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UNILATERAL INFUSION OF A DOPAMINE TRANSPORTER ANTISENSE INTO THE SUBSTANTIA NIGRA PROTECTS AGAINST MDMA-INDUCED SEROTONERGIC DEFICITS IN THE IPSILATERAL STRIATUM

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Abstract—The present study was designed to elucidate the consequences of antisense oligonucleotide-mediated knockdown of striatal dopamine reuptake transporters on 3,4-methylenedioxymethamphetamine (MDMA)-induced neurotoxicity. Antisense oligonucleotide complementary to the mRNA translational start site of the rat dopamine transporter was delivered by constant (7 days) intranigral infusion with an osmotic minipump. Delivery of the antisense oligonucleotide by this method resulted in a 70% reduction in the density of the dopamine transporter in the ipsilateral striatum, as measured by [^{3}H]mazindol binding. The effect of this transporter knockdown on MDMA-induced serotonergic neurotoxicity was then examined. MDMA (2×20 mg/kg, s.c., given 12 h apart) administered to control rats produced hyperthermia following the first dose and led to a 45–50% reduction in striatal serotonin, 5-hydroxyindoleacetic acid, and serotonin reuptake transporter density 1 week after the second dose. Conversely, in antisense-, but not missense-treated rats, a significant attenuation of MDMA-induced neurotoxicity was observed only in the ipsilateral striatum. The hyperthermic response elicited by MDMA was not altered by prior administration of antisense. *In vivo* microdialysis revealed that the antisense treatment attenuated MDMA-induced dopamine release in the ipsilateral striatum.

These results suggest that the dopamine transporter plays an essential role in the neurodegeneration induced by MDMA, and provides additional support for the hypothesis that extracellular dopamine is involved in the neurotoxic process, at least in the striatum.

Key words: hyperthermia, mazindol, neurotoxicity, serotonin transporter.

3,4-Methylenedioxymethamphetamine (MDMA) is a widely used recreational drug of abuse that has been shown to destroy selectively serotonin (5-HT) axon terminals in rodents and in non-human primates (Battaglia et al., 1988; Hatzidimitriou et al., 1999; McCann et al., 1998; Ricaurte et al., 1988). MDMA-induced 5-HT neurotoxicity is characterized by long-term reductions in 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Schmidt and Taylor, 1987), decreases in the density of 5-HT uptake sites (Battaglia et al., 1987), and reduction in tryptophan hydroxylase (TPH) activity (Stone et al., 1987). Furthermore, immunohistochemical studies have demonstrated that MDMA produces profound loss of 5-HT-containing neurons (Commins et al., 1987; O'Hearn et al., 1988; Slikker et al., 1988).

Although the exact details of the neurotoxic process have not been fully elucidated, there is substantial evidence that excessive dopamine (DA) release induced by MDMA might be an essential component of the neurodegenerative effect. MDMA can release DA by two mechanisms, a calcium-independent non-vesicular transporter-mediated process (Nash and Brodkin, 1991; Shankaran et al., 1999) and an impulse-dependent process (Gudelsky and Nash, 1996; Yamamoto et al., 1995). Pharmacological agents that interfere with dopaminergic transmission have been shown to be effective in attenuating the neurotoxic effects of MDMA. For example, depleting dopamine stores by tyrosine hydroxylase inhibition with α-methyl-*para*-tyrosine (AMPT), or blockade of the dopamine transporter (DAT) with GBR-12909 both protect 5-HT terminals from the neurodegenerative effects of MDMA (Nash and Brodkin, 1991). Similarly, Shankaran et al. (1999) used microdialysis to show that administration of mazindol, a DAT inhibitor, prior to MDMA (20 mg/kg, i.p.) attenuated extracellular DA in the striatum without altering the MDMA-induced hyperthermia, and also suppressed the MDMA-induced for-

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Abbreviations: 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; aCSF, artificial cerebrospinal fluid; AMPT, α-methyl-*para*-tyrosine; ANOVA, analysis of variance; AS, antisense; DA, dopamine; DAT, dopamine transporter; EDTA, ethylenediaminetetra-acetate; HPLC, high-performance liquid chromatography; MAO-B, monoamine oxidase B; MDMA, 3,4-methylenedioxymethamphetamine; MS, missense; ODN, oligodeoxynucleotide; ROS, reactive oxygen species; SNc, substantia nigra pars compacta; TPH, tryptophan hydroxylase.

mation of 2,3-dihydroxybenzoic acid (from perfused salicylic acid), a marker of free radical formation. The authors hypothesized that a DAT-mediated oxidative stress mechanism might result in MDMA-induced neurotoxicity. Sprague and Nichols (1995) recently offered a hypothesis that might explain how DA could be involved in 5-HT terminal damage that invokes an oxidative stress mechanism, by access of DA to the interior of 5-HT neuron terminals.

