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Brain Research 933 (2002) 150–163

BRAIN RESEARCH

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Research report

d-MDMA during vitamin E deficiency: effects on dopaminergic neurotoxicity and hepatotoxicity

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Accepted 5 December 2001

Abstract

The mechanism of 3,4-methylenedioxymethamphetamine (*d*-MDMA)-induced neurotoxicity may involve formation of toxic radical species. Endogenous defenses against toxic radical species include tissue stores of vitamin E, and thiols. We examined whether vitamin E deficiency could alter *d*-MDMA-induced neurotoxicity by administration of the drug to animals with diet induced vitamin E deficiency. Brain vitamin E levels in deficient mice were reduced 75% compared to sufficient animals. Animals received *d*-MDMA 5 or 10 mg/kg or saline (delivered every 2 h×4, s.c.). Diet slightly altered *d*-MDMA-induced temperature modulation. In brain, MDMA treatment reduced vitamin E, total antioxidant reserve and protein thiols 72 h after the first dose. In liver, MDMA treatment reduced glutathione and total antioxidant reserve at the same time point. The vitamin E-deficient group, treated with the low dose of *d*-MDMA, exhibited neurotoxic responses, including reduced striatal dopamine (47%) and elevated GFAP protein (3-fold): while the sufficient diet group was not altered. The higher *d*-MDMA dose caused neurotoxic responses in both diet groups. Liver toxicity was determined by histopathologic examination. *d*-MDMA caused hepatic necrosis that was more severe in vitamin E deficient than sufficient mice. These data indicate that (1) *d*-MDMA administration reduces antioxidant measures at a time coincident with *d*-MDMA-induced neuronal damage and (2) vitamin E deficiency increases susceptibility to *d*-MDMA-induced neurotoxicity and hepatic necrosis. Published by Elsevier Science B.V.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Dopamine; Striatum; Serotonin; *d*-MDMA; Alpha-tocopherol deficiency

1. Introduction

d-MDMA is a substituted amphetamine which causes dopaminergic neurotoxicity in mouse brain [24] and sustained depletions of serotonin neurotransmitter and serotonin uptake sites in rat brain [4], possibly by species dependent mechanisms [24]. Evidence of the dopaminergic neurotoxicity in mouse includes sustained depletions of striatal dopamine (DA) and it's homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) metabolites, accompanied by a loss of tyrosine hydroxylase protein (TH) and coincident astrogliosis, as evidenced by an increase in GFAP protein [32]. The mechanism of *d*-MDMA induced dopaminergic neurotoxicity in mouse brain is not known although evidence suggests that oxygen radical formation may be involved. Over-expression of copper/zinc superoxide dismutase in transgenic mice has been reported to protect against MDMA-induced striatal DA depletion [6,17]. Furthermore, the dopaminergic changes induced by MDMA are similar to those induced in mouse by amphetamine and methamphetamine [32]. The neurotoxic effects of methamphetamine in mouse also may be associated with formation of radical species since manipulations which protect mouse from neurotoxicity

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0006-8993/02/$ – see front matter Published by Elsevier Science B.V.
PII: S0006-8993(02)02313-2

also scavenge oxygen and nitric oxide radicals. For example, supplementation of diet with selenium, an essential cofactor of glutathione peroxidase protects mice, while diets deficient in selenium potentiate methamphetamine-induced DA depletion [15,16,19,20]. Our laboratory investigates how various stressors may alter toxicity. We previously have demonstrated alterations in *d*-MDMA neurotoxicity as a function of the physiologic status of the animal during exposure [18,28]. Since considerable work indicates that stress, induced by a variety of stimuli, can accelerate the consumption of antioxidant reserves [10,13,14,23,35,37,38], we were interested in the effects of MDMA in brain and liver in an antioxidant deficient condition. Accordingly, we examined how *d*-MDMA induced toxicity is altered after 20 weeks of a vitamin E deficient diet. We reasoned as follows: if *d*-MDMA is toxic through a mechanism involving the formation of oxygen radicals, then alimentary reduction of vitamin E in brain and liver should increase its toxic potential. We found that vitamin E deficiency reduces the levels of endogenous antioxidants in both organs. As well, our results indicate a direct effect of *d*-MDMA on depletion of antioxidant resources within brain and liver. Furthermore, the results support the hypothesis that *d*-MDMA-induced dopaminergic neurotoxicity and possible hepatotoxicity is mediated in part by radical species since *d*-MDMA-induced toxicity is enhanced in vitamin E deficient BALB/C male mice.

2. Materials and methods

2.1. Materials

The following drugs and chemicals were kindly provided by or obtained from the sources indicated: high-performance liquid chromatography (HPLC) standards (Sigma Chemical Co., St. Louis, MO); *d*-MDMA (Research Technology Branch, National Institute on Drug Abuse, Rockville, MD). Reagents used for HPLC were of HPLC grade (ESA, Chelmsford, MA).

2.2. Animals

All procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee and in accordance with the NRC *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996). Male BALB/c mice 3–4 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in a colony certified by the American Association for Accreditation of Laboratory Animal Care. Upon receipt, the mice were single housed in a temperature controlled (21±1 °C) and humidity controlled (50±10%) room, on a 12-h light/dark cycle and maintained either on a vitamin E sufficient pellet diet (sufficient, 50 IU vitamin E/kg diet) or vitamin E deficient pellet diet (deficient, less than 10 IU vitamin E/kg diet). Both diets were manufactured by Purina Mills (Richmond, IN). Deficient diet contained 3% crude fiber (solka floc), 22% protein (casein), 15% sucrose, 10% crude fat (tocopherol-stripped corn oil 5%, tocopherol-stripped lard 5%), 0.2% choline chloride, 43.65% starch (dextrin), mixed vitamins without vitamin E, 5.0% RP mineral mix, and 0.15% D,L-methionine. vitamin E sufficient diet contained the same as above, in addition to vitamin E 50 IU/kg. Food and water were available ad libitum.

2.3. Drug or vehicle administration

d-MDMA (5 or 10 mg/kg, calculated as base) or saline vehicle (0.9%) was administered s.c. in a volume of 1 ml/100 g body weight, every 2 h, for a total of four injections. Our preliminary experiments indicated that BALB/C male mice are more susceptible to the dopaminergic neurotoxicity of *d*-MDMA than are C57BL/6 mice. These doses are 25 and 50%, respectively, of the doses used in C57Bl/6J mice [28,32]. To minimize circadian influences on toxicity, the first injection was always given between 09:00 and 10:00 h. Male mice were used throughout the study. At the time of *d*-MDMA administration the animals were approximately 24 weeks (6 months) of age, and were young adults. BALB/C mice have a typical life expectancy of 30 months.

2.4. Temperature determinations

A baseline rectal temperature measurement was taken immediately prior to drug or saline injection and prior to each time an animal was injected with *d*-MDMA or saline. A final rectal temperature measurement was made approximately 24 h after the first injection. Rectal temperature was recorded with a Bat-10 thermometer coupled to a RET-3 mouse rectal probe (Physitemp, Inc., Clifton, NJ) lubricated with mineral oil. To facilitate temperature measurements mice were placed under a 'Quonset hut'-shaped piece of foam that was approximately the length of the mouse and that was blocked at the front end. Mice were held by the base of the tail while the temperature probe was inserted to a premarked depth of 2.0 cm, making sure that the probe touched the wall of the large intestine. This method minimized handling and in conjunction with the use of a fast-rise time rectal probe, made it possible to obtain reliable measurements of rectal temperature in less than 30 s per mouse.

The area under the temperature versus time curve (T_{AUC}) was determined using Simpson's rule as previously reported. In previous experience using C57Bl/6J mice, stress manipulations and co-administered drugs caused effects on T_{AUC} which were proportional to alterations of the neurotoxic effect of *d*-MDMA [18]. For example, manipulations

that reduced T_{AUC} also blocked the neurotoxic effect of *d*-MDMA.

