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(±)-3,4-Methylenedioxymethamphetamine (MDMA) Administration to Rats Does Not Decrease Levels of the Serotonin Transporter Protein or Alter its Distribution Between Endosomes and the Plasma Membrane

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Running Title: MDMA administration does not decrease 5-HT transporter protein

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Non-standard abbreviations. 3β-(4'-^{125}iodophenyl)tropan-2β-carboxylic acid methyl ester ([^{125}I]RTI-55), glial fibrillary acidic protein (GFAP), methamphetamine (METH), 5,7-dihydroxytryptamine (5,7-DHT), 5-HT transporter (SERT), heat shock protein 32 (HSP32, Heme Oxygenase-1), peripheral benzodiazepine receptor (PBR)

Abstract

We showed that the 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), reduces brain tissue 5-HT, decreases expression of 5-HT transporter (SERT) protein and increases expression of glial fibrillary acidic protein (GFAP). In contrast, doses of (±)-3,4-methylenedioxymethamphetamine (MDMA) that decrease brain tissue 5-HT fail to alter expression of SERT or GFAP. Using a new and highly sensitive anti-SERT antibody, we determined if MDMA alters the subcellular distribution of SERT protein by measuring SERT expression in endosomes and plasma membranes 2 weeks after MDMA administration. Rat brain tissues (caudate, cortex, hippocampus) were collected 3 days and 2 weeks after MDMA (7.5 mg/kg i.p., q 2hr x 3 doses) or 5,7-DHT (150 µg/rat, icv) administration. Representative results from cortex are as follows. At both 3 days and 2 weeks post-injection, MDMA decreased tissue 5-HT (60%) and had no effect on GFAP expression. MDMA increased HSP32 (a marker for microglial activation) expression (25%) at 3 days, but not 2 weeks. MDMA did not alter SERT expression at either time point and did not alter SERT levels in either endosomes or plasma membranes (2 weeks). 5,7-DHT decreased tissue 5-HT (80%), increased HSP32 expression at both time points (about 50%), and increased GFAP expression at 2 weeks (37%). 5,7-DHT decreased SERT expression (33%) at 2-weeks but not at 3-days. These findings indicate that a dosing regimen of MDMA that depletes brain 5-HT does not alter SERT protein expression or the distribution of SERT between endosomes and the plasma membrane, and does not produce detectable evidence for neurotoxicity.

Introduction

The club drug (±)-3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) is widely used as an illicit recreational drug (Banken, 2004). Numerous studies in rodents and nonhuman primates indicate that MDMA administration produces long-lasting decreases in markers of serotonergic nerve terminals (for review see (Sprague et al., 1998; Green et al., 2003; Lyles and Cadet, 2003)). The MDMA-induced changes in 5-HT markers include depletions of tissue 5-HT levels, reductions in 5-HT transporter (SERT) binding and function (Commins et al., 1987; Schmidt, 1987), and loss of tryptophan hydroxylase activity. Immunohistochemical analysis of 5-HT shows an apparent loss of 5-HT nerve terminals. The spectrum of decrements in serotonergic markers produced by MDMA administration is typically described as neurotoxicity.

More than 60 papers report that MDMA administration produces a long-lasting depletion of brain 5-HT (Green et al., 2003), but fewer studies have systematically examined the effect of MDMA administration on validated markers of neurotoxicity in the rat. The results from some studies examining markers of toxicity are not consistent with the hypothesis that MDMA-induced 5-HT depletion is accompanied by axotomy. One well established index of neurotoxicity is glial fibrillary acidic protein (GFAP), which is a structural protein that occurs in astroglia and not in neurons. O’Callaghan and associates carefully validated the use of GFAP to detect neuronal degeneration following administration of various drugs, including substituted amphetamines (O'Callaghan and Miller, 1993; O'Callaghan and Miller, 2002). Indeed, recent data supports the hypothesis that neuronal degeneration is accompanied by an increased expression of GFAP (O'Callaghan and Sriram, 2005). Their work and that of others (Bai et al., 2001; Pubill et al., 2003) shows that MDMA administration regimens that deplete

5-HT do not result in increased GFAP expression in the rat, indicating that neuronal degeneration has not occurred.

Recently, Thomas et al. (Thomas et al., 2004) suggested that neurotoxic amphetamines, in contrast to non-neurotoxic amphetamines, activate both microglia and astroglia. Perhaps this notion holds true for mice, but not necessarily for rats. Although Swiss Webster mice may be an exception (Achat-Mendes et al., 2005), it is generally well accepted that rats and mice are differentially affected by high-dose MDMA treatment, with rats exhibiting depletions of brain tissue 5-HT and mice exhibiting depletions of brain tissue DA (Green et al., 2003). To complicate matters further, when rats are treated with doses of MDMA or methamphetamine (METH) that deplete tissue 5-HT, activation of microglial and astroglial occurs only in the METH-treated rats (Pubill et al., 2003). Thus, 5-HT depletion and glial activation can occur independently. Although one could argue that the neurotoxicity markers used in the Pubill study were not sensitive enough to detect degeneration of nerve terminals, this possibility seems unlikely since these markers have been shown to detect degeneration of nerve terminals induced by METH and 5,7-DHT in other studies (O'Callaghan and Miller, 1993; O'Callaghan and Miller, 2002).

In reviewing the literature on the serotonergic neurotoxicity of substituted amphetamines, we noted that few studies measured the effect of these agents on the expression of the SERT protein, in contrast to the level of SERT binding. In our first study we determined the effect of D-fenfluramine and para-chloroamphetamine (PCA), administered according to a neurotoxic injection regimen, on SERT protein expression measured with Western Blots (Rothman et al., 2003). Surprisingly, despite pronounced decreases in tissue 5-HT levels and radioligand binding to SERT, there were no changes in SERT protein expression measured 2 days or 2 weeks after drug

administration. Consistent with previous reports, the substituted amphetamine D-fenfluramine also failed to affect expression of GFAP (Bendotti et al., 1994; O'Callaghan and Miller, 1994; Stewart and Slikker, 1999).

In light of our results with D-fenfluramine and para-chloroamohetamine, we administered MDMA according to a neurotoxic regimen and measured SERT and GFAP protein expression 2 weeks later (Wang et al., 2004). As a control, we administered the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) via the intracerebroventricular (i.c.v.) route in a separate group of animals. We hypothesized that both MDMA and 5,7-DHT would decrease SERT expression and increase GFAP expression. Interestingly, it was observed that MDMA had no effect on either SERT or GFAP expression, despite large decreases in tissue 5-HT. 5,7-DHT decreased SERT protein expression and increased GFAP consistent with the profile of neurotoxic injury.

One hypothesis to explain how MDMA can decrease SERT binding and function, but not decrease SERT protein expression, is that MDMA alters the distribution of SERT between the plasmalemma and endosome compartments. Presumably, plasmalemmal transporter will be functional whereas endosomal transporter might not be functional. In the present study we determined the effect of MDMA on the expression of SERT protein in subcellular fractions of brain tissue collected 2 weeks after MDMA administration. Additionally, the effects of MDMA on markers for astroglia, microglial and neurons were evaluated at 3 days and 2 weeks after drug treatment. Using a new and more sensitive affinity-purified anti-SERT antibody, the present findings indicate that MDMA treatment does not decrease SERT protein expression in endosomes or plasma membranes. Furthermore, MDMA does not alter the expression of glial markers in a pattern consistent with neurotoxicity.

Methods

Animals.

Male Sprague-Dawley rats, purchased from Charles River Laboratories (Wilmington, MA), weighing 280-320 g were double housed (lights on: 0700-1900 h) with food and water freely available. Rats were maintained in facilities accredited by the American Association of the Accreditation of Laboratory Animal Care, and the procedures described herein were carried out in accordance with the Animal Care and Use Committee of the National Institute on Drug Abuse (NIDA) Intramural Research Program (IRP).

Experimental design

Two large groups of animals (N=28 rats per group) were used in these experiments; one group was sacrificed 3 days after drug treatments while the other group was sacrificed 2 weeks after drug treatments. Within each large group of rats, there were 4 drug treatment conditions: 1) i.p. MDMA treatment (n=7), 2) i.p. saline treatment (n=7), 3) i.c.v. 5,7-DHT treatment (n=7), and 4) i.c.v. vehicle treatment (n=7). Post-mortem tissue levels of 5-HT were measured in the frontal cortices of all animals as the primary end-point marker to determine the level of 5-HT depletion produced by drug treatment. We did not perform extensive radioligand binding experiments in this study because it is known that SERT binding decreases in tandem with tissue 5-HT (Green et al., 2003). Based on the post-mortem tissue data, we chose 4 rats from the drug-treated groups that showed similar decreases in tissue 5-HT to generate a uniform

sample to assay with Western blots. The main purpose of including the 5,7-DHT treatment groups was to include a known neurotoxin as a positive control.