Despite a substantial body of evidence that supports a role for DA in the MDMA-induced neurotoxic process, there have been recent debates concerning the importance of DA per se. Many neuroprotective agents that attenuate the increased extracellular DA produced by MDMA also antagonize the hyperthermia produced by MDMA. For example, Malberg et al. (1996) showed that prior administration of AMPT attenuated not only the subsequent MDMA-induced serotonergic neurotoxicity but also the drug-induced hyperthermia. The neuroprotective effect of AMPT was lost, however, upon elevation of the body temperature, suggesting that its ability to attenuate the hyperthermic response might have been responsible for the neuroprotection. Similarly, Colado et al. (1999) have presented data suggesting that the neuroprotective effect of haloperidol and the potentiating effect of L-DOPA on MDMA-induced neurodegeneration may have resulted from altering MDMA-induced hyperthermia, rather than from direct effects on the dopaminergic system.

Therefore, a key question seemed to be: is DA per se functionally important in the neurotoxicity process, or is activation of the dopaminergic system just a correlative marker for some other process such as hyperthermia, which is the actual necessary event? In order to address this question, it was important to remove as many pharmacological variables from the study as possible. That is, ancillary neuroprotective effects might accompany any drug treatment that was employed. This concern has been circumvented, however, by the recent availability of a cloned sequence for the DAT, whereby targeted knockdown of the DAT in a region-specific manner using an antisense (AS) oligodeoxynucleotide (ODN) could be achieved. Reduced DAT levels should attenuate MDMA-induced carrier-mediated DA release without introducing additional pharmacological variables, thus providing insight into any functional relationship between DA release, neurotoxicity, and hyperthermia resulting from MDMA administration.

EXPERIMENTAL PROCEDURES

Animals

Adult, male Sprague–Dawley rats (Charles River, Portage, MI, USA) weighing 200–225 g were used. All animals were individually housed in a room maintained at 20–22°C on a 12-h light–dark cycle with food and water available *ad libitum*, and habituated to the animal facility for at least a week before use. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and were approved by the Purdue and Ohio Northern University Animal Care and Use Committees. All efforts were made to minimize the number of animals used and their suffering.

Drug and chemicals

MDMA hydrochloride was synthesized in our laboratory by standard procedures. The ODNs were purchased from Integrated DNA Technologies (Coralville, IA, USA). The ODNs were purified to approximately 96–98% by the manufacturer using high-performance liquid chromatography (HPLC). [^{3}H]Mazindol was obtained from Dupont NEN; all other reagents were purchased from Sigma (St. Louis, MO, USA). Stainless steel cannula tubing was purchased from Plastics One (Roanoke, VA, USA), and the osmotic minipump (model 1007D; 0.5 μl/h flow rate) was obtained from Alza (Palo Alto, CA, USA).

Surgical implantation of osmotic minipump

Rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). A cannula (28 gauge) connected to the osmotic minipump (0.5 μl/h, 7-day duration, Alzet model 1007D) through a 1-in length of silicone tubing that was previously filled with sterile ODNs was then stereotaxically implanted into the substantia nigra pars compacta (SNc) (A −5.8 mm, L +1.8 mm, V +8.0 mm from bregma; Silvia et al., 1997; Paxinos and Watson, 1986). Three stainless steel screws were threaded into the skull to anchor and prevent dislodging of the cannula, which was secured into place with dental acrylic. The pump was inserted into the subcutaneous intrascapular region. A topical antibiotic was applied to the incision area to prevent infection. In order to verify correct cannula placement post-experimentally, Methylene Blue injection or direct microscopic visualization of the cannula tract was utilized.

AS and drug administration

In the first set of experiments, the ODNs reported by Silvia et al. (1997) were employed. These comprised fully phosphorothioated AS-I (5′-AGA TTC AGT GGA TCC AT-3′) and missense (MS) -I (5′-AGC ATT GAA CAA GCC AT-3′). Silvia et al. (1997) reported that their AS ODN was targeted to the first 17 nucleotides in the coding region of the rat DAT, and the MS ODN was designed to have the same GC content as the AS ODN. As we note later however, their reported AS sequence had only 86% homology to the DAT mRNA.

In a second set of experiments, we designed AS-II ODN (5′-GCT CTT ACT CAT GGG TAG-3′) to be complementary to a sequence starting six bases upstream of the translational start site, and MS-II ODN (5′-TGC TCT TAC ATC AGG GAT-3′) was the scrambled version of AS-II. Based on preliminary experiments (unpublished), these sequences were fully phosphorothioated. MS ODN sequences were checked against the GenBank to ascertain that they did not match any other known sequence.

The ODNs were suspended at a concentration of 42 μM in sterile artificial cerebrospinal fluid (aCSF) (NaCl 124 mM, KCl 1 mM, KH_2PO_4 1.24 mM, $MgSO_4$ 1.3 mM, $NaHCO_3$ 26 mM, $CaCl_2$ 2.4 mM, glucose 10 mM) and used for infusion experiments. The ODNs were infused at a constant dose of 500 pmol/day for a period of 7 days. This treatment regimen was selected based on a previously described protocol for the central administration of AS ODNs by Silvia et al. (1997). In all experiments the animals were placed on a palatable diet (peanut butter cookies and grapes) for several days following surgery to minimize lethality that might result from severe anorexia that was observed in some post-surgical animals during preliminary experiments.