2.5. Brain dissection and tissue preparation

All brains were obtained 72 h after the initial *d*-MDMA injection, a time point at which striatal dopaminergic neurotoxicity is evident as indicated by markers of DA terminal integrity as well as decreases in DA content (see Ref. [32] for a complete time-course). Immediately after decapitation, whole brains were removed from the skull with the aid of blunt curved forceps. Striatum was dissected free-hand on a thermoelectric cold plate (model TCP-2; Aldrich Chemical Co., Milwaukee, SI) using a pair of fine curved forceps (Roboz, Washington, DC). Striata from the left and right sides of the brain were weighed, frozen on dry ice and stored at −80 °C for subsequent analysis of neurotransmitter content or preparation for GFAP assay, respectively.

2.6. Sample preparation for neurotransmitter analysis

Brain tissue homogenates were prepared by sonification (Kontes Micro ultrasonicator/cell disruptor) on ice using a 30-s pulse in 0.2 N perchloric acid, containing 3,4-dihydroxybenzylamine 1 μM as internal standard. The homogenate was centrifuged at 10 000×*g* for 15 min, and resulting supernatant immediately injected using the autosampler as described below. Each brain area was prepared in a standard volume (striatum 0.3 ml).

2.7. Preparation for GFAP assay

Right side striatum from each mouse was prepared for GFAP assay by sonification of frozen tissue in 10 Vol hot (90–95 °C) 1% SDS. The resulting preparation was frozen at −80 °C until use in the GFAP assay.

2.8. Neurotransmitter analysis by HPLC with electrochemical detection

The brain tissue concentrations of DA, DOPAC, HVA and other neurotransmitter substances were analyzed by HPLC with electrochemical detection using the following system: Supernatant prepared as described above (10 μl) was injected using a temperature controlled (4 °C) Waters 717Plus Autosampler (Waters Corporation, Milford, MA) connected to a Waters 515 HPLC pump. Sample passed over a reversed phase C_{18} column (Waters Symmetry, 4.6×250 mm, 5 μm, 100 A). Samples were detected using the Waters 464 pulsed electrochemical detector (range 10 nA, potential 700 mV) connected by means of the Waters bus SAT/IN module to a computer using Millennium Software 32. The mobile phase consisted of 75 mM sodium dihydrogen phosphate, 1.7 mM 1-octanesulfonic acid, 25 μM ethylendiaminetetraacetic acid, 10% v/v acetonitrile, all adjusted to pH 3.0 with concentrated phosphoric acid, pumped at a flow rate of 1 ml/min. Under these conditions the average run time is 30 min with representative retention times (in min) for 3,4-dihydoxybenzylamine (internal standard: 8.24), DOPAC (8.93), DA (11.28), HVA (19.77), 5-hydroxytryptamine (26.1). Quantitation was accomplished by the use of the internal standard (10 pmol DHBA per injection) method using daily standard curves of each analyte (0.5–25 pmol per injection). Results were expressed as μg/g wet tissue weight. The limit of detection was 0.5 pmol per injection, inter assay variation was ±3%

2.9. GFAP immunoassay

GFAP was assayed according to modifications [32] of a previously described sandwich ELISA [31]. Total protein concentration of the SDS homogenate used for the GFAP immunoassay was determined by the method of Smith et al. [39]

2.10. Histology

Mice were euthanized 72 h after treatment with *d*-MDMA. Livers were fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA) and dehydrated through a graded series of ethanol solutions. Samples were cleared with xylene (Fisher Scientific, Pittsburgh, PA, USA) and embedded in paraffin. Specimens were sectioned at 5 μm. The sections were deparaffinized and stained with haematoxylin/eosin (Fisher Scientific, Pittsburgh, PA) using an automatic stainer (GLX Shandon, Lipshaw, Pittsburgh, PA). Slides were examined and photographed using a light microscope (Olympus BX 40, Japan).

2.11. Preparation of brain and liver homogenates for determination of α-tocopherol, total antioxidant reserve, glutathione and protein thiols

The brain and liver were excised promptly after the mice were sacrificed by decapitation as described above, and brain (cortex) and liver samples for biochemical analysis were frozen at −80 °C until processed. The brain cortex or liver homogenates were prepared from frozen tissues with phosphate-buffered saline 1 ml (PBS, pH 7.4) using a tissue tearer (model 985-370, Biospec Products Inc., Racine, WI). Homogenates were stored at −80 °C until processed further. Total protein concentration in these homogenates was determined using the Bradford method [5] with Coomassie blue dye (BioRad, kit number #500-0006).

2.12. HPLC assay of α-tocopherol in brain and liver homogenates

Extraction of α-tocopherol from brain (cortex) homoge-

nates was performed using a procedure described by Lang et al. [21] Cortex was used for the determination of brain α-tocopherol because striatal tissue is limited and because of the expectation that vitamin E reserves would be depleted equally across all brain areas. A Waters HPLC system (Waters Associates, Milford, MA, USA) with 717 auto sampler, NOVA-PAK C_{18} column (3×3.9 mm×1500 cm), Waters 600 controller pump and an 474 fluorescence detector [wavelengths: 292 nm (excitation) and 324 nm (emission)] were used. The data acquired were exported from the Waters 474 detector using Millennium 2000 software. The method used for vitamin E detection in the study is sensitive with a detection limit of 1.5 nM corresponding to 0.1 pmol of α-tocopherol per mg of protein in tissue.

2.13. Fluorescence assay of glutathione (GSH) and protein thiols in brain and liver homogenates

GSH and total protein thiol concentration in samples was determined using ThioGlo-1™, a maleimide reagent, producing a highly fluorescent product upon its reaction with thiol groups [37]. This method has significant advantages in that it is simple, sensitive and is therefore used by a number of groups [22,37,40]. GSH content was estimated by an immediate fluorescence response registered upon addition of Thio Glo-1 to the homogenate [37]. Protein thiols were determined as an additional increase in fluorescence response after addition of sodium dodecyl sulfate (4.0 mM) to the same homogenate. A standard curve was established by the addition of GSH (0.04–4.0 μM) to 100 mM phosphate buffer, pH 7.4 containing 10 μM ThioGlo-1 (dimethyl sulfoxide solution). The detection limit was 0.3 nM, which corresponds to 6 pmol of glutathione per mg of protein in tissue. To validate the above method for determination of GSH and protein thiols the following was conducted: in a separate series of experiments, we performed ThioGlo-1™ assays in samples before and after treatment with GSH-peroxidase/H_2O_2 (0.2 U/ml/0.13 mmol/ml). We found that after pretreatment with GSH peroxidase/H_2O_2 the response to ThiGlo-1™ from low molecular weight thiols (in the absence of SDS) decreased by more than 95%, while that of protein thiol groups (after addition of SDS) did not undergo any substantial changes (97% of that in the presence of GSH-peroxidase). Given that GSH-peroxidase specifically oxidizes GSH [11,12,41] we conclude that assay with ThioGlo-1™ quantitatively determines both GSH (in the absence of SDS) and protein SH groups (after addition of SDS) in the mouse brain homogenates. A Shimadzu spectrofluorophotometer RF-5000U (Colombia, MD) was employed in the assay using wavelengths of 388 nm (excitation) and 500 nm (emission). The results were exported from the spectrofluorophotometer using RF-5301PC Personal Fluorescence Software (Shimadzu).