Drug Treatments

For MDMA-HCl treatments, rats received i.p. injections of 7.5 mg/kg every two hours for three doses. Control rats received i.p. injections of 1 ml/kg saline on the same schedule. Colonic temperature was measured in all rats that received i.p. injections. A temperature probe (Physitemp Instruments, Inc.) was inserted into the rectum immediately after each i.p. injection and temperature was recorded in degrees C. For 5,7-DHT treatments, rats were pretreated with nomifensine (15 mg/kg, i.p.) 30 min before receiving a single i.c.v. injection of 150 µg in 10 µl vehicle (0.1% ascorbic acid in saline). Control rats received a single i.c.v. injection of 10 µl vehicle. Rats were sacrificed 3 days or 2 weeks later. After sacrifice, the frontal cortex, caudate and hippocampus were dissected. Tissue was kept frozen at -70^0 C until the time of assay by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) or Western blot.

We developed the MDMA dose and injection schedule according to the principles of “effect scaling”, rather than “interspecies scaling” (Ricaurte et al., 2000), because the latter approach is hampered by significant limitations (de la Torre and Farre, 2004). Effect scaling involves the determination of drug doses required to evoke equivalent pharmacological responses in rats versus humans. Doses of MDMA (~1.5 mg/kg) that are discriminated readily by rats (Glennon and Higgs, 1992; Baker and Makhay, 1996) are identical to those misused recreationally by humans (Cole and Sumnall, 2003). We therefore administered a dose of MDMA that is 5 times higher than the discriminated dose three times in 6 hours, an effective dose 15 times higher than the dose typically

taken by a human. We chose a 2-week sacrifice time since this time point is commonly used in the literature as an appropriate time to measure long-term effects of MDMA. The 3-day sacrifice time was chosen to test for the possibility of MDMA-induced transient increases in glial markers.

Measurement of 5-HT.

Rat cortical tissue levels of NE, DOPAC, DA, HVA, 5-HT and 5-HIAA were measured using high pressure liquid chromatography with electrochemical detection as previously described (Baumann et al., 1998). All data were acquired and analyzed using a Millennium software system (Waters Millipore, Inc.).

SERT binding.

SERT binding assays and preparation of membranes from whole rat brain minus caudate proceeded with minor modifications of published procedures (Nandi et al., 2004). The assay conditions and procedures were as described except for the following changes: assay buffer (10 mM HEPES, 100 mM NaCl, pH 74), and incubation time (2 hr at 25^0 C). We used [^{125}I]RTI-55 (3β-(4'-^{125}iodophenyl)tropan-2β-carboxylic acid methyl ester) and a selective DA transporter blocker (100 nM 4-(2-benzhydryloxy-ethyl)-1-(4-nitro-benzyl)piperidine oxalate) to block DA transporter binding (Greiner et al., 2003).

Western blot analysis

Table 1 summarizes the antibodies used in this study. Preliminary experiments were conducted to determine the optimal amount of protein to load on the gel for each antibody. Neuronal markers (synapsin-1), glial markers (GFAP, HSP32) and SERT protein levels were assayed in crude homogenates. Tissues were homogenized by sonication in cold RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 10 mg/ml aprotinin, 1 mM sodium orthovanadate in PBS buffer, pH 7.4). Protein concentrations were determined using Pierce BCA Protein Assay Reagent Kit (Rockford, IL) and homogenates were diluted to a concentration of 2 mg/ml with 2X SDS-PAGE loading buffer (Invitrogen, Carlsbad, CA). Samples were boiled for 6 min. Sample protein (6 μg/lane for GFAP and 60 μg/lane for SERT assay) was separated on 8-16% polyacrylamide minigels (Invitrogen, Carlsbad, CA). Proteins separated by electrophoresis were transferred to Immobilon-PVDF membranes (Millipore Corp., Bedford, MA) using a semi-dry apparatus (Bio-Rad, Hercules, CA). Nonspecific binding to membranes was prevented by blocking for 60 min at room temperature in TBS solution, containing 5% nonfat dry milk. Membranes were then probed with various antibodies (Table 1). Membranes were rinsed three times with TBS, and then incubated with 1:5000 dilution of horseradish peroxidase-labeled secondary antibody in TBS solution, containing 0.25% nonfat dry milk for 90 min at room temperature. After washing three more times, antibody complex were visualized by chemiluminesence using a kit from Pierce Biotechnology (Rockford, IL).

To determine the intra-assay variation for the SERT protein assay, a sample of rat caudate was run in 9 lanes of the same gel. Changes in the immunoreactivity values were expressed relative to average value (defined as 100 value), resulting in a mean±SD = 100.00±2.67.

In separate experiments, levels of immunoreactive SERT protein were measured in subcellular fractions prepared from the 2-week group. All of these procedures used reagents pre-cooled at 4°C. Caudate, cortex and hippocampus were dissected out into 10 volumes of ice-cold 10 mM Tris/HCL 7.4 buffer, and homogenized by with a polytron. The homogenate (designated H1) was centrifuged for 20 min at 20,000 x g. The pellet was saved (designated P1) and the supernatant (designated S1) was centrifuged at 200,000 x g for 60 min to yield a pellet (designated P2) and a supernatant (designated S2). The subcellular fractions were stored at -80°C. The samples were then processed for Western blot analysis as described above.

Data analyses and statistics

Western blots were quantitated using standard methods (Jayanthi et al., 2002). Pooled control and treated samples were run side-by-side. The density of the SERT band in the treated sample was divided by the density of the SERT band in the control sample and multiplied by 100 to yield a percent of control. This was done in at least 3 independent experiments. Statistical significance of the SERT binding, 5-HT tissue data and the Western blot data was determined by the Students t-test.

Results

Characterization of the anti-SERT antibody.

The first set of experiments verified the sensitivity of the affinity-purified antibody generated against N-terminal residues 1-15 of the rat SERT. As reported in Fig. 1A, Western blots of rat brain tissue using the SERT antibody detected 2 bands of molecular weight 50 kDA and 70 kDA, which represent de-gylcosylated and glycoslated transporters, respectively (Yamamoto et al., 1998). To confirm this, we treated 16 µg of homogenate protein with PNGase for 4 hr at 37^{o} C and observed a substantial reduction in the 70 kDA band (data not shown). Importantly, immunohistochemical studies with the anti-SERT antibody labeled anatomically defined serotonergic cell bodies and nerve terminals (Fig 1B) (Jacobs and Azmitia, 1992).

Effects of MDMA and 5,7-DHT on tissue 5-HT

We administered MDMA and 5,7-DHT to separate groups of rats and determined tissue 5-HT in the frontal cortex. Based on these results, we chose 4 rats from each group that showed similar decreases in tissue 5-HT, so as to generate a relatively uniform sample to assay with Western blots. Table 2 reports the 5-HT depletions observed in the selected rats. MDMA reduced 5-HT to a similar extent in both the 3-day and 2-week groups (about 65%). It should be noted that rats treated with MDMA displayed significant hyperthermia; the mean colonic temperature of MDMA-treated rats after the third i.p. injection was 39.6±0.2 degrees C whereas the temperature of saline-treated rats after the third i.p. injection was 36.9±0.1 degrees C. 5,7-DHT reduced 5-HT to a greater extent at 2-weeks (84.2%) than at 3-days (50.7%), indicating an evolving neurotoxic response.

Effect of MDMA and 5,7-DHT on glial and neuronal markers.

We determined the effect of drug treatments on both glial and neuronal markers using a crude homogenate prepared from frontal cortex, caudate and hippocampus. Microglial activation has been monitored using HSP32 (Acarin et al., 2002) and the peripheral benzodiazepine receptor (PBR) (Casellas et al., 2002), although both can also be expressed in astroglia (Kuhlmann and Guilarte, 2000; Bechtold and Brown, 2003). MDMA administration increased HSP32 expression about 30% in cortex and caudate in the 3-day group, but not in the 2-week group. MDMA and had no effect on HSP expression in the hippocampus (Fig. 2A). In contrast, 5,7-DHT increased HSP32 expression by 50-70% in the cortex and caudate in both the 3-day and 2-week groups, and increased HSP32 expression in the hippocampus in the 2-week group (Fig 2B). As reported in Fig 3, MDMA administration failed to change GFAP expression in any brain region in either the 3-day and 2-week groups. Administration of 5,7-DHT increased GFAP expression by about 40% in the cortex and hippocampus in the 2-week, but not the 3-day group. In striatum, 5,7-DHT increased GFAP expression in the 3-day and 2-weeks groups. Neither MDMA nor 5,7-DHT altered PBR (Fig. 4) or synapsin-1 expression (Fig. 5).

Effect of MDMA and 5,7-DHT treatments on SERT protein expression.