In studies examining MDMA-induced 5-HT neurotoxicity, MDMA (2×20 mg/kg, s.c. given 12 h apart) or saline was administered following 7 days of ODN infusion. One week later the rats were killed by decapitation, and the brain regions

were rapidly removed by dissection and stored at −80°C until assay.

Surgical implantation of microdialysis cannulae

Rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and the microdialysis guide cannulae (CMA, Acton, MA, USA) were stereotaxically placed into the caudate putamen (A −0.5, L −3.0, V −5.0 from bregma; Paxinos and Watson, 1986) of control (sham) animals and those with mini-pumps inserted to deliver the AS ODN. The cannula was then fixed in place with dental acrylic supported by stainless steel screws threaded into the skull and glued into place.

For microdialysis the animals were placed in a Raturn® (BAS, Lafayette, IN, USA). The dialysis probe was connected to a Bee Hive® micro-infusion pump (BAS) calibrated to deliver aCSF at a rate of 1.0 µl/min. The probe was perfused for 30 min to allow for equilibration. Three baseline samples were then collected before the animals were treated with MDMA (20 mg/kg, s.c.). Samples were collected every 20 min for a total of 100 min post-treatment.

Measurement of DAT density

Mazindol binding procedure. A modified procedure of Silvia et al. (1997) was used to quantify DAT density. Striata from treated animals were rapidly dissected on a cold petri dish and quickly frozen in liquid nitrogen and stored at −80°C until the day of assay. For each experiment, a single striatum from each subject in the individual treatment groups was homogenized in 5 ml of ice-cold lysis buffer (50 mM Tris, 300 mM NaCl, 5 mM KCl; pH 7.9). The homogenate was centrifuged at 36 500×*g* for 10 min, with an intermittent wash of the pellet using the same volume of buffer. The pellet was then resuspended in 3 ml of binding buffer to achieve a protein concentration of 200 µg/ml. Non-specific binding was defined using 1 µM mazindol and 50 nM imipramine (Javitch et al., 1984). Following the addition of a single saturating concentration (15 nM) of [^{3}H]mazindol (Eisch et al., 1996), incubations were begun by adding 200 µl of tissue homogenates to tubes containing 1.5 ml of buffer to give a final volume of 2.0 ml. Tubes were allowed to equilibrate on ice for 60 min before adding 5 ml of ice-cold buffer and filtering through GF/B filters using a Brandel cell harvester. The tubes and filters were rinsed twice with 5 ml of ice cold buffer, and the filters were then placed in plastic vials containing scintillation cocktail. The vials were allowed to stand overnight prior to counting at an efficiency of 40%. Although we initially planned to make statistical comparisons between neurochemical levels in the ipsilateral and contralateral striatum in ODN-infused individual rats, we found that AS infusion led to small (ca. 14%), but significant reductions of DAT in the contralateral striatum. Thus, we employed separate groups of animals for each comparison. Data were analyzed using an analysis of variance (ANOVA) with post-hoc Student–Newman–Keuls tests. DAT levels in the prefrontal cortex remained unaffected by this treatment (control: 3.9 ± 1.7 fmol/mg; AS-II: 3.2 ± 0.84; $P > 0.10$); we did not attempt to measure DAT levels in hippocampus due to the relatively sparse dopaminergic innervation to this region from both the SNc and ventral tegmental area (Scatton et al., 1981).

Measurement of 5-HT and 5-HIAA levels

In brief, following rapid dissection on ice, the tissue was suspended in 0.5 ml of buffer containing 0.1 M $HClO_4$, 0.05% Na_2EDTA and 0.1% $Na_2S_2O_5$ and homogenized using a motor-driven Teflon pestle. Subsequently, the samples were centrifuged at 14 000×*g* for 5 min in a tabletop centrifuge. The supernatant was then added to a 22-µm nylon filter (Rainin, Woburn, MA, USA) and centrifuged at the same speed for 10 min. The filtrate was analyzed for monoamines and their metabolites by separation on an ESA MD-150 column with a flow rate of 0.7 ml/min using ESA MD-TM mobile phase. The HPLC-EC system consisted of an ESA pump (model 582) linked to an autosampler (Tosohaas, Philadelphia, PA, USA) with detection using a Coulochem 5200A electrochemical detector and a Coulochem 5011 detector cell. The Coulochem was operated in screen mode with the potential of the screening electrode set at 750 mV and the potential of the quantitating electrode set at −200 mV. The applied potential at the working electrode of the electrochemical detector was set at 610 mV. The current produced was integrated using Rainin software for determination of tissue 5-HT and 5-HIAA levels. Data were analyzed using an ANOVA with post-hoc Student–Newman–Keuls tests.