2.14. Chemiluminescence measurements of total antioxidant reserve in brain and liver homogenates

The antioxidant defense system has many components. Assessment of the total antioxidant capacity or antioxidant reserve involves integral measurements of all biological components present in biological fluid, tissue or cell homogenates that are capable of scavenging radicals generated by thermal decomposition of water- or lipid-soluble azo-initiators. This method has been widely used since it's introduction in 1985 [43]. For our experiments, we chose to use a water soluble azo-initiator that produces peroxyl radicals at a steady rate [30] and causes chemiluminescence of a reporter molecule, luminol. The water soluble azo-initiator was 2,2′-azobis(2-aminodinopropane)-dihydrochloride (AAPH). A delay in the chemiluminescence response, which is caused by interaction of endogenous antioxidants with AAPH-derived peroxyl radicals, is observed upon addition of homogenates. The delay before observation of the chemiluminescent reaction is proportional to the quantity of antioxidant present in the sample [43]. The incubation medium contained 0.1 M phosphate buffer, pH 7.4, at 37 °C, AAPH (50 mM), and luminol (400 μM). The concentration of protein in samples was 5–6 mg/ml for brain and 10–15 mg/ml for liver. Luminescent analyzer 633 (Coral Biomedical Inc., San Diego, CA) was employed for determinations. In our experiments, the lowest detectable level of scavenged peroxyl radicals was 13.5 μM, which corresponds to 2.2 nmol/mg protein in tissue

2.15. Statistical analysis

Results were analyzed by one-way (effect of diet) or two-way (effect of diet, effect of MDMA, and the interaction of diet and MDMA) analysis of variance followed by Tukey's multiple comparison post-hoc test (Sigma Stat, SPSS, Chicago, IL). Differences were considered significant at $P<0.05$

3. Results

3.1. Effects of vitamin E deficient diet on brain and liver vitamin E and other antioxidant measures

Twenty weeks of vitamin E deficient diet resulted in a significant reduction of vitamin E levels and three other antioxidant measures in the brain. Brain vitamin E was reduced by 75% while total antioxidant reserve was reduced by approximately 40%. Brain glutathione and protein thiols were reduced by approximately 40 and 33%, respectively (Fig. 1). Liver antioxidants also were reduced by the vitamin E deficient diet. Liver vitamin E and glutathione both were significantly reduced by 93 and 28%, respectively (Fig. 2). Liver total antioxidant reserve

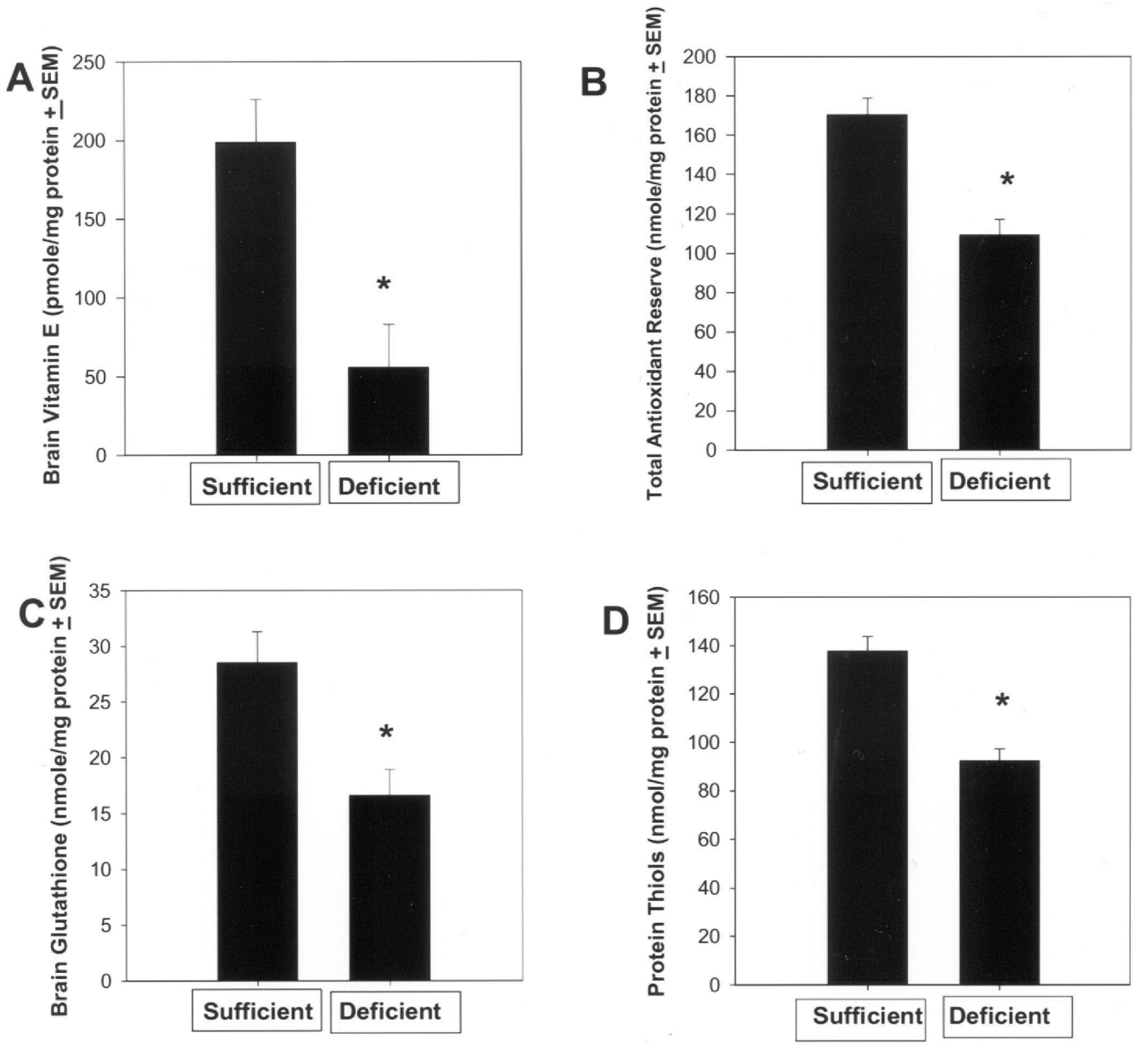

Fig. 1. Effect of vitamin E deficient diet on antioxidants of brain. Male BALB/c mice were maintained on control or vitamin E deficient diet for 20 weeks as described in Section 2. Mice were approximately 6 months old at the time of analysis. Brain vitamin E was reduced 75%, total antioxidant reserve was reduced 40%, brain glutathione was reduced 40% and brain protein thiols reduced 33%. * Indicates significant effect of diet $P<0.05$.

and protein thiols were not significantly affected by the diet.

3.2. Effects of vitamin E deficient diet on brain DA levels and measures of neurotoxicity

The vitamin E deficient diet alone did not alter striatal DA (see legend Fig. 3 for absolute values of DA concentrations). Similarly, the DA metabolites DOPAC and HVA were not altered by vitamin E deficient diet (data not shown). As well, vitamin E deficient diet did not alter basal levels of GFAP (see legend Fig. 4 for absolute values of GFAP levels for saline treated animals).

3.3. Effect of d-MDMA on brain DA and measures of neurotoxicity in vitamin E sufficient and deficient animals

d-MDMA dose dependently reduced striatal DA concentrations in the vitamin E sufficient fed animals with concomitant dose-dependent elevations of striatal GFAP protein. At the low dose of *d*-MDMA (5 mg/kg) striatal DA was not significantly reduced (less than 20% reduction), while the higher dose caused a significant 80% reduction (Fig. 3). Changes in levels of DA metabolites, were similar to the changes in DA (data not shown). Similarly, the low dose of *d*-MDMA did not significantly

