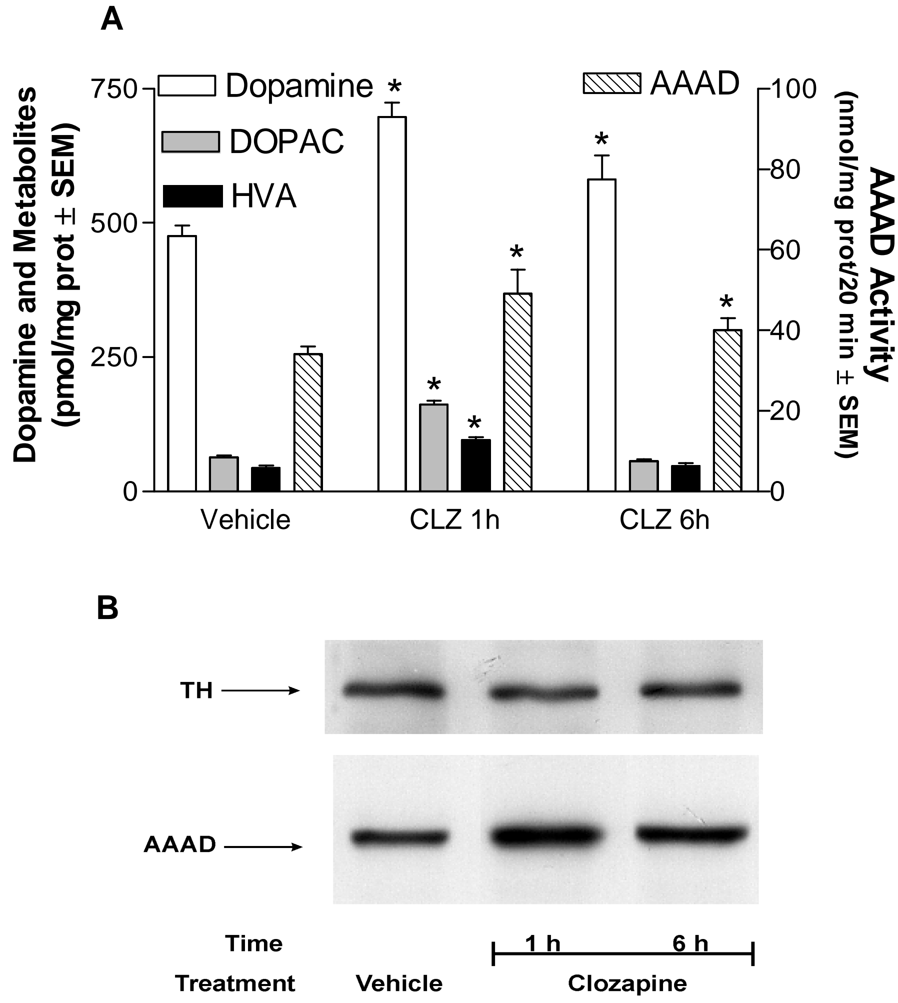

Figure 6

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