We determined the effect of MDMA and 5,7-DHT administration on SERT protein expression using crude homogenates (Fig. 6). As also noted in Fig. 1, the SERT antibody detected a 70 kDA and 50 kDA band. The graphs in Fig. 5 report the results

obtained with the 50 kDA band. Essentially identical results were obtained with the 70 kDA band. MDMA did not alter SERT expression in any brain region in either the 3-day or 2-week groups. In contrast, 5,7-DHT decreased SERT expression by about 40% in all 3 brain regions in the 2-week, but not the 3-day group.

Effect of MDMA and 5,7-DHT on SERT protein expression in sub-cellular fractions.

Because we failed to observe an effect of MDMA on SERT protein expression at either 3 days or 2 weeks post-injection, we examined the effect of MDMA on SERT protein expression in sub-cellular fractions enriched in plasma membranes or endosomes at 2 weeks after administration of MDMA. As reported in Fig. 7, the procedure we used to generate sub-cellular fractions yielded a good separation of markers for plasma membranes (Na^+/K^+ ATPase, (Schimmel et al., 1973)) and endosomes (EEA1, (Mu et al., 1995)). Fractions H1 and P1 are enriched with plasma membranes and almost undetectable levels of endosomes. Fraction P2 is highly enriched with endosomes and no detectable plasma membranes.

Interestingly, when probed with the SERT antibody, fractions H1 and P1 demonstrated both the 70 kDa and 50 kDA bands, whereas fractions P2 and S2 showed only the 70 kDa band (Fig. 8), indicating that the endosomal fractions are enriched with the glycosylated form of SERT. As reported in Fig. 8, treatment with MDMA did not alter SERT protein expression (50 kDa band) in any fraction prepared from the 3 brain regions sampled. Essentially identical results were obtained with the 70 kDa band (data not shown).

Effect of MDMA on SERT binding.

In order to verify that our MDMA injection paradigm decreases SERT binding, a separate group of rats were treated with MDMA or saline and sacrificed 2-weeks after treatment. Membranes were prepared from whole brain minus caudate and SERT binding sites were labeled with the cocaine analog, [^{125}I]RTI-55. The results, reported in Fig. 9, demonstrated that MDMA decreased SERT binding by 82%.

Discussion

Findings in this study.

The major findings from this study are as follows. First, administration of high-dose MDMA, which depletes brain tissue 5-HT and reduces SERT binding, does not alter expression of SERT or GFAP. Second, administration of the established 5-HT neurotoxin 5,7-DHT depletes brain tissue 5-HT, decreases SERT protein and increases GFAP. Thus, 5,7-DHT produces a profile of neuronal and glial responses that are consistent with neurotoxic damage, whereas MDMA does not. Finally, the differential effects of MDMA on SERT binding (i.e. reductions in binding) versus SERT protein expression (i.e. no change in SERT protein) can not be explained on the basis of altered SERT trafficking following MDMA treatment.

In our previous study (Wang et al., 2004), we reported that administration of MDMA at a dose that depletes 50% of brain 5-HT fails to decrease the expression of SERT protein, or increase expression of GFAP, an accepted marker for neurotoxicity. In contrast, administration of 5,7-DHT, a known neurotoxin, decreases SERT expression and increases GFAP expression. A central question raised by our previous work is how can MDMA administration decrease SERT binding and function without changing SERT protein expression. In view of data demonstrating the importance of intracellular trafficking of SERT (Blakely and Bauman, 2000), we hypothesized that administration of MDMA altered the distribution of SERT between the plasmalemma and endosomal compartments. It is assumed that SERT inserted into plasma membranes will be functional whereas SERT within endosomes may not be functional in vivo and not accessible to radioligands used to label SERT in vitro. In the present study, we used a

more sensitive affinity-purified antibody to extend our previous findings and to test the "altered intracellular trafficking" hypothesis.

The new antibody we used worked quite well for both Western blots and immunohistochemistry (Fig. 1). The antibody detected bands with MWs of 70 kDa and 50 kDA, which are the glycosylated and de-glycosylated forms of SERT, respectively (Yamamoto et al., 1998). The SERT antibody could be used to clearly identify serotonergic nerve terminals and fibers in immunohistochemical studies**.** We believe that these studies validate the selectivity and sensitivity of this antibody. The fractionation procedure we used effectively generated fractions enriched with plasma membranes without detectable levels of endosomal membranes (Fig. 7: H1 and P1) and a fraction enriched with endosomal membranes without detectable levels of plasma membranes (Fig. 7: S2). Interestingly, the fractionation procedure also physically separated the 70 kDa and 50 kDa forms of SERT, indicating that plasma membranes are enriched with the de-glycosylated SERT and that endosomes are enriched with the glycosylated SERT. Two weeks after MDMA administration, in the presence of a 68% depletion of cortical 5-HT, there were no changes in SERT protein expression in any brain region, in either endosomes or plasma membranes. These data appear to rule out changes in transporter trafficking as an explanation for why MDMA reduces SERT binding and function without altering SERT protein expression.

At the present time, it is clear that our investigations to-date have failed to determine why MDMA treatment causes a decrease in SERT binding and function, but not a decrease in SERT protein expression. We can only speculate that MDMA must ultimately either re-locate SERT into a compartment where it is non-functional in vivo and inaccessible to radioligand binding in vitro. A possible mechanism that remains to be explored include alterations in the activity of p38 mitogen-activated protein kinase

(Samuvel et al., 2005). Inhibition of p38 mitogen-activated protein kinase decreases the activity of its substrate, phosphoMAPKAPK, which leads to decreased SERT activity. Thus, it is possible that MDMA produces long-term changes in this regulatory pathway, resulting in long-term decreases in SERT function. Another possibility is that administration of MDMA results in less plasma membrane SERT being located in lipid rafts. Since SERT proteins not associated with lipid rafts are not functional, this also could provide a mechanism to reduce SERT activity but not SERT protein levels (Magnani et al., 2004).

Using our more sensitive anti-SERT antibody, we confirmed and extended our previous findings (Wang et al., 2004). Our previous work measured expression of SERT and GFAP 2 weeks after MDMA administration and failed to see any changes. Although the 2-week time point is generally accepted as being relevant to detecting neurotoxic effects of MDMA, it was possible that changes consistent with neurotoxicity had occurred earlier, and had normalized by 2 weeks. Thus, in the present study we measured all end-points at 3 days and 2 weeks after MDMA administration. We also assessed additional markers of neurotoxicity, including synapsin-1 to detect signs of neuronal degeneration (Monnet-Tschudi et al., 1995), HSP32 to detect signs of neuronal stress and microglial activation (Acarin et al., 2002; Bechtold and Brown, 2003) and the peripheral benzodiazepine receptor (PBR) to detect signs of microglial activation (Pubill et al., 2003). It must be mentioned that HSP32 (Bechtold and Brown, 2003) and the PBR (Kuhlmann and Guilarte, 2000) can be expressed on astroglia in addition to microglial. Thus, the results obtained with these markers must be interpreted in concert with changes in GFAP.

The results, detailed in Figs. 2-6, indicate that administration of MDMA failed to produce astrogliosis in either the 3-day or the 2-week group, or alter expression of the

PBR, indicating no evidence of microgliosis. The transient increase in HSP32 expression is most likely related to the occurrence of hyperthermia in the MDMA-treated rats, which is known to increase HSP32 expression (Bechtold and Brown, 2003). As noted in the Results section, administration of MDMA according to the dosing regimen employed here caused a sustained hyperthermic response that lasted at least 4 hours post-injection. Importantly, MDMA treatment did not alter expression of the SERT protein in either the 3-day or 2-week groups. The data obtained with synapsin-1 were not revealing, since 5,7-DHT treatment failed to decrease expression of synapsin-1. The simplest explanation of this is that the proportion of synapsin-1 in the degenerating 5-HT nerve terminals was too small a fraction of the total to be detected. The findings with MDMA contrast sharply with the effects of the known neurotoxin, 5,7-DHT. Administration of 5,7-DHT decreased SERT expression, increased GFAP expression and increased HSP32 expression in both the 3-day and 2-week groups. Interestingly, 5,7-DHT decreased SERT expression at 2-weeks, but not 3-days, indicating that some time must elapse for the lesion to fully develop. Viewed collectively, these data support the hypothesis that 5-HT depletion induced by MDMA might not be due to axotomy.

Literature review.