Measurement of DA

Microdialysis samples of 20 µl were analyzed using a modified procedure of Chapin et al. (1994) to determine DA levels in the striatum. The aqueous portion of the mobile phase consisted of 0.05 M sodium phosphate, 0.03 M citric acid, 0.1 mM Na_2EDTA, and 0.042% sodium octyl sulfate dissolved in HPLC grade water. The pH of the aqueous portion of the mobile phase was in the range of 2.7–2.9. The mobile phase consisted of 80% aqueous phase and 20% methanol. A Waters HPLC (Model 600) with electrochemical detection (Waters 464) and a C_{18} reverse phase analytical column (4-µm spheres; 3.9×300 mm; Waters) was used. The electrode was set at +750 mV, and the flow rate set at 0.7 ml/min.

Millennium® software was used to integrate and analyze the raw data for the determination of DA levels as compared to internal standard curves. For *in vivo* microdialysis samples, the effect of MDMA treatment alone on DA levels was compared with the AS/MDMA treatment results using Student's *t*-test ($P < 0.05$) at each individual time point. Each treatment group was then compared to basal DA levels within the group using a Student's *t*-test ($P < 0.05$).

Measurement of 5-HT transporter density

[^{3}H]Paroxetine binding procedure. A modified procedure of Marcusson et al. (1988) was employed to measure the density of 5-HT uptake sites. A buffer consisting of 50 mM Tris–HCl with 120 mM NaCl and 5 mM KCl at pH 7.4 was employed for both tissue homogenization and incubations. Tissue samples were weighed and homogenized in 5 ml of the buffer described above with a Brinkman polytron (setting 6, 2×20 s). The homogenate was centrifuged twice at 30 000×*g* for 10 min with an intermittent wash and the pellet was resuspended in the same volume of the buffer described above. Because it has been previously reported that only the B_{max} and not the K_D values are altered after MDMA treatment (Battaglia et al., 1987), it is possible to estimate the number of 5-HT uptake sites with a saturating concentration of [^{3}H]paroxetine. Therefore, for this experiment we used only a high single concentration (1 nM) of [^{3}H]paroxetine. Specific binding was defined as that displaced by 1 µM fluoxetine. Incubations were started by adding 150 µl of tissue homogenate to each tube to give a final volume of 1.65 ml. Tubes were allowed to equilibrate at 24°C for 1 h before adding 4 ml of ice-cold buffer and filtering through Whatman GF/C filters using a Brandel cell harvester. The tubes were washed twice with 5 ml of ice-cold buffer and the filters were placed in plastic vials containing 10 ml of scintillation cocktail. The vials were allowed to stand overnight prior to counting at an efficiency of 40%. Data were analyzed using an ANOVA with post-hoc Student–Newman–Keuls tests.

Measurement of body temperature

Temperature was recorded using a digital thermometer (CMA/150 Carnegie Medicin; Stockholm, Sweden). The probe was lubricated and inserted 3 cm into the rectum and the rat was gently held. A steady readout was obtained following 30 s of probe insertion. Body temperature was recorded hourly for the next 7 h. Data were analyzed using an ANOVA with post-hoc Student–Newman–Keuls tests.

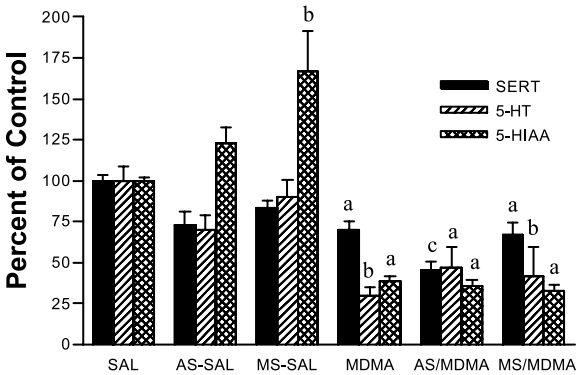

Fig. 1. The MDMA-induced neurotoxic response in the ipsilateral striatum after 7 days of treatment with DAT AS-I ODN. Animals were given constant unilateral infusion of DAT AS-I or MS ODNs (500 pmol/day) for 7 days. Starting on day 8, animals were administered two s.c. injections of 20 mg/kg 12 h apart. One week following the last dose of MDMA rats were killed and the infused side was analyzed to assess the degree of MDMA-induced 5-HT neuronal loss. Saline control values for striatum: serotonin reuptake transporter (SERT) 16.0 ± 0.55 fmol/mg wet wt, 5-HT 548 ± 23.4, 5-HIAA 1010 ± 17.3 pg/mg wet wt. Each value is the mean ± S.E.M. for $n = 4$–7 animals. Significant when compared to saline: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

RESULTS

Effects of the AS sequence on DAT density in the striatum

The AS-I reported by Silvia et al. (1997) produced a 40% reduction in [^{3}H]mazindol-labeled sites in the ipsilateral striatum following 7 days of continuous infusion of AS-I ODN into the SNc (Table 1). These results parallel those reported by Silvia et al. (1997). AS-II was then examined using similar procedures, which produced a 70% reduction in the density of DAT in the striatum (Table 1). DAT density in the striatum was not altered by the MS sequence, confirming sequence specificity.