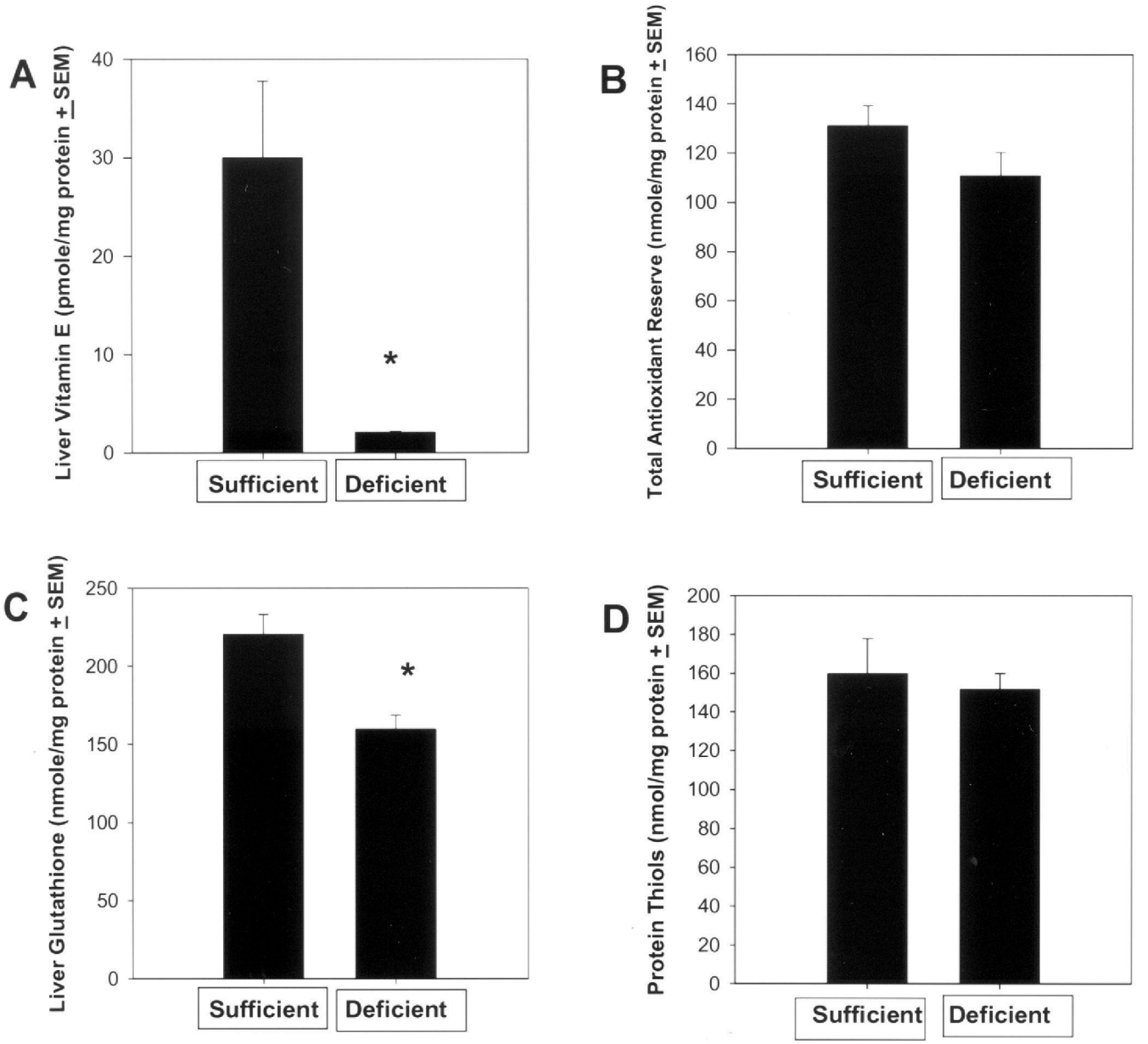

Fig. 2. Effect of vitamin E deficient diet on antioxidants of liver. Male BALB/c mice were maintained on control or vitamin E deficient diet for 20 weeks as described in Section 2. Mice were approximately 6 months old at the time of analysis. Liver vitamin E was reduced approximately 93% and liver glutathione was reduced 28%. * Indicates significant effect of diet $P<0.05$.

elevate striatal GFAP protein levels while the high dose of *d*-MDMA elevated GFAP to 300% of the saline-treated control value (Fig. 4).

In vitamin E deficient fed animals, *d*-MDMA dose-dependently reduced striatal DA and elevated striatal GFAP by margins of larger magnitude than those caused by the same doses in vitamin E sufficient animals. For example, with deficient animals, a 47% reduction in striatal DA was observed using *d*-MDMA 5 mg/kg which was clearly greater than the effect observed in sufficient fed animals described above (Fig. 3). The effect of the high dose of *d*-MDMA (10 mg/kg) was similar in both diet groups, approximately an 80% depletion in each case (Fig. 3).

Striatal GFAP was elevated by the low dose of *d*-MDMA in the deficient fed group to a level 200% of control which was a much larger effect than that observed in sufficient fed animals (Fig. 4). The GFAP elevation caused by the high (10 mg/kg) MDMA dose was about the same in both diet groups (250% of saline-treated group in deficient versus 300% in sufficient group).

3.4. Effects of d-MDMA on brain and liver vitamin E levels and other antioxidant measures in vitamin E sufficient and deficient animals

d-MDMA administration to vitamin E sufficient animals caused dose-dependent reductions of three of the brain

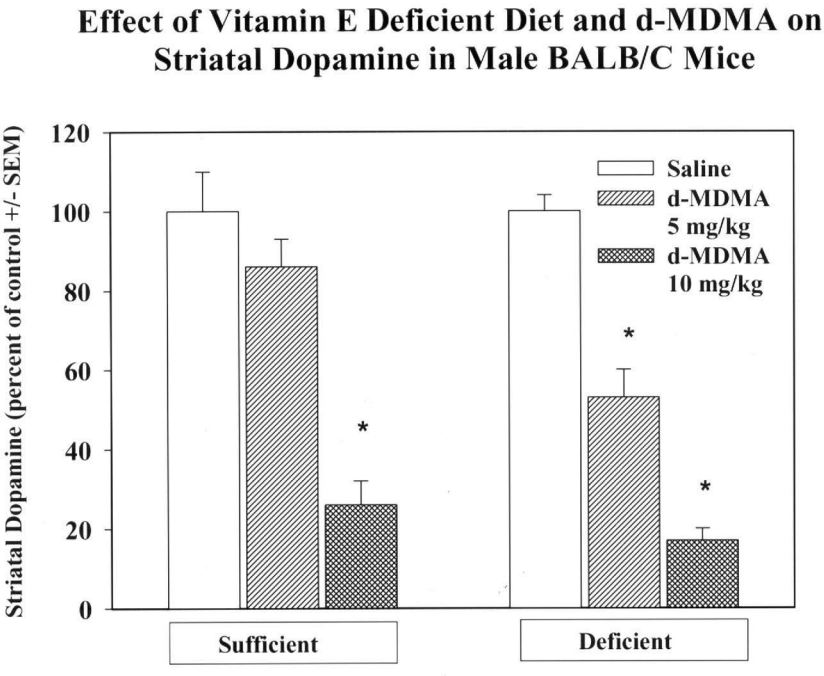

Fig. 3. Effect of vitamin E deficient diet and *d*-MDMA on striatal DA in male BALB/C mice. The vitamin E deficient diet had no effect on striatal DA levels in saline treated mice (compare groups 1 and 4, absolute levels were 2.08±0.22 and 1.95±0.09 μg/g tissue, respectively). *d*-MDMA treatment dose dependently decreased striatal DA levels in both diet groups, but the effect was more pronounced in the vitamin E deficient diet animals. Drug treatments were as follows: groups 1, 4 received saline 200 μl s.c. every 2 h×4; groups 2, 5 received *d*-MDMA 5 mg/kg s.c. every 2 h×4; groups 3, 6 received *d*-MDMA 10 mg/kg s.c. every 2 h×4. Numbers of animals analyzed in each group were as follows: groups 1 and 3, $n=4$ in each group; groups 2, $n=6$; group 4, $n=10$; group 5, $n=14$; group 6, $n=8$. Data were analyzed by a two-way ANOVA with the main effects of diet and MDMA dose and the interaction diet×*d*-MDMA dose. Each main effect and the interaction were significant ($P<0.05$). Post-hoc tests indicated that the decrease in striatal DA in vitamin E deficient fed animals was significantly greater than in vitamin E sufficient diet, 5 mg/kg *d*-MDMA treated animals ($P<0.05$). Animals treated with 10 mg/kg MDMA exhibited the same level of DA depletion whether fed sufficient or vitamin E deficient diet. * Indicates significant difference from saline-treated animals of corresponding diet ($P<0.05$).

antioxidant measures. The highest dose of *d*-MDMA (10 mg/kg) reduced brain vitamin E (56%), total antioxidant reserve (33%) and protein thiols (24%). A non-significant trend toward decreasing brain glutathione concentrations was also observed (Fig. 5). In liver, *d*-MDMA administration caused dose-dependent reductions of two of the measures. At the highest dose, *d*-MDMA (10 mg/kg) reduced total antioxidant reserve (28%) and liver glutathione (21%). However, at the same dose, liver vitamin E levels and protein thiols were not significantly reduced (Fig. 6).