Many studies demonstrate that MDMA administration produces long-term reductions in markers of the serotonergic nerve terminal (Green et al., 2003). Although Swiss Webster mice may be an exception (Achat-Mendes et al., 2005), it is generally well accepted that rats and mice are differentially affected by high-dose MDMA treatment, with rats exhibiting depletions of brain tissue 5-HT and mice exhibiting depletions of brain tissue DA (Green et al., 2003). The effects of MDMA on 5-HT

markers in rats include depletions in tissue 5-HT levels, decreases in SERT binding and reduction of tryptophan hydroxylase activity (Sprague et al., 1998; Ricaurte et al., 2000; Lyles and Cadet, 2003). Evidence that these changes result from death of nerve terminals (axotomy) is based primarily on immunohistochemical analysis of 5-HT (O'Callaghan and Miller, 2002). However, the use of semi-quantitative immunohistochemical methods with intact nerve terminals possessing considerably less 5-HT could produce misleading results if not supported by other neurotoxicity markers. Thus, there is clearly a need to test the hypothesis that MDMA produces axotomy using additional measures of neurotoxicity.

Several published studies examined the effect of MDMA administration on validated markers of neurotoxicity. O'Callaghan and associates determined the effect of MDMA treatment on expression of GFAP in rats (O'Callaghan and Miller, 1993; O'Callaghan and Miller, 2002). They observed no change in GFAP expression except at exceedingly high doses (at least 75 mg/kg twice daily for 4 days). Moreover, this group reported that when MDMA and 5,7-DHT produce a similar decrease in 5-HT depletion (about 70%), only 5,7-DHT increased GFAP (O'Callaghan and Miller, 1993). Pubill et al. (Pubill et al., 2003) administered MDMA (20 mg/kg bid x 4 days) and assessed SERT binding and glial activation 3 and 7 days post-treatment. Despite the expected decreases in SERT binding, there were no changes in several measures of glial responses. For example, MDMA administration failed to increase [^{3}H]PK-11195 binding, which labels the PBR, or induce expression of OX-6, both markers for microglial activation. Similarly, MDMA administration failed to induce astroglial activation, as indicated by Western blot measurement of GFAP and HSP27 expression. In support of these findings, Bai et al. (Bai et al., 2001) reported that MDA administration (10 mg/kg) failed to increase GFAP expression. In contrast, Aguirre et al. (Aguirre et al., 1999)

reported that a single dose of MDMA (20 mg/kg, i.p.) increased GFAP expression in the hippocampus. The reasons for these discrepant results are unclear, but may related to the route of MDMA administration or specific strain of rats tested in each study. Most of the studies cited above administered MDMA via the s.c. route (O'Callaghan and Miller, 1993; Bai et al., 2001; O'Callaghan and Miller, 2002; Pubill et al., 2003), whereas Aguirre et al. (Aguirre et al., 1999) administered MDMA via the i.p. route. MDMA administration to female C57BL6/J mice increased striatal GFAP expression (O'Callaghan and Miller, 1994; Miller and O'Callaghan, 1995). However, MDMA persistently depletes dopamine in the mouse, and the observed increase in GFAP expression could reflect dopaminergic axotomy. Taken together, the available findings indicate that MDMA-induced 5-HT depletion in the rat can occur without significant changes in GFAP expression.

Although MDMA administration fails to increase glial markers of neurotoxicity, it is well known that MDMA administration, even a single relatively low dose, results in neuronal damage as assessed by silver staining (Commins et al., 1987; Jensen et al., 1993). Similar results are obtained with the newer stain, Fluoro-Jade B (Schmued and Hopkins, 2000). As reviewed in detail elsewhere (Jensen et al., 1993), much of the staining is not associated with serotonergic nerve terminals, indicating that MDMA can produce some degree of non-serotonergic brain damage. This is not surprising, since MDMA could damage non-serotonergic neurons via its hyperthermic and sympathomimetic effects (Cole and Sumnall, 2003; Green et al., 2003).

The findings reported here for the rat demonstrate that a dosing regimen of MDMA that produces long-lasting depletion of brain 5-HT fails to alter either expression of SERT or expression of astroglial and microglial markers. The dosing regimen we used, which was rationally chosen based on “effect scaling” (see Methods), uses lower

doses than those used by some other investigators. However, our dosing regimen produces acute hyperthermia and long-term 5-HT depletions that are comparable to other studies employing much higher doses (Battaglia et al., 1987; Commins et al., 1987; Scanzello et al., 1993). In our experience, administration of 3 sequential doses of MDMA at 7.5 mg/kg constitutes the upper limit of dosing that can be used without killing ~15-20% of rats. The decrease in SERT expression and increase in GFAP expression induced by i.c.v. administration of 5,7-DHT, a known neurotoxin, shows that the Western blot assays can detect changes expected to occur following neurotoxic injury. It is interesting that SERT expression level in 5,7-DHT-treated rats is higher than expected based on the degree of 5-HT depletion. A possible explanation is that whereas 5-HT in compromised nerve terminals is cleared rapidly via metabolic pathways, large integral membrane proteins are probably cleared much more slowly. It seems unlikely that we are measuring proteolytic fragments of the SERT, since the bands we quantitated occurred at the expected molecular weight.

As noted in several reviews (Sprague et al., 1998; Ricaurte et al., 2000; Lyles and Cadet, 2003), numerous variables affect MDMA responses, including dosage, species, ambient and body temperature, and route of administration (Bai et al., 2001; Esteban et al., 2001). Under the conditions used here, MDMA administration failed to produce changes consistent with axotomy. These findings are similar to those we found with using d-fenfluramine and para-chloroamphetamine (Rothman et al., 2003). The simplest explanation of our data is that, using our dosing regimen, administration of MDMA, d-fenfluramine or para-chloroamphetamine leads to functional inactivation of SERT, not serotonergic axotomy (Molliver and Molliver, 1990).

In conclusion, this study demonstrates the MDMA administration to rats does not decrease SERT expression or increase validated markers of neurotoxicity. These data

suggest that the putative serotonergic toxicity of substituted amphetamines should be re-examined using immunological methods to measure SERT protein changes, and explanations other than axotomy should be sought to explain the long-lasting changes produced by MDMA on SERT binding and function.

Acknowledgements

The authors note the excellent technical assistance of Christina M. Dersch for performing the SERT binding assays.

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Legends for Figures

Figure 1. A. Typical Western blot for SERT in rat cortex reveals 70 kDa and 50 kDa bands. B. Affinity-purified anti-SERT antibody clearly labels cortex (1: low magnification, 2: high magnification) and striatum (3: low magnification, 4: high magnification). Magnification bar equals 4 μm for 1 and 2, and 2 μm for 3 and 4.

Figure 2. Western blot analysis of HSP-32 immunoreactivity in rat cortex, caudate and hippocampus 2 weeks and 3 days after treatment with MDMA (Panel A) or 5,7-DHT (Panel B) and corresponding control groups (n=3). Blots were digitized, and quantified using densitometric analysis (NIH IMAGE software). Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100 value). *p<0.01 when compared to control (Student t-test). Each value is the mean±SD.

Figure 3. Western blot analysis of GFAP immunoreactivity in rat cortex, caudate and hippocampus 2 weeks and 3 days after treatment with MDMA (Panel A) or 5,7-DHT (Panel B) and corresponding control groups (n=3). Blots were digitized, and quantified using densitometric analysis (NIH IMAGE software). Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100 value). *p<0.01 when compared to control (Student t-test). Each value is the mean±SD.

Figure 4. Western blot analysis of PBR immunoreactivity in rat cortex, caudate and hippocampus 2 weeks and 3 days after treatment with MDMA (Panel A) or 5,7-DHT (Panel B) and corresponding control groups (n=3). Blots were digitized, and quantified using densitometric analysis (NIH IMAGE software). Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100 value). Each value is the mean±SD.

Figure 5. Western blot analysis of Synapsin 1 immunoreactivity in rat cortex, caudate and hippocampus 2 weeks and 3 days after treatment with MDMA (Panel A) or 5,7-DHT (Panel B) and corresponding control groups (n=3). Blots were digitized, and quantified using densitometric analysis (NIH IMAGE software). Changes in immunoreactivity

values were expressed relative to their corresponding control (defined as 100 value). Each value is the mean±SD.

Figure 6. Western blot analysis of SERT immunoreactivity in rat cortex, caudate and hippocampus 2 weeks and 3 days after treatment with MDMA (Panel A) or 5,7-DHT (Panel B) and the corresponding control groups (n=3). Blots were digitized, and quantified using densitometric analysis (NIH IMAGE software). Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100 value). *$p<0.01$ when compared to control (Student t-test)

Figure 7. Western blot analysis of the plasma membrane marker, Na/K-ATPase, and the endosomal marker, EEA1 in H1, P1, P2 and S2 different fractions of rat brain samples (n=4).

Figure 8. Western blot analysis of SERT immunoreactivity in the H1, P1, P2 and S2 fractions from rat cortex, caudate and hippocampus 2 weeks after vehicle and MDMA treatment (n=3). Blots were digitized, and quantified using densitometric analysis (NIH IMAGE software). Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100 value).