Effects of AS-I DAT knockdown on MDMA-induced serotonergic toxicity one week later

MDMA (2×20 mg/kg, s.c., 12 h apart) given to control rats produced a significant reduction in 5-HT markers 1 week following the last dose of MDMA. AS-I infusion failed to provide protection against the neurotoxic effects of MDMA in the striatum (Fig. 1). The [^{3}H]paroxetine binding site density, which is considered to be an indirect marker of the structural integrity of serotonergic terminals, was also reduced 40–50% in the striatum. In a parallel manner, rats that were pretreated with AS-I exhibited the same magnitude of MDMA-induced 5-HT and 5-HIAA depletion in the striatum.

Following these experiments, we analyzed (using GenBank) the published AS sequence of Silvia et al. (1997) and discovered that it was not 100% complementary to any portion of the rat DAT. Indeed, it had only 86% identity to a portion of the sequence from the translational start site for the rat DAT mRNA. In view of this discrepancy, and the relatively modest reduction in DAT density, we sought AS candidates that might lead to more marked knockdown of the DAT. AS-II was then characterized and found to reduce [^{3}H]mazindol binding by nearly 70% (Table 1). Subsequently, all remaining studies utilized the AS-II ODN targeting the DAT.

Table 1. Effect of AS ODN infusion on striatal [^{3}H]mazindol binding site density

Treatment	AS-I (% of control)	AS-II (% of control)
aCSF (I)	100 ± 2.4	ND
Saline (I)	ND	100 ± 5.9
Saline (C)	ND	100 ± 5.0
AS (I)	62 ± 2.9**	32 ± 4.5**
AS (C)	ND	86 ± 5.0*
MS (I)	93 ± 2.2	87 ± 4.6
MS (C)	ND	100 ± 2.3

Continuous infusion with DAT AS produced significant decreases in mazindol binding both on the infused side and on the untreated (contralateral) side. Continuous infusion of MS ODN failed to produce any appreciable differences in mazindol binding in either side of the brain. I = ipsilateral side; C = contralateral side; ND = not determined. Values given are the mean ± S.E.M., for $n = 5$–9. Significant difference from saline control: *$P < 0.05$, **$P < 0.001$, ANOVA.

Effect of AS-II on MDMA-induced hyperthermia

Following the first dose of MDMA (2×20 mg/kg, s.c., 12 h apart) an immediate and significant rise in rectal temperature was evident within 1 h and maintained for 3 h post-treatment (Fig. 2). This initial and significant rise in rectal temperature was also seen in the AS/MDMA and MS/MDMA treatment groups. The AS/MDMA and MS/MDMA treatments also produced a rise (albeit non-significant) in rectal temperature that did not differ from MDMA alone at the 2- and 3-h time points. There was no significant difference in basal temperature between any of the treatment groups.

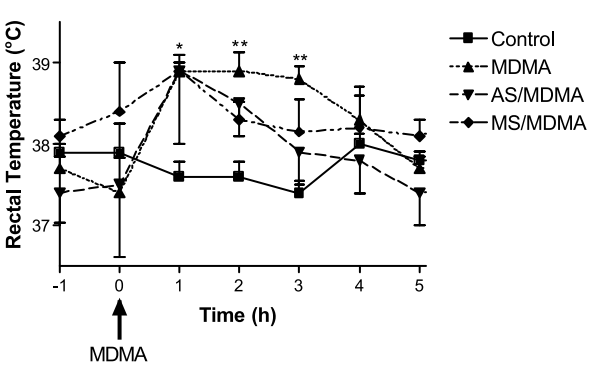

Fig. 2. Rectal temperature in rats administered MDMA that had previously received intranigral infusion of AS-II ODN directed to the DAT. AS or MS (500 pmol/day) was infused into the SNc by an osmotic minipump for 7 days prior to MDMA (2×20 mg/kg, 12 h apart). Results shown are mean ± S.E.M., $n = 3$–6. There was no significant difference in the basal temperature between groups. The group treated with MDMA alone exhibited a significant (**$P < 0.05$) rise in body temperature compared with the saline-treated group 1–3 h after MDMA administration. Significant hyperthermia (*$P < 0.05$) was only observed in the AS/MDMA and MS/MDMA treatment groups 1 h after treatment, although there was a trend towards increased temperature at the 2-h time point.

Effects of AS-II on MDMA-induced DA release in the striatum

MDMA treatment resulted in increased ($P = 0.02$) extracellular DA 60 min after treatment; a rise in DA release occurred after 40 min and was maintained throughout the 100-min monitoring period. The AS/MDMA treatment significantly attenuated the 60-min rise in DA release and reduced the overall increase in DA release seen over the 100-min monitoring period (Fig. 3).