In vitamin E deficient animals, *d*-MDMA administration caused a further significant reduction in antioxidant reserve in both the brain and liver of approximately 30% (Figs. 5 and 6). However, with respect to vitamin E levels, glutathione levels and protein thiol levels, *d*-MDMA administration did not cause a further reduction below that caused by vitamin E deficient diet alone (Figs. 5 and 6). It should be noted, since liver vitamin E was so severely reduced by the vitamin E deficient diet, a further reduction by MDMA would have been difficult to detect. Brain vitamin E levels were less severely depleted by the vitamin E deficient diet and were well within detection limits. MDMA treatment of vitamin E deficient fed animals did not further reduce brain vitamin E levels.

3.5. Effects of vitamin E sufficient and deficient diets on the thermoregulatory response induced by d-MDMA

Since the neurotoxin activity of *d*-MDMA treatment is closely linked to the body temperature elevating properties of the drug it was important to determine whether the vitamin E deficient diet fed animals responded to the thermoregulatory properties of *d*-MDMA differently than vitamin E sufficient animals. Typically, the doses used in other strains of mice to induce dopaminergic neurotoxicity are higher than the doses used here. In C57Bl/6J mice, neurotoxic doses of *d*-MDMA (15–20 mg/kg every 2 h×4) cause body temperature elevations [18,28]. BALB/c mice are more sensitive to the neurotoxin and require lower doses such as the ones used here. The thermoregulatory properties of *d*-MDMA appear to be dose dependent such that the 5 mg/kg dose caused a lowered body temperature in the sufficient fed mice compared to saline at most of the time points (Fig. 7A) The same pattern emerged for the 10 mg/kg dose in sufficient fed animals. A different pattern emerged in the deficient fed groups, *d*-MDMA 5 mg/kg caused body temperatures not significantly different from saline. However, in the deficient fed group, the 10 mg/kg dose caused body temperatures that were elevated compared to saline at the 4- and 6-h

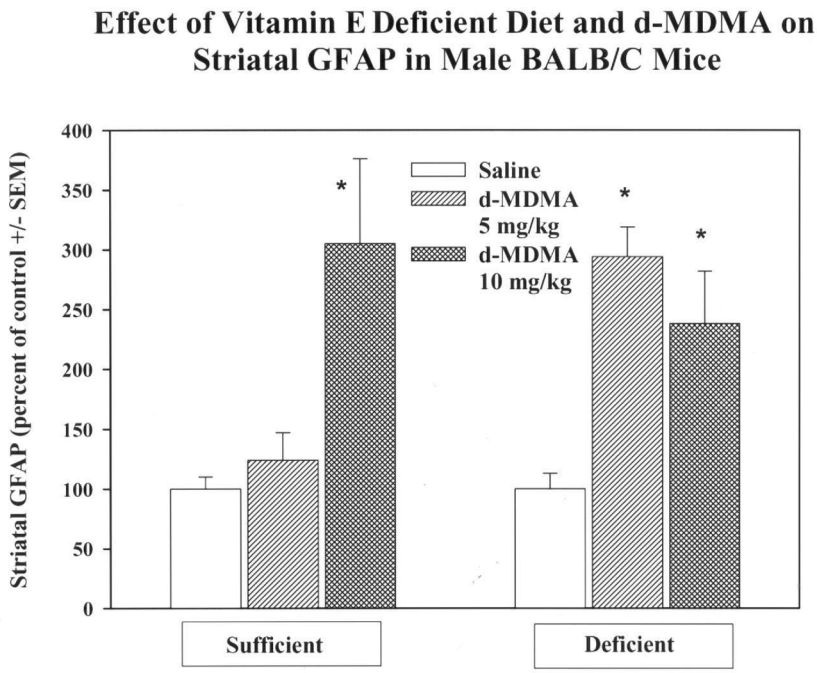

Fig. 4. Effect of vitamin E deficient diet and *d*-MDMA on striatal GFAP in male BALB/C mice. The vitamin E deficient diet had no effect on striatal GFAP levels in saline treated mice (compare groups 1 and 4, absolute levels were 0.211 ± 0.015 and 0.158 ± 0.048 μg/mg total protein, respectively). Vitamin E sufficient diet animals included groups 1–3, vitamin E deficient animals include groups 4–6. Drug treatments and numbers of animals analyzed in each group were as described in Fig. 3. Data were analyzed by a two-way ANOVA with interaction with the main effects of diet and MDMA dose and the interaction diet×*d*-MDMA dose. The main effect of MDMA dose and the interaction were significant ($P<0.05$) while diet was not. In sufficient diet fed animals only the 10 mg/kg *d*-MDMA treated group exhibited a significant elevation of GFAP ($P<0.05$) while in the vitamin E deficient animals both the 5 and 10 mg/kg doses of *d*-MDMA resulted in a significant elevation of GFAP ($P<0.05$). * Indicates significant difference from saline-treated animals of corresponding diet ($P<0.05$).

time-points. The area under the temperature curve was integrated using Simpson's rule for the segment from zero to 8 h. In the sufficient fed group, at both doses, *d*-MDMA caused lower T_{AUC} values compared to saline, while in the deficient fed group T_{AUC} values for *d*-MDMA treated animals were not different from saline (Fig. 7B)

Comparing sufficient fed to deficient fed at the 10 mg/kg *d*-MDMA dose, the T_{AUC} for the sufficient fed group was lower than for the deficient fed group. At the 5 mg/kg *d*-MDMA dose, there was no difference in the T_{AUC} between sufficient fed and deficient fed.

3.6. Effects of vitamin E deficient diet and d-MDMA on liver: cytoarchitecture

Livers of animals fed the vitamin E sufficient diet exhibited normal architecture (Table 1 and Fig. 8). Livers of animals that received the vitamin E deficient diet showed normal architecture in 90% of the group. One animal exhibited a large necrotic focus of liver cells close to a very large bile duct but no inflammatory cells were observed.

Treatment with *d*-MDMA in sufficient diet fed animals resulted in a dose dependent increase in micro-vesicles replacing the cytoplasm of hepatocytes. Treatment with *d*-MDMA 5 mg/kg caused a low incidence of mild microvesicles while *d*-MDMA 10 mg/kg caused numerous microvesicles replacing most of the cytoplasm of the hepatic cells.

Treatment of animals fed the vitamin E deficient diet with *d*-MDMA 10 mg/kg caused multiple foci of frank liver cell necrosis and areas with multiple microvesicles in the cellular cytoplasm. A lesser degree of necrosis was observed in the livers of animals treated with *d*-MDMA 5 mg/kg.