Figure 9. Effect of MDMA treatment on SERT binding. [^{125}I]RTI-55 (0.1 nM) was used to selectively label SERT in membranes prepared from saline and MDMA-treated rats 2-weeks after drug treatment. Each value is the mean±SD (n=6). *$p<0.01$ when compared to control (Students t-test).

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Table 1

Summary of Antibodies Used in This Study

Antibody	Source	Catalog Number	Dilution/ Incubation Condition	Protein loaded(μg)
GFAP	Research Diagnostics Inc, Flanders, NJ	RDI-GFAPabm-2e1	1:500/4^{o} C overnight	2
HSP32	Stressgen Bioreagents, Victoria, BC Canada	OSA-111	1:1000/4^{o} C overnight	24
SERT	Prosci Inc, Poway, CA	Custom preparation[1]	1:100/4^{o} C overnight	16
Synapsin I	EMD Biosciences Inc, San Diego, CA	574777	1:1000/4^{o} C overnight	2
EEA1	BD Biosciences, San Jose, CA	610457	1:1000/4^{o}C overnight	24
Na/K ATPase	BD Biosciences, San Jose, CA	610915	1:1000/4^{o} C overnight	2
Peripheral benzodiazepine receptor	Gift from Dr. Vassilios Papadopoulos, Georgetown University Medical Center	(Papadopoulos et al., 2005)	1:200/4^{o} C overnight	60

[1]Affinity purified antibody, antigen = residues 1-15 from the N-terminus of the rat SERT

Table 2

5-HT Depletion Produced by MDMA and 5,7-DHT Administration

Treatment	3-Day Group (n=5) (Percent Depletion±SD)	2-Week Group (n=4) (Percent Depletion±SD)
MDMA	64.6±7.2*	68.4±9.4*
5,7-DHT	50.7±5.6*	84.2±9.7*

As described in Methods, tissue 5-HT was determined in samples of frontal cortex and the percent depletion calculated relative to rats injected with vehicle. *$p<0.05$ when compared to control. The control 5-HT level was 358±37 pg/mg tissue.

Figure 1

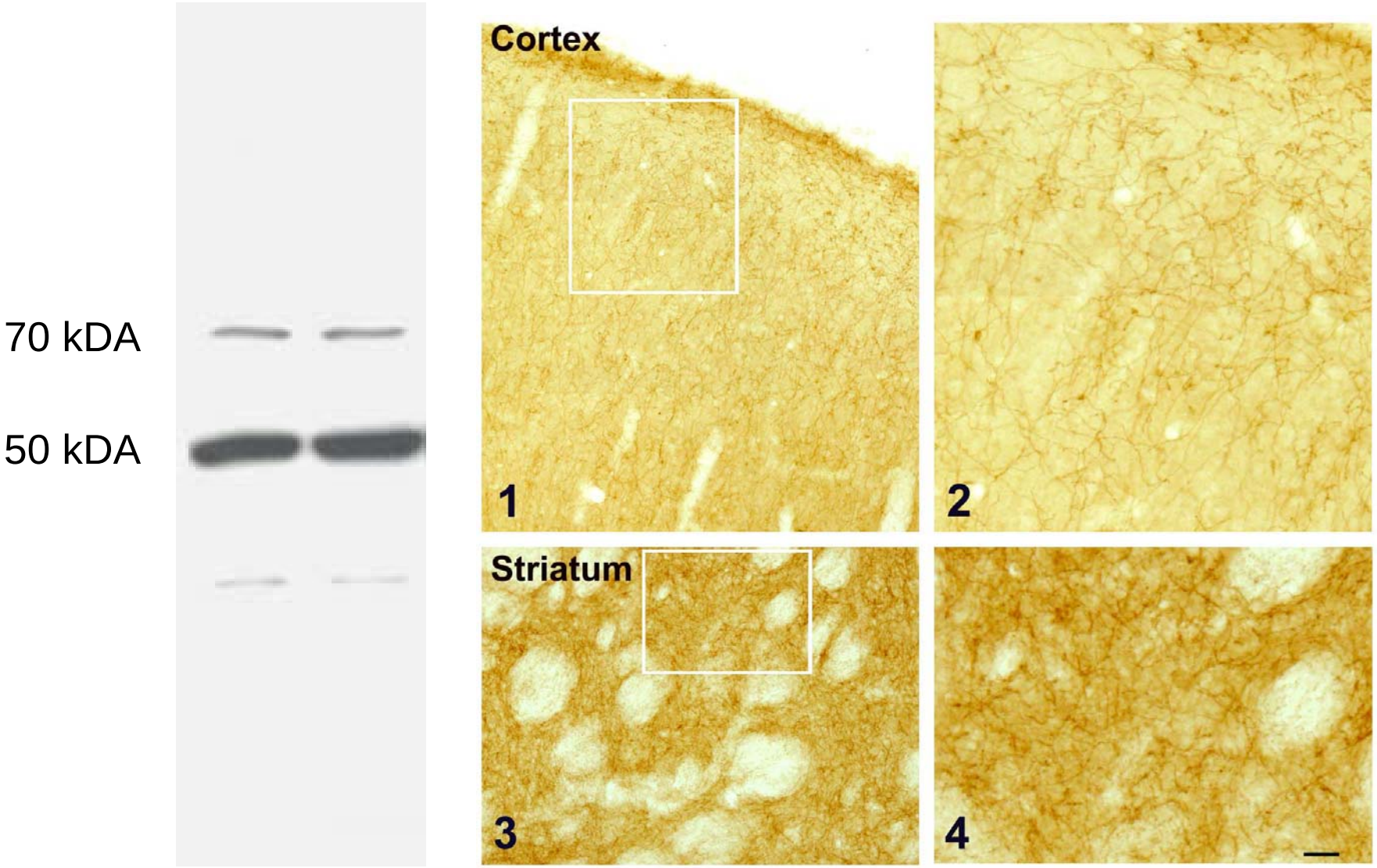
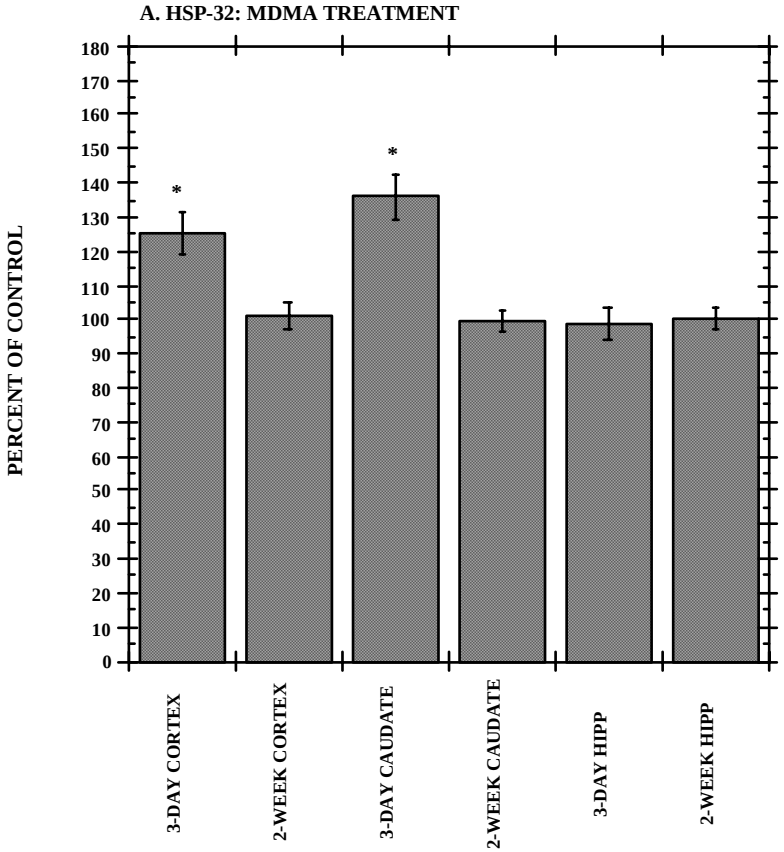
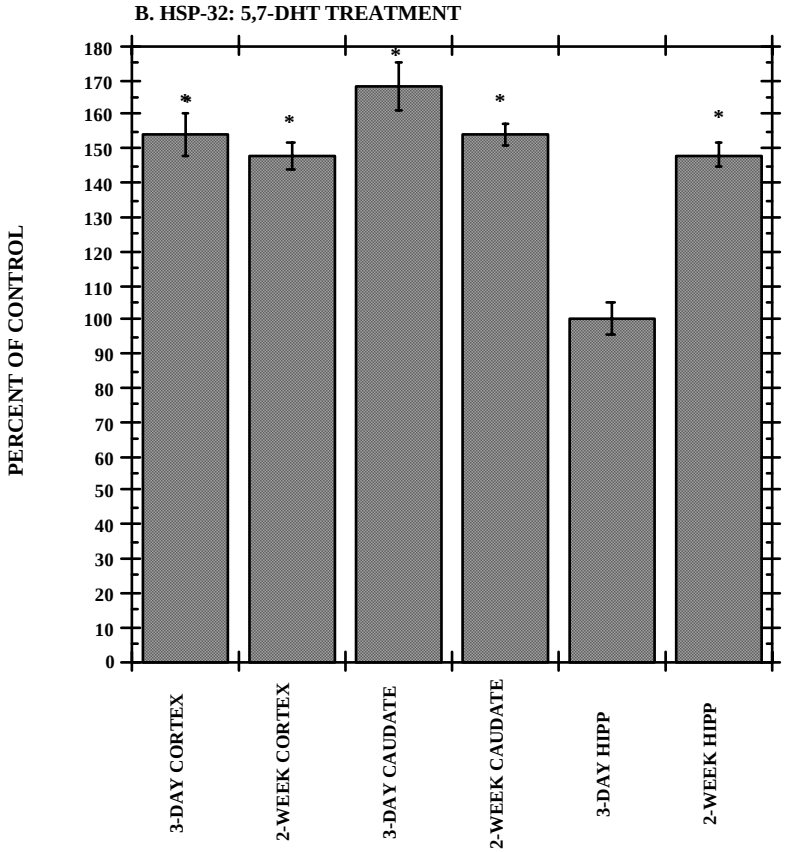
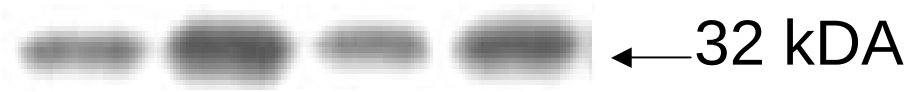