Effects of AS-II on MDMA-induced serotonergic toxicity one week later

Animals that received a 1-week infusion of AS-II prior to MDMA (2×20 mg/kg, s.c., given every 12 h) showed a dramatic reversal of MDMA-induced reduction in 5-HT markers in the striatum (Fig. 4). By contrast, no protection occurred in the hippocampus or cortex (data not shown), consistent with the failure of the AS treatment to alter DAT density in the cortex and with the virtual absence of DAT in hippocampus. Clearly, the attenuation of serotonergic deficits induced by MDMA only in striatum, a target region for SNc projections, is consistent with a role for the DAT in the MDMA-induced loss of 5-HT terminal integrity in that region. The absence of any significant change in MDMA-induced loss of 5-HT markers in MS-treated rats illustrates the specificity of AS-mediated DAT knockdown on MDMA-induced serotonergic deficits. The AS or MS sequences given alone, without MDMA administration, produced no significant changes in 5-HT or 5-HIAA levels or 5-HT transporter site density.

DISCUSSION

These results confirm and extend previous findings that the MDMA-induced alteration of dopaminergic function by interactions with the DAT might be involved in producing the observed long-term serotonergic deficits (Nash and Brodkin, 1991; Shankaran et al., 1999). The dosage and continuous infusion protocol were based on

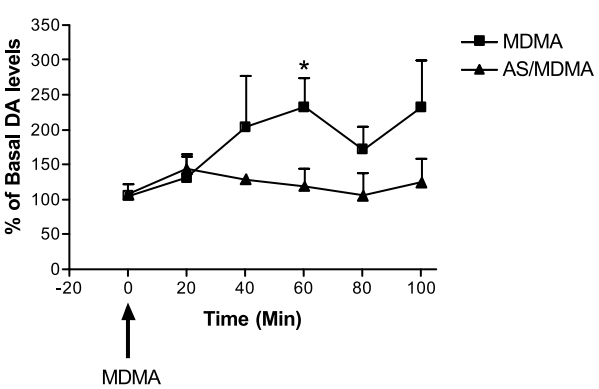

Fig. 3. Effects of the AS-II ODN to DAT on MDMA-induced DA release in the striatum. Each value is the mean ± S.E.M., $n = 4$. *Significantly different from MDMA's baseline ($P = 0.02$) and from AS/MDMA at 60 min ($P = 0.05$).

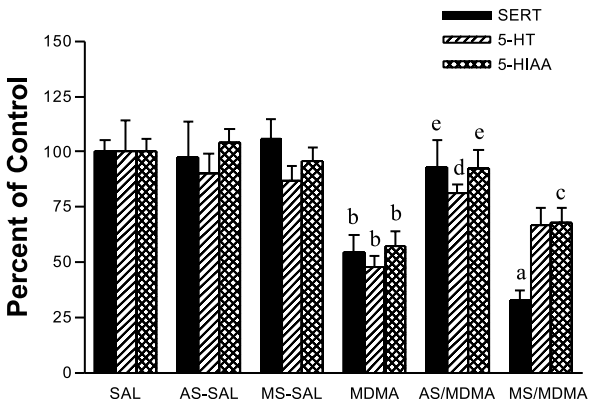

Fig. 4. The MDMA-induced neurotoxic response in the ipsilateral striatum after 7 days of treatment with DAT AS-II ODN. Animals were given constant unilateral infusion of DAT AS-II or MS ODNs (500 pmol/day) for seven days. Starting on day 8, animals were administered two s.c. injections of 20 mg/kg 12 h apart. One week following the last dose of MDMA rats were killed and the infused side was analyzed to assess the degree of MDMA-induced 5-HT neuronal loss. Saline control values for the ipsilateral striatum were: serotonin reuptake transporter (SERT) 13.6 ± 0.68 fmol/mg wet wt, 5-HT 326 ± 48.6, 5-HIAA 252 ± 14.1 pg/mg wet wt. Each value is the mean ± S.E.M. for $n = 4$–7 animals. Significant when compared to saline: $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$. Significant when compared to MDMA: $^{d}P < 0.05$, $^{e}P < 0.01$, ANOVA.

previous studies demonstrating that short-term microinjections into the SNc and lower doses of ODNs failed to produce the desired levels of DAT reduction (Silvia et al., 1997). In this context, several studies have highlighted the fact that the turnover rate of the protein is a critical determinant of attaining maximal reduction of the target (Silvia et al., 1997; Albert and Morris, 1994). Thus, based on studies demonstrating that DAT turnover time is approximately 6.3 days (Fleckenstein et al., 1997), we followed the protocol that involved a 1-week constant infusion.