4. Discussion

Our report of the four-fold reduction in brain vitamin E levels after 20 weeks of a vitamin E deficient diet is consistent with previous reports using other rodent species. Monji et al. [29] reported a 4-fold reduction of brain alpha-tocopherol levels in Sprague–Dawley rats after 12–20 weeks of vitamin E deficient diet which provided only 1 IU/kg of diet, 10-fold lower vitamin E levels than the current report. Thus the brain depleting effect of a vitamin E deficient diet is similar across rodent species. With regard to effects on liver vitamin E levels after the vitamin E deficient diet, we report an approximate 15-fold reduction, which is similar to that reported by others after only 40 days on a deficient diet [3,7,26]

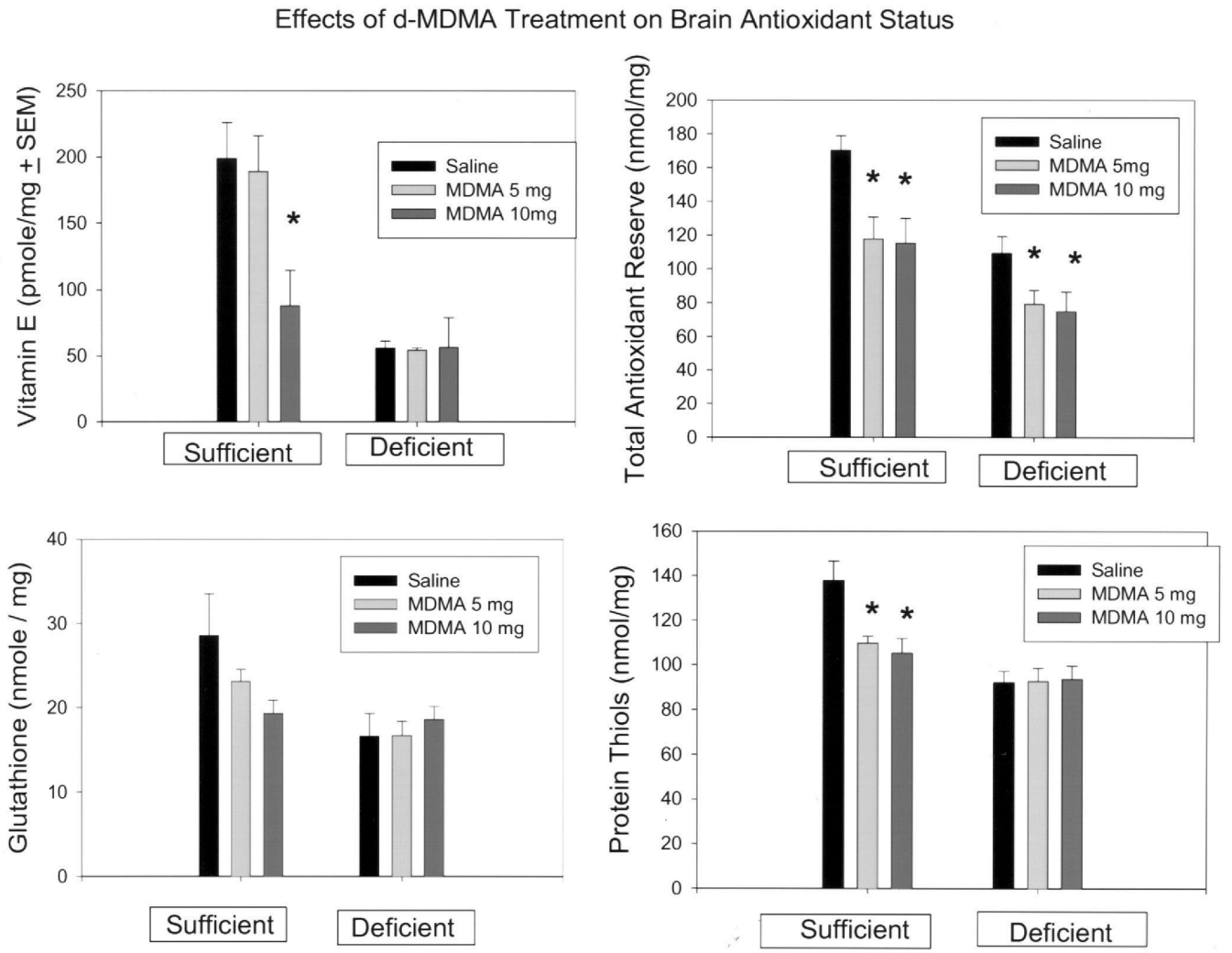

Fig. 5. Effects of MDMA treatment on brain antioxidant status. Animals in both the sufficient and deficient diet groups were treated as described in Fig. 3. Seventy-two hours later (a time shown in previous work to be coincident with maximal damage by MDMA), tissues were dissected and analyzed for antioxidant content (see Section 2). MDMA caused a significant reduction of brain vitamin E, total antioxidant reserve and protein thiol content in the sufficient diet group ($P<0.05$). MDMA treatment also further significantly reduced total antioxidant reserve in the deficient diet fed group ($P<0.05$). * Indicates significant difference from saline-treated animals of corresponding diet ($P<0.05$).

In both brain and liver, the vitamin E depleting effects of the diet were much greater than those of *d*-MDMA, yet *d*-MDMA treatment resulted in significant reductions in three of the brain antioxidants measured. Others have reported that MDMA causes a temporary reduction of brain vitamin E and ascorbate in rat brain, which returns to control levels by 12 h after the dose [36]. It appears that the effect of *d*-MDMA may be more long lasting in mouse brain since our measurements were made at 72 h after the dose and showed significant depletions of vitamin E, total antioxidant reserve and protein thiols. Experiments to examine the time course of the reduction and possible recovery should be conducted in mouse. In addition it appears that in mouse, there is a floor effect on brain vitamin E such that the level could be reduced either by *d*-MDMA (see sufficient diet animals) or with vitamin E deficient diet (saline-treated animals) but the combined treatments provided no additional reduction. These data suggest that a mechanism exists by which brain vitamin E levels are maintained during periods of oxidative stress, perhaps at the expense of vitamin E levels in other tissues (e.g. liver). A large body of work has established that ascorbate, vitamin E, glutathione and other cellular antioxidants function together as an antioxidant network to protect cells from oxidative stress. During oxidative stress, oxidized ascorbate and vitamin E are reduced (recycled) by glutathione and other antioxidants through both enzymatic and non-enzymatic pathways to maintain adequate levels of antioxidant reserve [33,44]. Thus the MDMA-induced reductions of vitamin E and ascorbate observed by Shankaran et al. [36], albeit of short duration, are consistent with the longer lasting MDMA-induced depletions of vitamin E, antioxidant reserve and thiols observed in our study. Early MDMA-induced depletion of cellular vitamin