Figure 2







Control 5,7-DHT Control MDMA

3-Day Caudate Samples

Figure 3

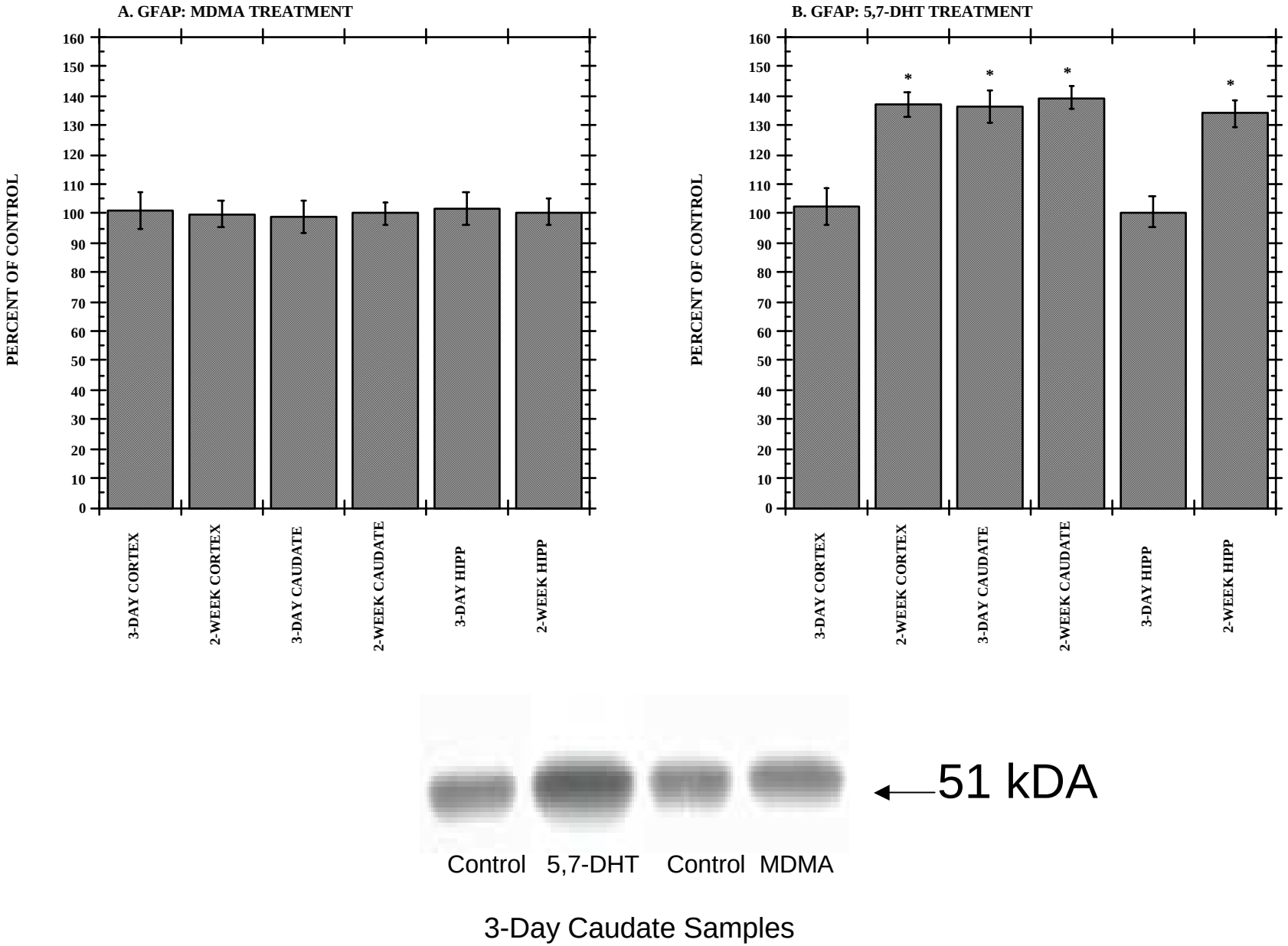
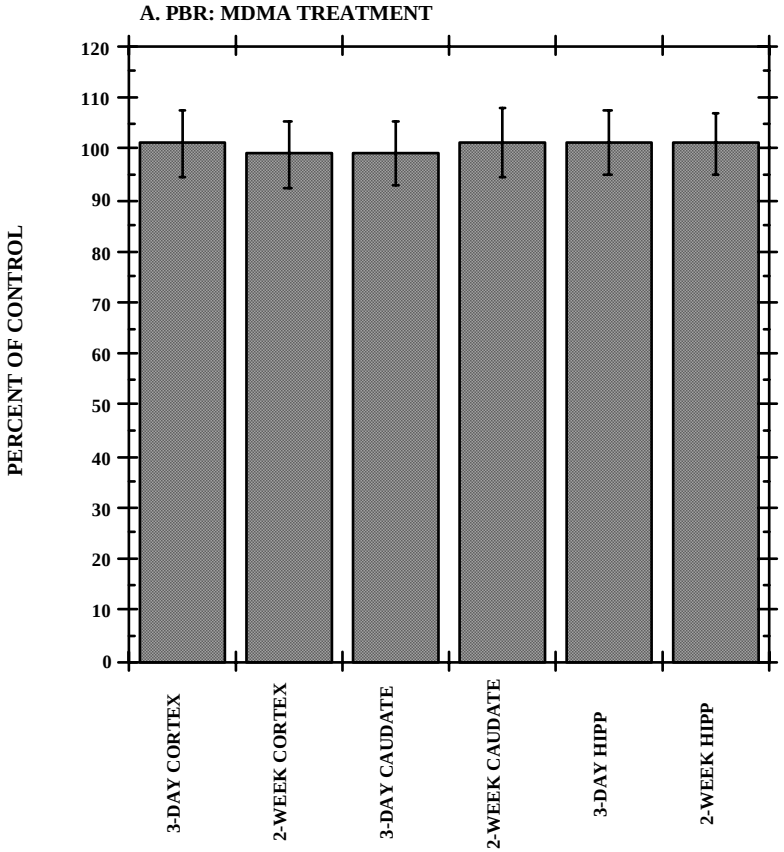
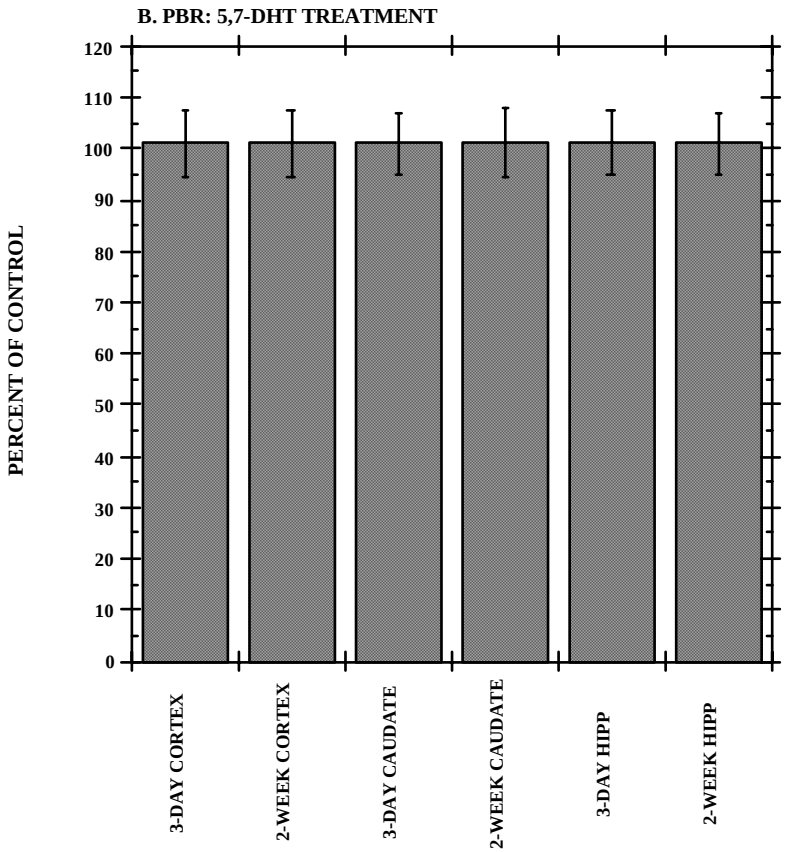
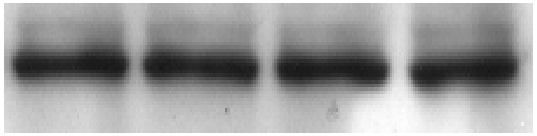