In rats whose striatal DAT levels were dramatically reduced by AS-mediated translational arrest, a significant attenuation of the neurotoxic response was also evident. AS infusion failed to prevent the hyperthermic response elicited by MDMA (Fig. 2), but the DAT knockdown was also limited to one striatum. Clearly, however, the neuroprotective effect of DAT knockdown cannot be attributed to attenuation of MDMA-induced hyperthermia. Furthermore, MDMA induced a sustained increase in striatal DA release that was attenuated by the AS ODN to DAT (Fig. 3). Taken together, the data support the view that excessive dopaminergic transmission induced by MDMA is involved in a series of neurotoxic events that culminate in the loss of 5-HT terminal integrity, at least in the striatum. In this view, hyperthermia may potentiate the neurotoxic process, but does not enable it.

In the first set of experiments, we tested the efficacy of AS-I. Constant infusion of AS-I into the SNc produced a 40% reduction in striatal DAT density, consistent with the results of Silvia et al. (1997). Based on their report that a 40% reduction in DAT was sufficient to attenuate the amphetamine-induced locomotor response, we tested the efficacy of AS-I in reducing the neurotoxic response

elicited by MDMA. Surprisingly, no significant attenuation of MDMA-induced neurotoxicity was observed (Fig. 1). Thus, a modest reduction in DAT is sufficient for modulating the effects of amphetamine (Silvia et al., 1997), but not for blocking the neurotoxic effects of MDMA. We subsequently discovered that AS-I had only 86% homology to the DAT mRNA. In the absence of 100% complementarity to the DAT mRNA, the 40% reduction in DAT sites might also represent some non-specific cytotoxic effect of the ODN.

In the second study, AS-II targeted to the translational start site resulted in the maximal reduction (67%) of DAT density in the ipsilateral striatum, although a significant reduction (14%) also occurred on the contralateral side. Indeed, AS sequences that are complementary to the translation initiation codon and surrounding sequences are believed to be most effective (Crooke, 1992). Consistent with previous observations, MDMA alone produced significant long-term reductions (40–50%) in serotonergic markers, namely 5-HT, 5-HIAA, and serotonin reuptake transporter, whereas indicators of dopaminergic function remained unchanged (data not shown).

There is considerable evidence demonstrating the involvement of non-vesicular, calcium-independent release of DA, presumably via a carrier-mediated 'exchange-diffusion' process in the psychomotor stimulant-induced release of DA (Fischer and Cho, 1979). Pretreatment with DAT inhibitors was previously shown to attenuate increased efflux of DA in the striatum following local (Nash and Brodkin, 1991) or systemic administration (Shankaran et al., 1999) of MDMA. For example, using microdialysis studies, Shankaran et al. (1999) demonstrated that both sustained MDMA-induced increases in the extracellular concentration of reactive oxygen species (ROS) and long-term depletion of 5-HT in the striatum were attenuated by mazindol pretreatment. These results point to a role for the DAT in mediating the acute release of DA and consequent formation of ROS, a potential mechanism for long-term loss of 5-HT terminals in the striatum (Sprague and Nichols, 1995). In a similar fashion, pretreatment with mazindol or GBR-12909, a DAT inhibitor, was found to inhibit significantly the increase in extracellular DA produced by local infusion of MDMA into the striatum (Nash and Brodkin, 1991). The authors concluded that, similar to amphetamine, mazindol blocks the binding of MDMA to the DAT in the axon terminal, thereby preventing the release of DA believed to be mediated by the transporter. This interpretation is confounded, however, by the fact that mazindol is also a potent inhibitor of norepinephrine uptake (Javitch et al., 1984). This potential problem is circumvented in the current study by the use of a specific DAT AS and confirms a significant role for DAT in MDMA-induced DA release in the striatum.

DAT AS-II treatment led to almost complete reversal of the long-term reductions in striatal 5-HT markers, whereas MS-II treatment failed to alter the MDMA-induced neurotoxic effects. Taken together, the results of the current work, in conjunction with the above-mentioned studies, support the hypothesis that excessive DA release, mediated through the DAT, serves as an intermediate for the formation of neurotoxic species that might ultimately lead to 5-HT terminal loss.

The significance of non-vesicular DA in MDMA-induced long-term deficits is further illustrated by the following studies. Johnson et al. (1991) demonstrated that a potent non-vesicular releaser of 5-HT, 5-methoxy-6-methyl-2-aminoindan, produced marked long-term reductions in 5-HT markers *only* when combined with amphetamine, a non-vesicular DA releaser, whereas neither drug alone produced neurotoxicity. Conversely, increasing DA synthesis by administration of both amphetamine and the 5-HT_2 receptor agonist *R*-DOI was not sufficient to produce long-term serotonergic deficits in the striatum (Huang and Nichols, 1993). Those studies led to the hypothesis that the process of 5-HT depletion from the axon terminal somehow renders it susceptible to the neurotoxic effects of MDMA. Sprague et al. (1994) subsequently showed that pretreatment with the 5-HT precursors tryptophan and 5-hydroxytryptophan protected against MDMA-induced serotonergic neurotoxicity. In a recent report, Aguirre et al. (1998) demonstrated that administration of MDMA to rat pups at postnatal day 21, where the rat is believed to have a poorly developed dopaminergic system, failed to produce neurotoxicity. Pretreatment with L-DOPA followed by MDMA, however, did produce persistent reduction of 5-HT markers in the hippocampus and frontal cortex in the rat pup. Likewise, at 35 days, the rat pups' dopaminergic systems were developed and MDMA-induced serotonergic neurotoxicity was observed. Conversely, in that study 6-hydroxydopamine-lesioned rats failed to display long-lasting reductions in 5-HT markers following MDMA, but administration of L-DOPA restored the neurotoxic effects of MDMA. These results seem to confirm the obligatory requirement of a fully functional DA system in order to sustain 5-HT neuronal injury.