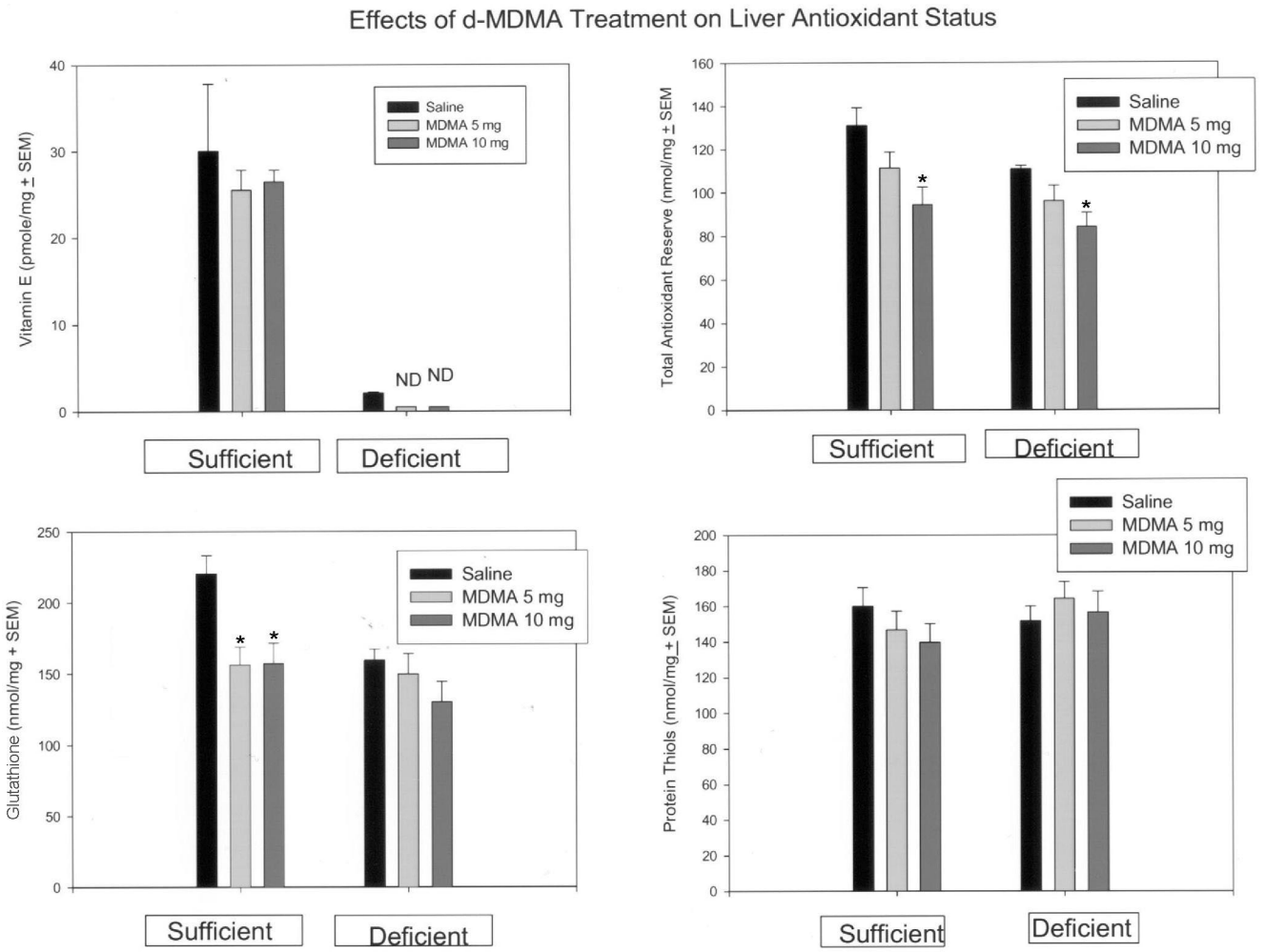

Fig. 6. Effects of MDMA treatment on liver antioxidant status. Animals in both the sufficient and deficient diet groups were treated as described in Fig. 3. Seventy-two hours later (a time shown in previous work to be coincident with maximal damage by MDMA), liver tissue was dissected and analyzed for antioxidant content (see Section 2). MDMA caused a significant reduction of liver glutatione and total antioxidant reserve in the sufficient fed group ($P<0.05$). MDMA caused a further reduction of liver total antioxidant reserve in the deficient fed group ($P<0.05$). * Indicates significant difference from saline treated animals of corresponding diet ($P<0.05$).

E and ascorbate may be followed by later depletion of antioxidant reserve and protein thiols which are consumed by recycling mechanisms to restore vitamin E and ascorbate to pre-stress levels.

The impact of the vitamin E deficient diet in reducing brain antioxidant levels was larger than that of *d*-MDMA exposure. This statement also holds true for the effects of diet and *d*-MDMA on liver antioxidant measures. In general, the vitamin E deficient diet caused greater reductions of antioxidant measures than did *d*-MDMA treatment alone. We observed an additional reduction of antioxidant measures in animals treated with the combination of treatments in one measure, the total antioxidant reserve in brain. Thus, despite the apparent independent ability of each manipulation to cause reductions in these antioxidant measures, the combined application of *d*-MDMA with vitamin E deficient diet did not cause an additive reduction in the other anti-oxidant measures. This finding is in contrast to the result of combined vitamin E deficient diet with *d*-MDMA treatment on striatal integrity as reflected by striatal DA and GFAP measures (see below).

Placing animals on the vitamin E deficient diet caused a shift in the dose response curve for neuronal damage caused by *d*-MDMA. Previously, we have shown that the neurotoxicity profile of *d*-MDMA in the mouse consists of loss of DA, induction of reactive gliosis (as assessed by an increase in GFAP) and terminal degeneration (as assessed by cupric silver degeneration staining and loss of tyrosine hydroxylase protein [32]. Based on these established criteria, our present data indicate that the low dose of *d*-MDMA is not neurotoxic to the normal diet mice but is neurotoxic to the vitamin E-deficient animals.

Striatal DA levels in rats have been reported to be reduced by vitamin E deficient diet [1,9]. In contrast with those reports, we saw no depletion of DA that could be attributed to any effect of diet in striata of vitamin E

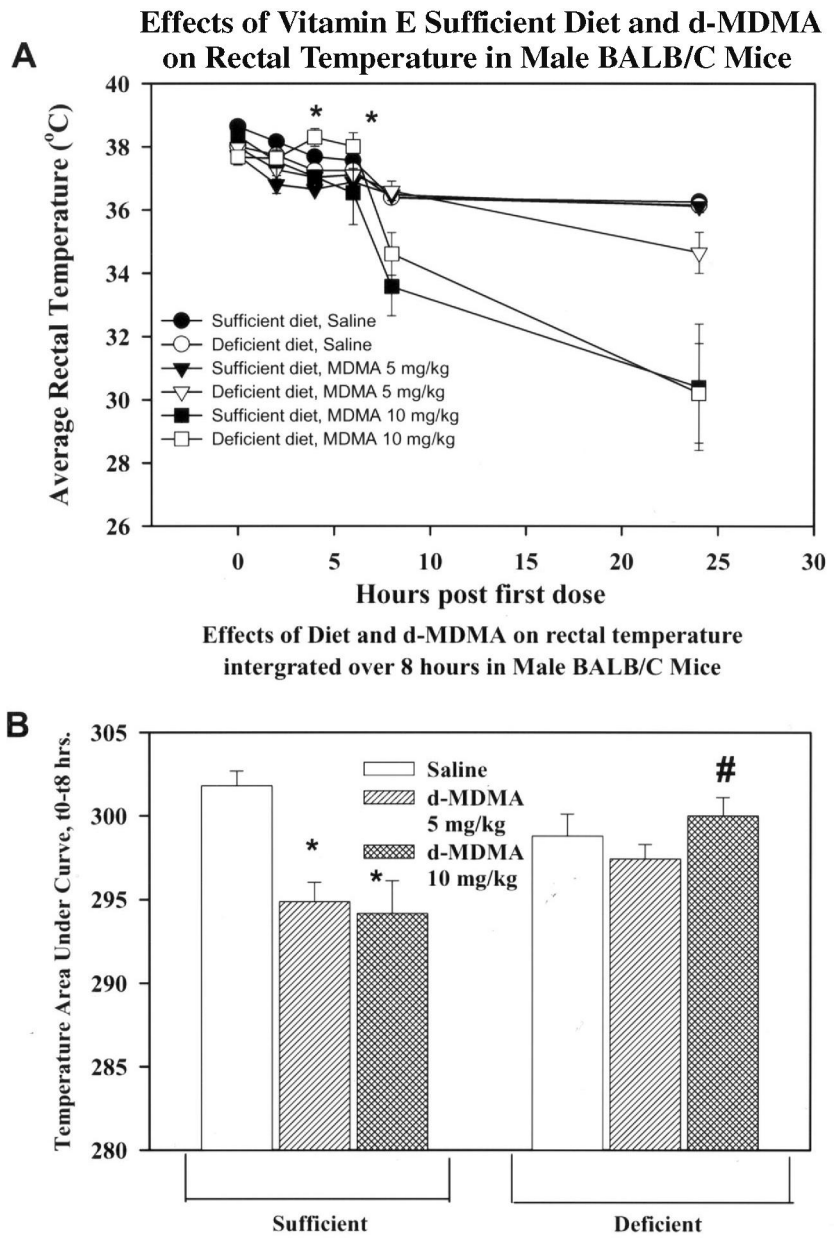

Fig. 7. Panel A: effects of vitamin E sufficient and deficient diets combined with saline or *d*-MDMA on average rectal temperature in male BALB/c mice. Temperatures were taken using a rectal probe as described in Section 2. Temperatures were determined just before the first injection (0 h), just before each subsequent injection (2, 4, 6 h), 2 h after the last injection (8 h) and 24 h after the first injection (24 h). Error bars represent the S.E.M. * Indicates significant temperature elevation compared to same diet, saline-treated animals. Panel B: effects of diet and *d*-MDMA on rectal temperature integrated over 8 h in male BALB/C mice. The same raw data used to construct panel A above was integrated using Simpson's rule (temperature area under the curve (T_{AUC}). T_{AUC} was then plotted as a bar-graph with error bar representing S.E.M. of T_{AUC}. Groups 1–3 are sufficient diet; groups 4–6 are vitamin E deficient diet. Groups 1, 4 received saline; groups 2, 5 received *d*-MDMA 5 mg/kg every 2 h×4; groups 3, 6 received *d*-MDMA 10 mg/kg every 2 h×4. The integrated AUC parameter reveals that temperature is reduced over the 8-h period in groups 2, 3 relative to saline (indicated by * $P<0.05$), while groups 4–6 are not different from each other. Group 6 is elevated compared to group 3 (indicated by $^{\#}$ $P<0.05$); however, groups 2 and 5 are not different from each other.

deficient saline treated mice. However, consistent with our previous work, we observed that *d*-MDMA dramatically lowers striatal DA in mice and that this effect is potentiated by the vitamin E deficient condition. We interpret this effect as an increase in neurotoxicity because it was accompanied by an increase in GFAP.