Figure 4





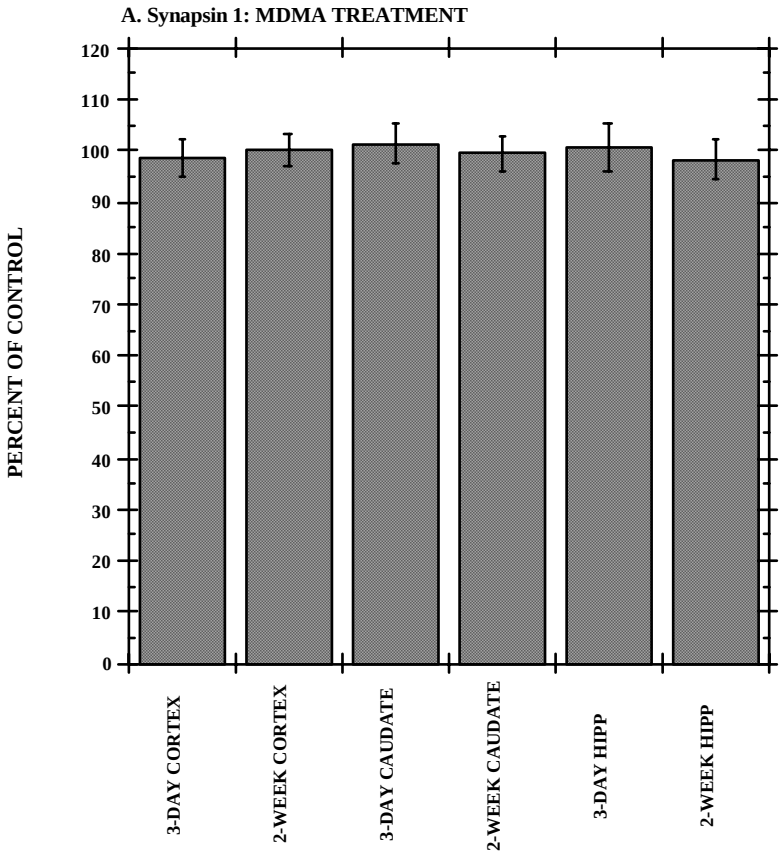


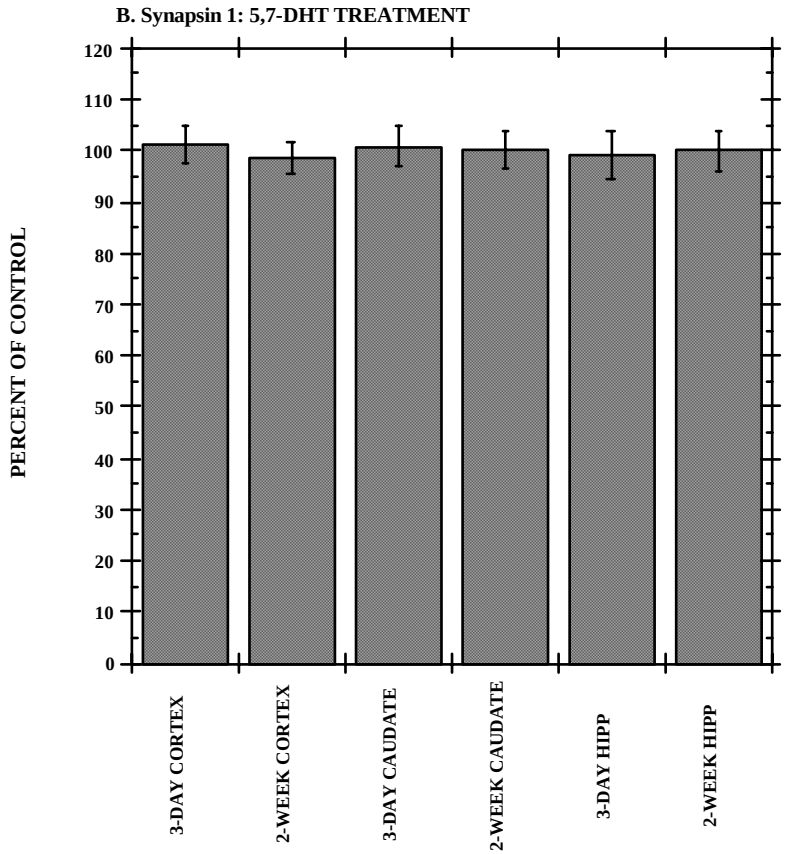
70 kDA

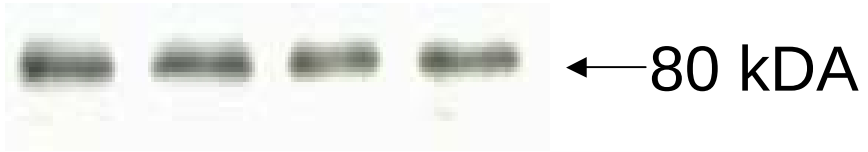
Control 5,7-DHT Control MDMA

3-Day Caudate Samples