In this context, numerous studies have implicated DA as a potential neurotoxic agent. For example, DA can undergo enzymatic oxidation via monoamine oxidase B (MAO-B) to generate H_2O_2 with subsequent formation of superoxide or hydroxy radicals (Halliwell, 1992). DA can also undergo autooxidation to the corresponding chemically reactive quinones (Monks et al., 1992). Thus, DA might gain access to the interior of the 5-HT terminal and damage the 5-HT terminal through an oxidative stress mechanism. Indeed, Sprague and Nichols (1995) demonstrated MDMA-induced formation of thiobarbituric acid reactive substances, considered to be indirect markers of membrane lipid peroxidation. L-Deprenyl, an inhibitor of MAO-B, prevented subsequent loss of 5-HT markers in the striatum, possibly implicating oxidative deamination products of DA in the neurotoxicity process. More recently, the use of an AS ODN targeted at MAO-B was shown to protect against the serotonergic toxicity induced by MDMA (Falk et al., 2002) and MAO-B knockout mice have been shown to be resistant to this toxicity as well (Fornai et al., 2001).

In a recent *in vitro* study, Kuhn and Arthur (1998) demonstrated the detrimental effects of DA in mediating the loss of TPH activity. The authors hypothesized that TPH-quinoprotein could be formed *in vivo* under conditions of elevated extracellular DA levels or elevated DA synthesis. Additional support for the ROS-mediated inactivation of TPH following MDMA administration arises from studies showing that MDMA-induced depletion of 5-HT and 5-HIAA and loss of TPH activity were attenuated by reducing agents such as dithiothreitol or Fe^{2+} (Stone et al., 1987), and L-cysteine (Schmidt and Taylor, 1990). Collectively, DA might serve as an important source of ROS that could lead to oxidative damage of 5-HT terminals.

A neurotoxic dosing regimen of MDMA generally produces hyperthermia in experimental animals. A number of previous studies have shown that attenuation of MDMA-induced hyperthermia or pharmacological manipulations that induce hypothermia diminish the neurotoxic effects of MDMA (Colado et al., 1997, 1999). In the present studies, neuroprotection occurred without alteration of the drug-induced hyperthermic response. Indeed, our results are consistent with those of Shankaran et al. (1999), who showed that pharmacological blockade of the DAT using mazindol prior to MDMA had no significant effect on MDMA-induced hyperthermia. Those authors showed that hydroxy radical formation and subsequent loss of 5-HT levels in the striatum were, however, attenuated. In a similar fashion, Aguirre et al. (1998) recently demonstrated that even in the presence of hyperthermia, rat pups were not vulnerable to the neurotoxic effects of MDMA, emphasizing that DA rather than hyperthermia is an essential factor for the expression of neurotoxicity.

In an analogous fashion, although fluoxetine prevented the long-term loss of 5-HT markers, the drug failed to reverse the hyperthermia produced by MDMA (Malberg et al., 1996). Taken together, it seems reasonable to conclude that hyperthermia per se cannot be the critical factor (but is a confounding factor) in MDMA-induced neurotoxicity. These findings are consistent, however, with the hypothesis that hyperthermia may enhance the toxic process under conditions where the antioxidant status of the 5-HT neuron is overwhelmed or exhausted.

CONCLUSIONS

In summary, the results of the current study are consistent with the hypothesis that DAT-induced DA release is a critical mediator of MDMA-induced neurotoxicity and provide further evidence for the involvement of the DA uptake carrier, at least in the striatum. When expression of the DAT in striatum was reduced by AS ODN, attenuation of MDMA-induced long-term reductions in serotonergic markers was observed only in that brain region. Moreover, the occurrence of neuroprotection, in spite of MDMA-induced hyperthermia, argues against a role for hyperthermia as a critical determinant of neurotoxicity. Regardless of the mechanism of action, hyperthermia and neurotoxicity are not inextricably linked. The current study also underscores the feasibility of using site-directed, temporally limited knockdown of the target protein by constant AS infusion to facilitate the delineation of the role of a particular protein in a neurochemical pathway. As a final note, the studies here are focused specifically on the role of DA and the DAT in the striatum. Based on the patterns of innervation in the cortex and hippocampus, it seems unlikely that similar conclusions could be readily applied to those brain areas. Indeed, it is possible that the mechanism of neurotoxicity in each brain region may be mediated by different processes.

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