Our data suggest that a low dose of *d*-MDMA, which is not neurotoxic in the sufficient diet mouse becomes neurotoxic in the vitamin E deficient mouse. Others have reported that vitamin E deficiency can increase the severity of a variety of toxic exposures. For example, the lesion observed after focal cerebral ischemia in vitamin E de-

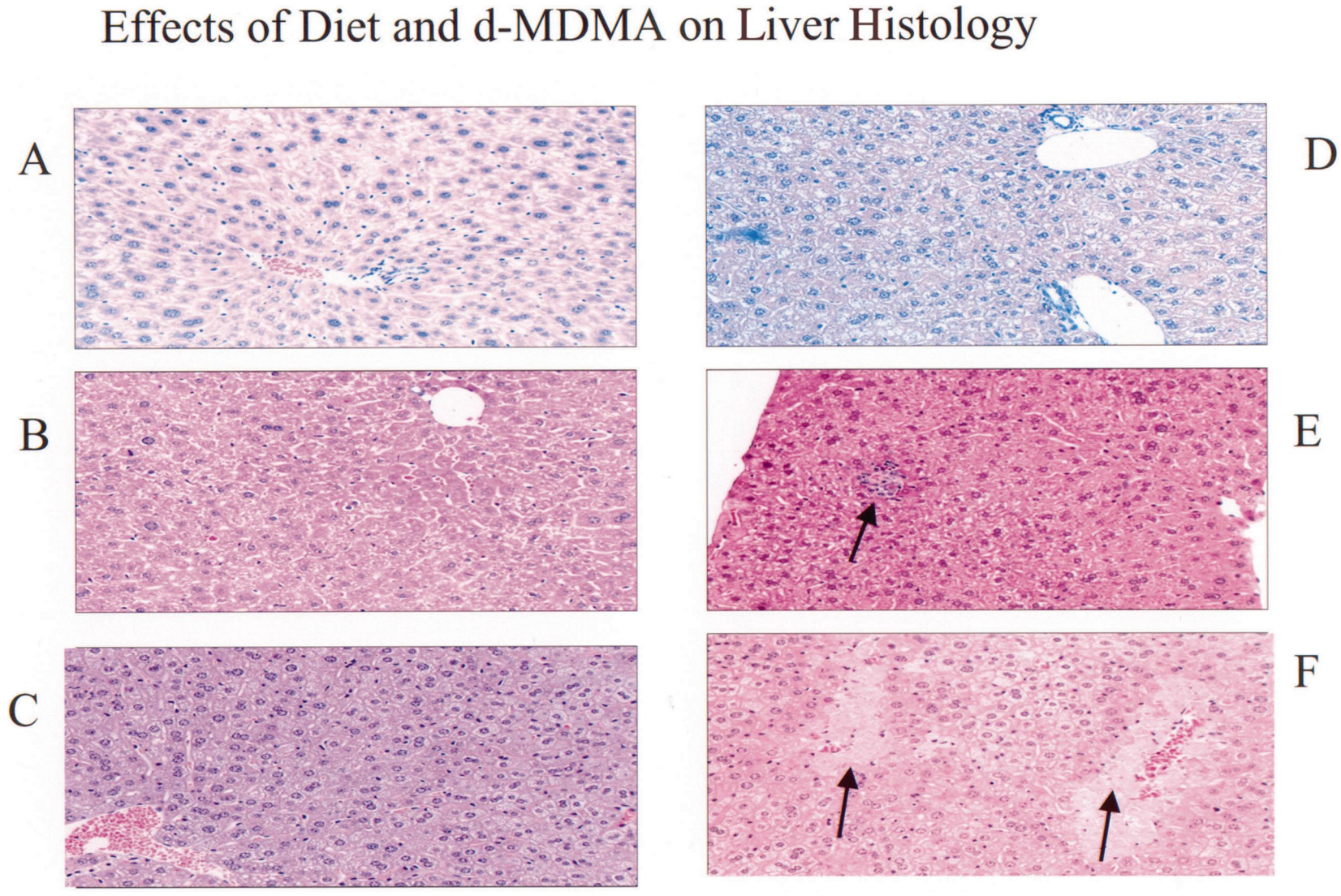

Fig. 8. Panels A–C are sufficient diet fed; panels D–E are deficient diet fed. Panels A, D are saline treated; panels B, E are *d*-MDMA 5 mg/kg every 2 h×4 treated; panels C, F are *d*-MDMA 10 mg/kg every 2 h×4 treated. Arrows point to areas of necrosis.

Table 1
Effects of vitamin E sufficient or deficient diet, combined with saline or *d*-MDMA treatment, on liver histology of male BALB/C mice

Group	Hepatic cells	
	Micro vesicles	Necrosis
Sufficient diet, Saline	+	–
Sufficient diet, MDMA 5 mg/kg	+	
Sufficient diet, MDMA 10 mg/kg	+ + +	
Deficient diet, Saline		+
Deficient diet, MDMA 5 mg/kg	+ +	+ +
Deficient diet, MDMA 10 mg/kg	+ + +	+ + +

ficient rat is approximately twice as large as that in control rats [42]. Vitamin E deficient mice suffer from potentiated acetaminophen-induced lethality which is linked to increased liver toxicity [34]. Similarly, vitamin E deficient mice exhibit potentiated carbon tetrachloride or *d*-galactosamine induced hepatotoxicity [45]. Our observed increase in necrotic foci in the liver of MDMA-treated–vitamin E depleted mice suggests a potentiation of MDMA hepatotoxicity.

According to the hypothesis we tested, elevated brain levels of antioxidants, such as ascorbate and vitamin E, should ameliorate the neurotoxicity of administered amphetamines. However, the results of short-term administration of vitamin E prior to or during methamphetamine administration have yielded conflicting results in rodents [2,8; unpublished data, Johnson and Miller]. It has subsequently been demonstrated that supplementation of diet for at least 2 months is required to cause elevations of brain vitamin E levels and that a ceiling of vitamin E accumulation in brain occurs with diet supplementation, e.g. diets supplemented with 60 mg vitamin E/kg of diet versus 500 mg vitamin E/kg result in similar rodent brain levels of vitamin E [27]. Furthermore, in an earlier study, Machlin and Gabriel [25] demonstrated that uptake of vitamin E supplied by a highly fortified diet continues in the tissues of rats up to 20 weeks. These data indicate that the extent of alteration of brain antioxidant capacity after vitamin E administration is limited but requires an extended duration of dietary exposure. This information indicates, as well, that future experiments designed to determine the effect of vitamin E supplementation on *d*-MDMA toxicity in mouse brain will require prolonged vitamin E supplementation.

In summary, the data reported herein support the hypothesis that *d*-MDMA-induced neurotoxicity in mouse may be mediated in part by formation of radical species since antioxidant defenses in mouse brain are depleted by *d*-MDMA at least as long as 72 h after the first dose. These species, which may normally be detoxified by membrane associated vitamin E, cause a larger degree of neuronal damage to mouse brain when depleted of vitamin E by diet.

Acknowledgements

The authors gratefully acknowledge Fang X. Ma, Mary Ann Hammer, Monica Graziani, Brenda Mutnansky, Christopher Felton and Sherri Friend for their expert technical assistance.

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