Figure 5







3-Day Caudate Samples

Figure 6

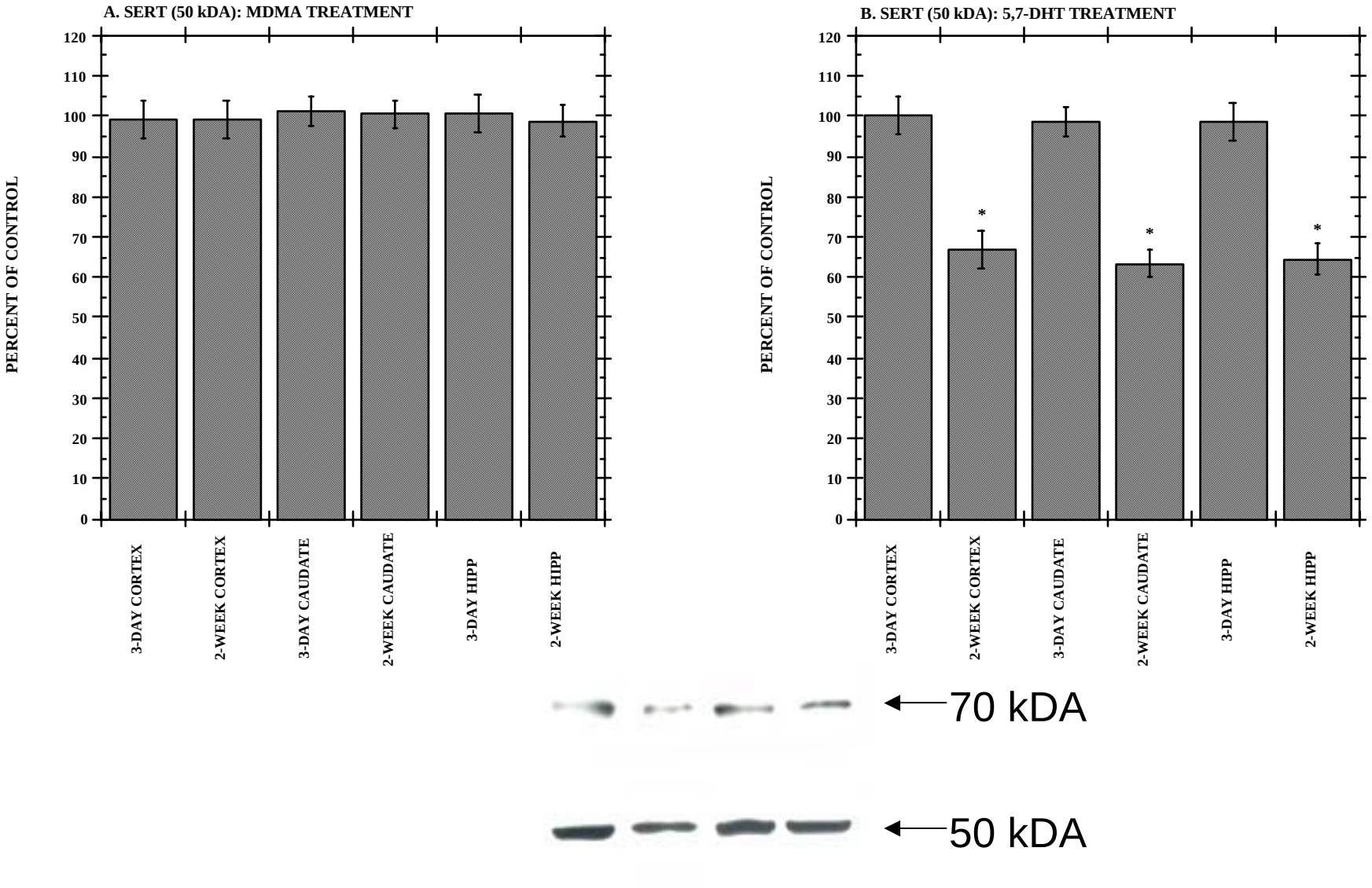


Figure 7

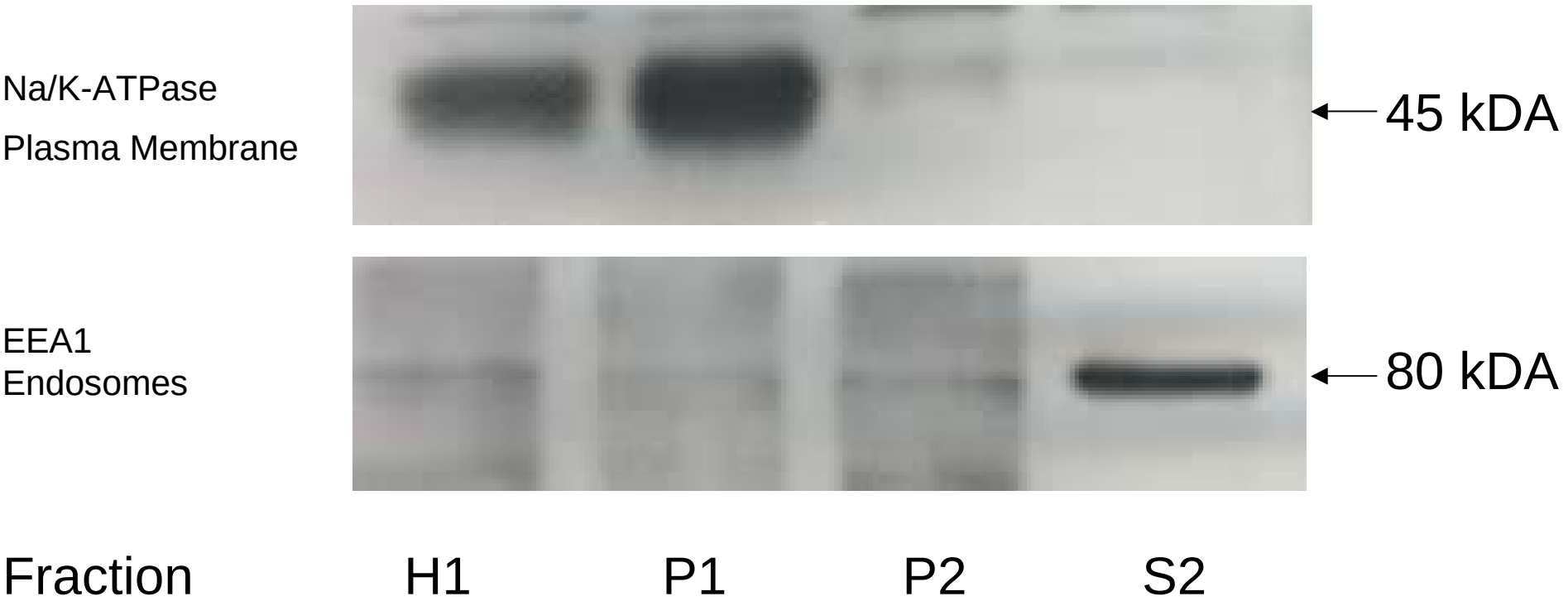


Figure 8

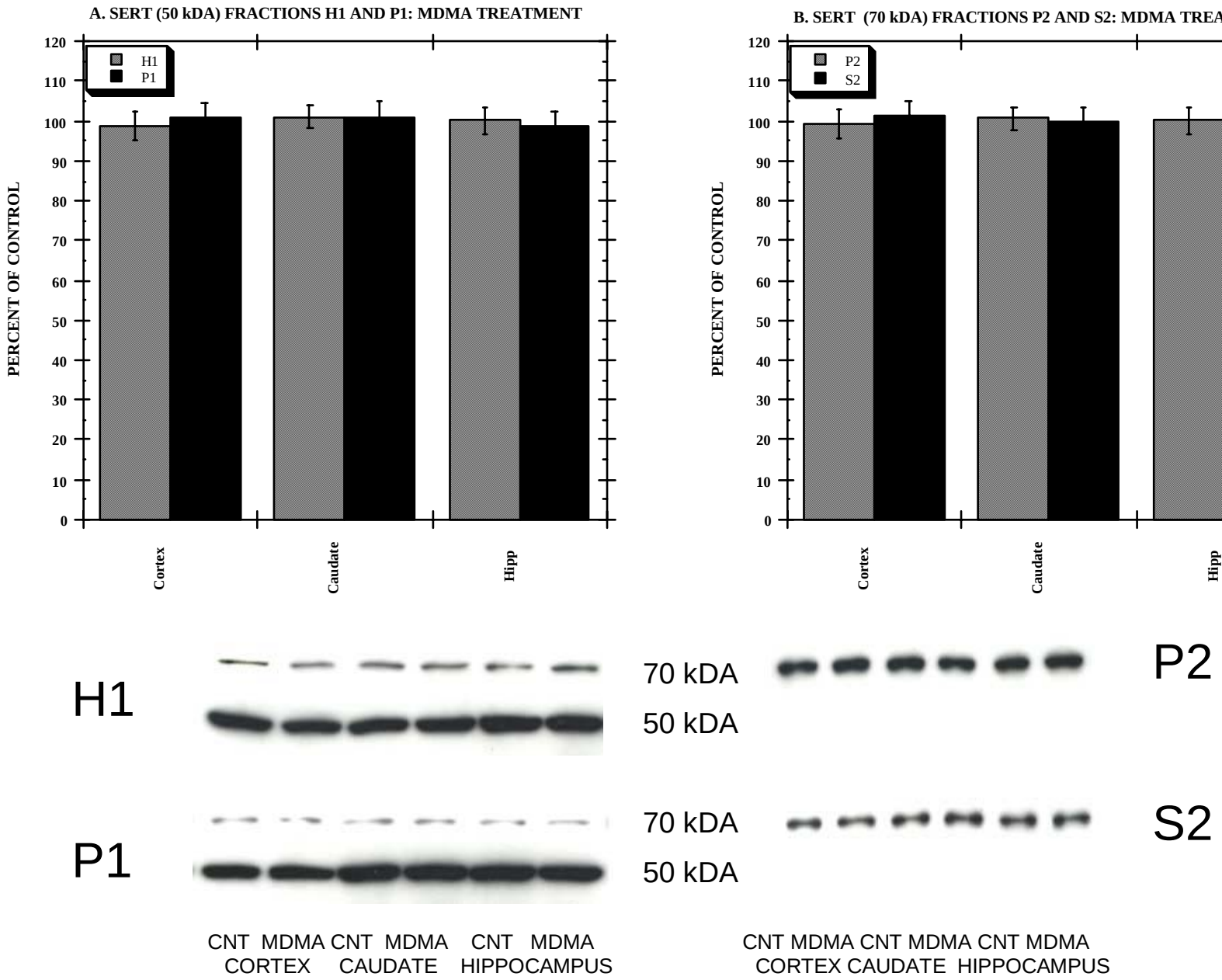


Figure 9